Available online at www.pharmacie-globale.info ISSN 0976-8157

Review Article

PHARMACIE GLOBALE

INTERNATIONAL JOURNAL OF COMPREHENSIVE PHARMACY

Catharanthus roseus (L.) G. Don. AN IMPORTANT DRUG: IT'S APPLICATIONS AND PRODUCTION

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Received: 18 August 2010; Revised: 20 October 2010; Accepted: 29 October 2010; Available online: 1 November 2010

ABSTRACT

Catharanthus roseus is an important drug. Traditionally, different parts of it used in the treatments of various diseases (viz. diabetes, menstrual regulators, hypertension, cancer and antigalactagogue etc.), in numbers of countries (Australia, Brazil, China, Cook Island, Dominica, England, Europe, France, French Guina, India, Jamaica, Kenya, Mexico, Mozambique, North Vietnam, Pakistan, Peru, Philippines, South Africa, South Vietnam, Taiwan, Thailand, USA, Venda, Vietnam, West Indies etc.). Moreover, more than 130 alkaloids have been isolated from different parts; amongst which two important alkaloids (Vinblastine and Vincristine used in cancer treatment) present in very low concentrations. Keeping these views; researcher continuously using different approaches to enhance the level of important alkaloid to meet the required demand. Thus, in present communication various *in vitro* biotechnological and biochemical approaches (viz. effect of media composition, plant growth regulators, pH of the culture medium, temperature, light, aeration, elicitors, mutagenesis, high cell density culture, selection of superior cell lines, bioreactor and immobilization methods, hairy root culture, somatic embryogenesis, biosynthesis of alkaloids, metabolic and genetic engineering in alkaloids biosynthesis, coupling method for alkaloids biosynthesis, cellular compartmentation etc.) have been used worldwide; which directly concerning with the *in-vitro* micropropagation and the enhancement of important secondary metabolites present in different parts of *Catharanthus* and being used in the treatment of various diseases.

Keywords: *Catharanthus roseus*, secondary metabolites, disease, *in-vitro* micropropagation.

INTRODUCTION

Catharanthus roseus is a renowned medicinal plant, belonging to the family Apocynaceae; and is a rich source of alkaloids, which are distributed in all parts of the plant. The alkaloid content of *C. roseus* varies considerably in various parts; the maximum being in the root bark which ranges from 0.15 to 1.34 % and even up to 1.79 in some strains.[1] The plant contains about 130 alkaloids of the indole group out of which 25 are dimeric in nature. Two of the dimeric alkaloids vinblastine and vincristine mainly present in the aerial parts, have found extensive application in the treatment of human neoplasma. Among the monomeric alkaloids ajmalicine (raubacine) found in the roots has been confirmed to have a broad application in the treatment of circulatory diseases, especially in the relief of obstruction of normal cerebral blood flow.

Vinblastine sulphate (sold as Velban) is used particularly to treat Hodgkin's disease besides lymphocarcoma, choriocarcinoma, neuroblastoma, carcinoma of breast, lungs and other organs in acute and chronic leukemia. Vincristine sulphate (sold as Oncovin) arrest mitosis in metaphase and is very effective for treating acute leukaemia in children and lymphocytic leukemia. It is also used against Hodgkin's disease, Wilkins's tumor, neuroblastoma and reticulum cell sarcoma. Today India is the third largest manufacture of Vinblastine and Vincristine in the world and is exporting these alkaloids to European countries. High demand and low yield of these alkaloids in the plant has led to research for alternative means for their production. Vinblastine is also modified structurally to yield deacetyl vinblastine amide (Vindesine) introduced recently as Eldisine for use in the treatment of acute lymphoid leukemia in children. Biochemical coupling of alkaloids Catharanthine and Vindoline to get dimeric compounds is also achieved.

Beside these, tissue culture technique is developed for the development of these dimeric alkaloids.[2] In the present communication a detailed application of *C. roseus* including traditional uses in various developed and developing countries, pharmacological activities and the application of various biotechnological tools viz.

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Optimization of Media Composition, Phytohormones, pH, Temperature, Light, Aeration, Elicitors, Mutagenesis, High Cell Density Culture, Selection of Superior cell lines, Bioreactors and Immobilization Methods, Hairy root culture, *In Vitro* Somatic embryogenesis, Biosynthesis of alkaloids in *Catharanthus,* Metabolic and Genetic Engineering in alkaloids biosynthesis, Coupling method for Alkaloids biosynthesis, Cellular Compartmentation has been applied for the enhancement of important secondary metabolites present in different parts of *Catharanthus*.

TAXONOMY, HABIT AND HABITAT OF *Catharanthus roseus*

Catharanthus roseus is an important medicinal plant, commonly known as Madagascar periwinkle. It is a perennial, evergreen herb, 30-100 cm height that was originally native to the island of Madagascar. It has been widely cultivated for hundreds of year and can now be found growing wild in most warm regions of the world. The leaves are glossy, dark green (1-2 inch long), oblong – elliptic, acute, rounded apex; flowers fragrant, white to pinkish purple in terminal or axillar y cymose clusters; follicle hairy, many seeded, 2-3 cm long; seeds oblong, minute, black. The plant is commonly grown in gardens for beddings, borders and for mass effect. It blooms througout the year and is propagated by seeds or cuttings. The bloom of natural wild plants are pale pink with a purple eye in the centre, but horticulturist has developed varieties (more than 100) with colour ranging from white to pink to purple.

TRADITIONAL USE OF *C. roseus*

The plant has historically been used to treat a wide assortment of diseases. It was used as folk remedy for diabetes in Europe for centuries.[3] In India, juice from the leaves was used to treat wasp stings. In Hawaii, the plant was boiled to make a poultice to stop bleeding. In china, it was used as an astringent, diuretic and cough remedy.[4]

In central and south America, it was used as a homemade cold remedy to ease luncongestion and inflammation**.** Throughout the Caribbean, an extract from the flowers was used to make a solution to treat eye irritation and infections. It also had a reputation as magic plant, European thought it could ward off evil spirits, and the French referred to it as "violet of the sorcerers." Western researchers finally noticed the plant in 1950's when they learn of a tea Jamaican were drinking to treat diabetes. They discovered that the plant contains a motherlode of useful alkaloids (130 in all at last count). Some, such as catharanthine, leurosine sulphate, lochnerine, tetrahydroalstonine, vindoline and vindolinine lower blood sugar level, however, others act as haemostatics (arrest bleeding) and two others, vincristine and vinblastine have anticancerous properties. Periwinkle also contains the alkaloids reserpine and serpentine, which are powerful tranquilizers. Table **1.** Summarized traditional uses of the *C. roseus* in different countries.

Table 1 . Traditional Medicinal Uses of *Catharanthus roseus in* various developed and developing countries.

Country	Used as
Australia	Hot water extract of dried leaves is taken orally for menorrhagia, diabetes and extract of root bark is taken orally as febrifuge[146,147]
Brazil	The hot water extract of dried entire plant is taken orally by human for diabetes mellitus[148,149].
China	Hot water extract of the aerial parts is taken orally as a menstrual regulators.[150, 151]
Cook Island	Decoction of dried leaves used orally to treat diabetes, hypertension and Cancer[152]
Dominica	Hot water extract of leaves is taken orally by pregnant woman to combat primary inertia in childbirth and the boiled leaves are drink to treat diabetes[153]
England	Hot water extract of dried entire plant is taken orally for the curing of diabetes[154]
Europe	Decoction of dried leaves is taken orally for diabetes mellitus[24].
France	Hot water extract of entire plant is taken as an antigalactagogue [150].
French Guina	Hot water extract of entire plant is taken orally as a cholagogue [155].
India	The hot water extract of dried entire plant is taken orally by human for cancer. Hot water extract of dried leaves is taken orally to Hodgkin's disease. The root extract is taken orally for menorrhagia [151, 156].
Jamaica	Hot water extract of dried leaves is taken orally for diabetes [158]
Kenya	Hot water extract of dried leaves is taken orally for diabetes[158]
Mexico	Infusion of whole plant is taken orally for stomach problem [159]
Mozambique	Hot water extract of leaves is taken orally for diabetes and rheumatism and the root extract is taken orally as hypotensive and febrifuge [160].
North Vietnam	Hot water extract of the aerial parts is taken orally as a menstrual regulator [150, 151].
Pakistan	Hot water extract of dried ovules is taken orally for diabetes[161].
Peru	Hot water extract of dried entire plant is taken orally by human adults for cancers, heart disease and leishmaniasis[162].
Philippines	Hot water extract of root is taken orally by pregnant women to produce abortion[151, 163, 164].
South Africa	Hot water extract of dried leaves is taken orally for menorrhagia and diabetes [146].
South Vietnam	Hot water extract of the entire plant is taken orally by human adults as an antigalactagogue [150,151].
Taiwan	Decoction of dried entire plant is used orally by human adults to treat diabetes mellitus[164] and liver disease [165].
Thailand	Hot water extract of dried entire plant is taken orally for diabetes[166].
USA	Hot water extract of leaves are smoked as a euphoriant [167].
Venda	Water extract of dried root is taken orally for venereal disease [168].
Vietnam	Hot water extract of dried aerial parts is taken orally as drug in Vietnamese traditional medicine, listed in Vitnamese pharmacopoeia (1974 Edition) [169].
West Indies	Hot water extract of leafy stems is taken orally for diabetes [170].

Antitumor Activity: Ethanol (70%) extract of leaves was administered intraperitoneally to female mice[5], and prove to be highly active on CA-Ehrlich ascites. Alkaloid fraction of dried leaves was also used externally was actives. Nineteen patients with either flat, verruca vulgaris, plantar or genital warts were treated in this study. Six patients had all warts disappears, seven had the majority of their warts disappears, five had 50% disappears and one showed no response.[6] Chloroform extract of leaves was active on Leuk-P388[7]. The plant contains about 130 alkaloids (Table **2**) of the indole group, out of which 25 are dimeric in nature. Total alkaloids of the entire plant administered to mice intraperitoneally at a dose of 10.0 mg/kg and orally at a 75.0 mg/kg were active on Leuk-P1534[8].

Antispermatogenic Effect: Hot water extract of dried leaves administered intraperitoneally to male mice at a dose of 0.2 ml/animal produced weak activity.[9] At 10.0

mg/ animal, regressive changes in seminiferous tubules and Leydig cells, increased cholesterol in testes and degeneration of all germinal elements.[10] Total alkaloids of entire plants administered intraperitoneally to male rats were active.[11]

Table 2. Alkaloids isolated from different parts of *Catharanthus roseus* (largely based on Dictionary of Natural Products, version 8.2, 2000).

Alkaloids	Class[1]	Extracted from
β-carboline	M	Leaf
tryptamine,*N*,*N*-dimethyl	M	Cell suspension culture
tryptamine,*N*b-acetyl	M	Cell suspension culture
apparicine	A	Leaf, flower
ammocalline	U	Plant extract, root
anthirine	C	Plant extract, cell suspension culture
akuammicine	S	Plant extract, leaf, root, callus
iochrovicine	U	Leaf
pericyclivine	C	Plant extract, leaf
pleiocarpamine	C	Cell suspension culture
cavincine	C	Plant extract, leaf, root, callus culture, hair y root
iochnerine	C	Cell suspension culture
tubotaiwine	A	Callus culture, cell suspension culture
rosicine	P	Leaf
catharanthine	I	Plant extract, leaf, flower, seedlings, callus culture, cell suspension culture, shoots
tabersonine	P	Plant extract, leaf, seedlings, seed, callus culture, cell suspension culture
venalstonine	P	Root
akuammicine,12-hydroxy	S	Cell suspension culture
perivine	C	Plant extract, leaf, flower, root, callus culture, cell suspension culture
vinervine	S	Cell suspension culture
coronaridine	I	Flower
vincadifformine	I	*
cyclolochnerine,21-hydroxy	C	Callus culture, cell suspension culture, shoots, hairy root
Iochneridine	S	Leaf, callus culture, cell suspension culture, hairy root
alstonine	C	Root, callus culture
serpentine	C	Leaf, root, seedlings callus culture, cell suspension culture, shoots, ,hairy root
cathenamine	C	Plant extract
vallesiachotamine	V	Callus culture, cell suspension culture
isovallesiachotamine	V	Callus culture, cell suspension culture
ajmalicine	C	Callus culture, cell suspension culture
ajmalicine,19-*epi*,3-*iso*	C	Plant extract, callus culture, cell suspension culture
ajmalicine, 3-*epi*	C	Plant extract, callus culture ,cell suspension culture
akuammigine	C	Cell suspension culture
akuammiline, O-deacetyl	C	Leaf, callus culture
Iochnericine	P	Plant extract, leaf, cell suspension culture
minovincine	P	Plant extract
preakuammicine	S	Seedlings
rosamine	U	Leaf
tabersonine,19-hydroxy	P	Cell suspension culture
tetrahydroalstonine	C	Plant extract, flower, root, callus culture, cell suspension culture, shoots, hairy root
vindolinine, *N*b-oxide	P	Plant extract, cell suspension culture
vindolinine,19-*epi*,*N*-oxide	P	Cell suspension culture
fluorocarpamine, *N* -oxide	M	Plant extract, leaf
perividine	U	Plant extract
isositsirikine, 19,20-*cis*-16 (R)-	C	Plant extract, cell suspension culture
isositsirikine, 19,20-*trans*-16 (R)-	C	Plant extract, cell suspension culture
isositsirikine, 19,20-*trans*-16 (S)-	C	Plant extract, leaf, cell suspension culture
minovincinine	P	Cell suspension culture
sitsirikine	C	Plant extract, leaf, callus culture, cell suspension culture, shoots
yohimbine	C	Plant extract, leaf, root, callus culture, cell suspension culture, hairy root
sitsirikine,dihydro-	C	Plant extract, leaf, root, callus culture, cell suspension culture
perimivine	C	Plant extract, root
tabersonine,11-methoxy	P	Plant extract, flower
almalicine, 7-hydroxy - indolenine	C	Callus culture
ajmalicine pseudo-indoxyl	C	Callus culture
akuammiline,10- hydroxy- deacetyl	C	Callus culture
epimisiline,19(s)	P	Hairy root
horhammericine	P	Cell suspension culture, shoots
mitraphyllline	C	Flower, callus culture
vincoline	P	Plant extract, leaf
vindolinine	P	Plant extract, leaf, cell
vindolinine,19-*epi*	P	Plant extract, leaf, cell suspension culture
vincolidine	U	Plant extract, leaf
akuammine	C	Plant extract
lochnerinine	S	Plant extract, leaf, cell suspension culture
lochrovidine	U	Plant extract
tabersonine,19- hydroxy-11- methoxy	P	Plant extract
iochrovine	U	Plant extract
vindolidine,*O*-deacetyl-	P	*
akuammiline	C	Plant extract, cell suspension culture
horhammericine,11-methoxy	P	Cell suspension culture, shoots
vincarodine	E	Plant extract, leaf
vinosidine	U	Root
vindoline,deacetoxy-	P	Cell suspension culture, leaf, seedlings
tabersonine,19- acetoxy-11- hydroxy-	P	Plant extract, leaf, cell suspension culture
vindoline,deacetyl-	P	Plant extract, leaf
iochnerinine	U	Leaf, root
tabersonine,19-acetoxy-11- methoxy	P	Cell suspension culture
cathovaline	P	Leaf
vindolidine	P	Plant extract, flower
strictosidine lactam	V	Cell suspension culture, shoots, hairy root
vindoline	P	Plant extract, leaf, flower, seedlings, shoots
akuammicine, xylosyloxy-	S	Cell suspension culture
strictosidine	D	Plant extract, leaf. Root, seed, callus culture, cell suspension culture
bannucine	P	Plant extract, leaf
leurosivine	B	Leaf
leurosine,17-deacetoxy-	B	Plant extract
vinblastine,4-deacetoxy-	B	Plant extract, leaf
vinblastine, deacetyl-	B	Plant extract
vinsedine	B,U	Seed
leurosinine	B,U	Plant extract
vinsedicine	B,U	Seed
vinblastine,3',4'-anhydro-	B	Leaf, shoots
vingramine	B	Seed
vinblastine,4'-deoxy-	B	Plant extract, leaf
vinosidine	B,U	Plant extract
vinblastine, *N*-demethyl-	B	Plant extract

vingrmine, methyl-	B	Seed
catharanthamine	B	Plant extract, leaf
leurosine	B	Plant extract, leaf, shoots
roseadine	B	Plant extract, leaf
vincathicine	B	Plant extract, leaf
roseamine	B,U	Plant extract
vinblastine	B	Plant extract, leaf, flower, seedlings, cell suspension culture
vinblastine,20'-*epi*-	B	Plant extract, leaf
catharicine	B	Plant extract, leaf, flower
catharine	B	Plant extract, leaf, shoot
leurosine, 5'-oxo-*	B	Leaf
carosine	B	Plant extract, leaf, flower
leurosine,*N* b'-oxide	B	Leaf
vinamidine	B	Plant extract, leaf
vincristine	B	Plant extract, leaf
leurosidine, *N* b-oxide	B	Plant extract
vinblastine,14'-hydroxy-	B	Plant extract
vinblastine, 15'hydroxy-	B	Plant extract
neoleurocristine	B	Plant extract, leaf
vindolidine	B	Plant extract
leurosinone	B	Leaf
neoleurosidine	B	Plant extract, leaf
neoleurosidine,*N* b-oxide	B	Plant extract, leaf
vindolicine	B	Plant extract, leaf
ammorosine	U	Root
cathalanceine	U	Root
cathindine	C	Leaf, root, cell suspension culture
cavincidine	C	Plant extract, leaf, root, callus culture, cell suspension culture
lochneririne	U	Leaf, root
maandrosine	U	Plant extract, root
perosine	C	Plant extract, leaf, root, callus culture
rovindine	B,U	Plant extract, leaf
vinaphamine	B,U	Plant extract, leaf
vinaspine	B,U	Plant extract, leaf
vincamicine	B,U	Plant extract, leaf

[1]**Class** (Biogenetic class according to [18]: A = aspidospermatan, B = bisindole, C = corynanthean, D = vincosan, E = eburnan, I = ibogan, M = miscellaneous, P = plumeran, S = strychnan, V = vallesiachotaman, U = unknown structure.

Animutagenic Effect: Hot water extract of dried leaves was active on red blood cells. A reduction in number of micro- nucleated polychromatic red blood cells caused by various mutagens showed ant mutagenic effect.[12]

Antifertility Activity: Methanol/water (1:1) extract of dried leaf and stem administered orally to male rats was active.[13]

Antihypertensive Activity: Total alkaloids of root administered intravenously to dogs at a dose 4.0 mg/kg was active.[14]

Antifungal Activity: Acetone and water extracts of dried aerial parts at a concentration (50%) on agar plate was inactive on *Neurospora crossa.*[15] Hot water extract of dried leaves in broth culture was active on *Trichophyton mentagrophytes.*[16] Hot water extract of dried stem in broth culture was active on *T. mentagrophytes* and weakly active on *T. rubrum.*[17] Leaves and roots on agar plate were active on *Pythium aphanidermatum*.[18]

Antimitotic Activity: Ethanol (70%) extract of leaves was administered to female mice was active on CA-Fhrlich ascites vs induction of metaphase arrest in ascites cells. Dosing was applied four days after tumor cell inoculation ascetic samples removed 2, 4, 6 and 24 hours post treatment.[19]

Anti-Inflammatory Activity: Ethanol extract (95%) of dried leaves was administered intraperitoneally to rats at a dose of 4000.0 mg/kg was active 65% inhibition was noticed in Edema.[20]

Antihyperchcholesterollemic Activity: Hot water extract of dried leaves were taken orally to rabbits was active.[21]

Antidiuretic Activity: Alkaloid fraction of the entire plant was given subcutaneously to male rats at a dose of 50.0 mg/kg was active.[22]

Antimalerial Activity: Chloroform extract of root at a dose 400.0 mg/kg along with water extract at a dose 4.42 gm/kg was administered orally to chicken produced weak activity on *Plasmodium gallinaceum*.[23]

Antihyperglycemic Activity: Dried leaves in the ration of male in mice at a concentration of 6.25% of the diet for 28 days was inactive vs streptozotocin –induced hyperglycemia.[24] Hot water extract of dried aerial parts administered intragastrically to dogs at a dose of 50.0 gm/kg (dry weight of plant) was inactive; a dose of 10.0 gm/kg in case of rabbits was active vs alloxan induced hyperglycemia.[25] Water extract of fresh cells administered intragastically to male rats was active vs streptozotocin induced hyperglycemia, ultimately 60% decreased in blood sugar was observed.[26]

Antibacterial Activity: Benzene extract of dried flowers at a concentration of 50% on agar plate was active on *Proteus*, *Pseudomonas*, *Shigella* and *Stphylococcus* species, however, benzene extract of leaves at a concentration of 50% on agar plate was active on *Proteus*, *Pseudomonas*, *Salmonella*, *Shigella* and *Staphylococcus* species.[27] Ethanol (70%) extract of dried leaves on agar plate was active on *Bacillus megaterium* and *Staphylococcus albus* and inactive on *Bacillus cereus* and *Staphylococcus aureus.*[28] Total alkaloids of root at a concentration of 500.0 mcg/ml in broth culture were inactive on E.coli, *Salmonella lyphosa* and *Shigella dysenteries.*[14] Water extract of entire plant on agar plate at a concentration of 1:4 was inactive on *Salmonella paratyphi*.[22]

Antiviral Activity: Water extract of callus tissue in cell culture was active on Tobacco Mosaic Virus.[29]

Cardiotonic Activity: Ethanol (70%) extract of leaf and stem administered intravenously to rats at a dose of 120.0 mg/kg was active.[14]

CNS Depressant Activity: Total activity of root was administered intraperitoneally to rats at a dose of 120.0 mg/kg was active.[14]

Cytotoxic Activity: Alkaloid fraction of dried leaves in cell culture was active on CA-9KB, ED50 0.0435 mcg/ml.[30] Chloroform extract and culture filtrate of callus tissue in cell culture at dose of 50.0 gm (dry wt of plant) were active on Leuk-L12 10 culture, water extract.

ROLE OF BIOTECHNOLOGICAL APPROACHES IN *CATHARANTHUS* MICROPROPAGATION AND ENHANCEMENT OF PHARMACEUTICAL-LY ACTIVE COMPOUNDS BEING USED IN THE TREATMENT OF VARIOUS DISEASES

Due to the pharmaceutical importance and the low content in the plant of vinblastine and vincristine *Catharanthus roseus* became an important model system for biotechnological studies on plant secondary metabolism. Researchers are focusing their attention to enhance the alkaloids yield by various ways (chemically, enzymatically, synthetically or by cell culture method).

The plant cell can be cultured at large scale[31], but the yield of alkaloids production is too low and limits commercial applications. In recent times, however, two strategies have been commonly used for the enhancement of alkaloids.

a) *In vitro* cultivation of shoot via organogenesis and somatic embryogenesis, callus or suspension by the optimization of media, phytohormones, temperature, pH, light, aeration etc. In addition, high cell density culture, elicitor's treatment, mutagenesis, bioreactors and immobilization are also practiced to improve alkaloids yield.
b) Genetic engineering and over expression of biosynthetic rate limiting enzymes in alkaloid biosynthesis pathways.

In-vitro STUDIES

In tissue culture, the response of culture has been influenced by a number of factors which in turn regulate alkaloids yield. Some of them are discussed in brief

Media Composition: The yield of alkaloids in suspension culture is directly influenced by the surrounding environmental conditions and genetic constitution of the concerned plant material. Over the years efforts have been made in numbers for optimization of culture media for better biomass and alkaloids production, some patents have also been filed.[32-35] Carbon sources and inorganic compounds play a significant role in indole alkaloid production. It was earlier reported that nitrogen and phosphate both promoted growth but had an adverse effect on alkaloids yield[36,37] The inhibitory effect of nitrogen on alkaloid production has not always been observed.[38] The effect of nitrogen on alkaloids production is dependent on carbon availability to the cells which makes the carbon-to-nitrogen ratio (C/N ratio) an important factor to be taken into account. By the determination of the cellular C/N ratio,[39] identified three distinct growth phases: an active growth phase, an accumulation phase, and a biomass decline phase (endogenous metabolism). They also noticed that phosphate (0.56 Mm), nitrate (12.97 Mm) and low concentration of ammonia were beneficial for maximum growth and increased alkaloids production. Similarly higher concentration of sucrose only enhanced biomass, the optimized glucose (500Mm), ammonium and phosphate (0- 12Mm) were previously used for higher alkaloids yield.[40]

Medium composition and day's interval had direct effect on induction and accumulation of indole alkaloids.[41] A medium added with 6% sucrose is favourable for both biomass and alkaloids production in *Catharanthus*.[42] Liquid medium with 3-6% maltose was also found to be highly effective for production of somatic embryos.[43] It has been[44] reported that agitated liquid media added with BAP (1.0 mg/l) was very productive for large-scale plant regeneration. Alteration in macro and micronutrient of MS medium[45] has also been used to promote growth and subsequent alkaloid production.[46]

Surface methodology[47], has been used for the rapid biomass growth and increase in ajmalicine production in hairy root cultures. Similar results in cell suspension culture have been noticed.[40] Hairy root culture is a unique system, often used for root specific indole alkaloids production[48] Recently,[49] have observed an increase in growth and terpenoids indole alkaloids (ajmalicine and serpentine) yield when left and right termini-linked Ri T-DNA gene integration were made in hairy root cultures of *C. roseus*.

Phytohormones: The role of plant growth regulators in alkaloids production of *C. roseus* has been extensively studied, but the response varies with genetic makeup of the used explant, type and quantity of phytohormones.[33,50] The cytokinin applied exogenously either alone or in combination with auxins to suspension cultured cells enhanced alkaloids accumulation in tumorous and non-tumorous cell lines.[51,52] Enzyme peroxidase play a significant role in alkaloids biosynthesis, addition of 2,4-D to the culture medium however, reduced the peroxidase activity.[53]

An increase,[54] in vindoline and catharanthine concentration by using 0.1 mg/l BAP and 0.1 mg/l NAA added MS medium had been reported. Exogenously supplied cytokinin increased ajmalicine and serpentine content in untransformed callus from cotyledons[55]. At the protein level it was shown that endogenously produced cytokinin did not mimic the effect of exogenously applied cytokinin in *Catharanthus*,[56] and they also noticed that the protein pattern of Ipt transgenic callus lines were insensitive to exogenously used cytokinin. A28 KD polypeptide and simultaneous Ajmalicine accumulation was noted on omission of 2, 4-D in medium and by the use of NaCl treatments.[57, 58]

pH *of Culture Medium:* *In-vitro* biomass and alkaloid production are directly influenced by the pH values of the medium; values with a range of 5.5-6.5 did not have much effect on alkaloids yield. The value 5.5 was found to be optimum for serpentine production.[59] It has been[60] reported that alkaloids produced by suspension culture were stored in vacuole and simultaneously storage capacity changed as the changes of pH in the medium and vacuole take place. Low and higher values of pH were used to release intracellular alkaloids into the culture medium.[61] It is quite known that the optimized value (5.5-5.8) occasionally fluctuates during culture time and influences *in-vitro* responses including alkaloid yield.

Temperature: For *in-vitro* study temperature range from 20-30 has been considered best for better biomass and growth of cultures, but contradictory information have been reported about the alkaloids yield. Temperature in low range had inhibitory[62], stimulatory[63], and no effect on alkaloid yield. In the tested cell lines under different temperature range (20,25,30°C), highest serpentine production was[64] recorded at 25°C and, no effect was recorded at temperature 17,23 and 32°C while in hair y root culture low temperature enhanced alkaloid yield.[65]

Light: Light is an important factor for both *ex vitro* and *in-vitro* morphogenetic study. Its presence, absence, time and intensity directly influence anabolic and catabolic processes, particularly secondary metabolism.[66,67] Most of the study of the effect of light was made on serpentine and ajmalicine where serpentine content was directly related to the intensity of light in *Catharanthus*[68], same was true for vindoline[69] and however, another alkaloid catharanthine was decreased in the absence of light. But it has also been reported[70], that light did not affect yield but it affect the accumulation site. However, 15h per day exposure instead of 24 improved serpentine accumulation. Although, dark-grown culture was much better in comparison to light grown, where serpentine and ajmalicine content were decreased (serpentine from 79%-14% and ajmalicine 78%-18%). Gradual transfer of

dark grown culture of *Catharanthus* towards the light increased serpentine content, however, continuous exposure of light decreased serpentine level.[64] It has been optimized that 12h light period[54], for better callus growth and alkaloid production, however, dark period more than 12h decreased alkaloid contents. It has been found[71], that an increased chloroplast number and enhanced chlorophyll accumulation in response to light influenced serpentine production. Besides, exposure of monochromic light such as blue (450 nm) or red (670 nm) did not affect growth and alkaloid accumulation, showed constant ajmalicine and serpentine synthesis which decreased further under white light.[54, 71]

Aeration: Different types of gases, mainly CO_2 and ethylene, are usually evolved within the culture. In many cases these gases reduce O_2 level in close vessels, inhibit plant culture growth and secondary metabolism. High dissolved oxygen and improved gaseous permeability at aerated condition stimulated secondary metabolism as observed by [72], when ajmalicine production was increased with high oxygen level. Improved oxidative metabolism at rich O_2 level is believed to be the reason for better product conversion. Aeration has been provided in culture to influence the alkaloids synthesis and to make it more efficient modern stirring devices have been employed along with traditional shake flask.[73,64,74,76,77] Different types of fermenters have also been used; shikonin and ginseng, the two important secondary metabolites have been commercially produced by the use of fermenters. Several researchers[78, 79] have suggested the use of bioreactors in secondary metabolites production in plant cell culture of *Catharanthus*. An impeller with a speed of 100 rpm was most appropriate for the accumulation of alkaloids**;** however, higher impeller speed increased callus/suspension growth. The rate of ajmalicine production was studied[80] by using different vessels including shake flask and bioreactors. He found that biomass was not affected by different culture vessels; however, ajmalicine production was decreased with over feeding of biomass in shake flask and fermentor.

Elicitors*:* New groups of triggering factors which are better known as elicitors have been reported to stimulate the secondary metabolites.[81] The substance used as elicitors may be of biotic and abiotic in origin. Biotic elicitors include microbial filtrates (Yeast, *Pythium* and other fungal filtrate), while abiotic elicitors comprise of simple inorganic and organic molecules (vanadyl sulphate, oxalate, UV irradiation etc.).

It has been reported[82] that addition of *Phytium aphanidermatum* filtrate increased the accumulation of phenolic compounds instead of alkaloids production. Effect of different concentrations of *Pythium vexans* extract was studied by[83], who had noticed that low elicitor concentration increased serpentine production but no effect was on catharanthine yield. Addition of nicotinamide (8.2 mm) in *C. roseus* cell lines was used to enhance the anthocyanin accumulation.[84] The extract of *Pythium aphanidermatum* in a hormone free cell lines responded well and induced enzymes {(TDC and anthranilate synthase (AS)} which catalyse the biosynthesis of several intermediates and subsequently accumulated tryptamine.[34] Several inorganic compounds (sodium chloride, potassium chloride and sorbitol) had also a positive effect on catharanthine accumulation.[85]

The addition of vanadyl sulphate[86] to cell suspension culture increased catharanthine, serpentine and tryptamine production but was concentration dependent. At 25 ppm, catharanthine and ajmalicine were primarily accumulated, and at 50-75 ppm tryptamine accumulation was only noticed. Moreover, the effect of heavy metal was studied[87] where addition of copper (200μm) increased total indole alkaloid accumulation which was correlated with decreased tryptamine concentration.

Several stress factors (fungal elicitor, vanadyl sulphate and potassium chloride) were used and it was found that the alkaloids accumulation was concentration dependent[88] The optimal concentration (29,1.45 and 145 mg g-l dry weight) of fungal elicitor, vanadyl sulphate and potassium chloride into medium increased alkaloids accumulation, however, higher concentration had toxic effects and resulted in the loss of cell viability. Two fold increase in alkaloids yield was noticed added tryptophan, fungal elicitor and vanadyl sulphate to the culture production medium.[89]

Exposure of 2,2-azobis dehydrochloride (AAPH, an oxidative stress agent) and UVB irradiation to *C. roseus* increased nicotinamide and trigolline content.[90] Simultaneously phenylalanine ammonia lysate (PAL) activity was also increased. The increase in PAL activit y caused by 2μm AAPH was prevented by 0.1 mm 3-amino benzomide, which is an inhibitor of poly (ADP-ribose) polymerase. This suggests that nicotinamide and its metabolites function as signal transmitter in response to the oxidative stress, since poly-polymerase has defensive metabolic functions. The level of vinblistine and leurosine increased in response to irradiation with near (370 nm) ultraviolet light[91, 92] in shoot culture of *C. roseus*; however, catharanthine and vindoline content were decreased. Leaves were more sensitive to dimeric alkaloid accumulation in comparison to shoot, however,[93] near ultraviolet's irradiation in whole plant of *C. roseus,* accumulation of dimeric alkaloids was increased.

Yeast extract induces transcription of the biosynthetic gene encoding strictosidine (STR) in cultured *C. roseus* cells and alkalinization of the culture medium. The active principle from yeast extract was partially purified and found to be of a proteinacious in nature.[94] Age of culture is very important factor for the elicitor's to be effective[34, 95]; addition of elicitors is preferred after a few days of inoculation of the culture when the cells are rapidly dividing.

Mutagenesis*:* Mutagenesis plays a potent role in the alteration of the genetic constitution which leads to produce new varieties. *Penicillium* is the most classic example, with many other successful cases. Process of mutagenesis in diploid plants is very complex. Mutagenesis enhance alkaloids yield but the route of biosynthesis and the necessary regulation procedure are not elucidated yet clearly. Therefore, mutation at target site in duplicate is really difficult. In spite of several limitations in this process, scientists in numbers have used mutagens.[96] Some p-flurophynylalanine resistant cell lines of *Nicotiana tabacum and* N. *glauca* accumulated higher level of phenolics.[96] In case of *Catharanthus,* he noticed that a tryptophan analog resistant mutant accumulated catharanthine in both growth and production medium. Similarly several research groups used x-rays where more serpentine was produced. Beside these examples, some successful reports are available in other group of crops where mutagenesis improved

metabolic accumulation.

High Cell Density Culture: In order to increase secondary metabolites production, high cell density culture feeding has been attempted with or without much success. Study on *Catharanthus* in relation to high cell density was found to be unsuccessful. Ajmalicine production was very low when inoculam potential was increased to 2:8 from 1:9 mg/g.[70] Moreover,[97] low-density cultures increased alkaloids yields. It has also been remarked[72] that low oxygen level and inadequate nutrient uptake are among the possible causes for low metabolic accumulation during high cell density culture.

Selection of Superior Cell Lines: Isolation and selection of superior lines from the heterogeneous cell populations help to improve the yield of alkaloids. These cells show genetic variability which was further diversified by the use of various mutagenic agents. Ajmalicine and serpentine level were increased in *Catharanthus* by the selection of superior cell lines.[98]

Bioreactor and Immobilization: In tissue culture, for alkaloids production research has been mainly focused on suspension culture which requires a rotatory shaker. For large-scale production, however, large size culture vessel fermenter/bioreactor is most important. In both types of systems a stirring devices is provided for improved aeration.[70,99,100] There are several important vessels fitted with compressors which provide filtered air. For plant culture growth and productivity, it is recommended that bioreactors with low shear stress are much more suitable than those of high shear stress. Bioreactors with improved mechanical designs are regularly introduced in bioreactors industry with innovated impeller which helps to regulate shear agitation.[101]

In *Catharanthus,* immobilization of plants cells has been suggested for better accumulation of terpenoids.[61,102,103,104] Immobilization not only maintains the cells viable for a longer period of time but also helps in extracellular alkaloids accumulation. Alginate mediated immobilized cells enhanced the accumulation of tryptamide, ajmalicine and serpentine.[98,105] The use of agar and agarose are found to be effective for long-term maintenance of cells. In the last few years surface immobilization has been proposed using different types of matrices for large- scale production of alkaloids.[106, 107] In some other cases, negative influence of immobilization on cell was noticed;[104] gel or matrices entrapment on polysaccharide sheet is employed in many plant systems and in *Catharanthus* it is fairly successful.

Hairy Root Culture: Root contains a variety of secondary metabolites which produce alkaloids. High rooting can be induced by genetic transformation using *Agrobacterium rhizogenes.* Induced roots grew with a faster rate in hormone free medium with high accumulation of secondary metabolites in *Catharanthus.*[66] In transgenic *Catharanthus* root, a significant increase in ajmalicine and catharanthine was noticed.[49,108] Other groups used various types of bioreactors/fermenters to improve the growth of hairy roots and then for better production of secondary metabolites.[109, 110]

In-vitro Somatic Embryogenesis: Although somatic embryogenesis (SE) has been reported in a wide variety of plant genera;[111,112] in *Catharanthus roseus* it has been reported for the first time.[113] Earlier, a preliminarily study on plant regeneration from immature zygotic embryo was reported in *Catharanthus.*[114] The advantage of SE is that the initial cell populations can be used as a single cellular system and their genetic manipulation are easy and are similar to microorganisms.

BIOSYNTHESIS OF ALKALOIDS IN *C. roseus*

Beside alkaloids, many other secondary metabolites have been isolated from *C. roseus*, which include monoterpenoids, glucosides (loganin, secologanin, deoxyloganin, dehydrologanin) steroids (catasteron, brassinolides), phenolics, flavaonoids and anthocyanins. Metabolites are in fact the end products of a complex process comprising the involvement of several enzymes, genes, regulatory genes and (transport through) intra-and inter-cellular compartments. The TIA (terpenoids indole alkaloids) are condensation products of two biosynthetic routes which require coordination of the amount of the intermediates supplied by both pathways. The biosynthesis of vinblastine requires the participation of at least 35 intermediates, 30 enzymes, 30 biosynthetic agents, 2 regulatory genes and 7 intra and intercellular compartments.

The first study on the biosynthesis of alkaloids was performed at the end of the 1950s for *Catharanthus*. Plants were grown in an atmosphere containing $^{14}CO_2$ and after the extraction of alkaloids; many labeled alkaloids have been detected by using column and paper chromatography. Among the isolated alkaloids vinblastine and vincristine were found only in a very low quantity. Thereafter, to increase the level of vincristine and vinblastine, cell cultures of *Catharanthus* were used. Biosynthesis of alkaloids by *in vitro* cell culture has the advantages to manipulate the physiological (rapid growth, ease of precursor feeding, etc) and genetical process. During the biosynthesis of alkaloids of *C roseus* various types of proteinaceous compounds have been reported in different biosynthetic pathways (Table **3**).

Table 3. List of proteins isolated during the alkaloids biosynthesis in *Catharanthus roseus.*

Name	Abbreviation	References
Proteins involved in regulation		
Octadecanoid-responsive *Catharanthus*	ORCA2	94
Octadecanoid-responsive *Catharanthus*	ORCA3	172
Box P Binding Factor	CrBPF-1	173
Proteins from the indole pathway		
anthranilate synthase	ASA	134
chorismate mutase	CM	134
isochorismate synthase	ICS	174
tryptophan decarboxylase	TDS	174
Proteins from the terpenoids pathway		
1-deoxy-D-xylulose -5-phosphate reductoisomerase	DXR	175
2C-methyl-D-erythritol -2,4-cyclodiphosphate synthase	MECS	175
1-deoxy-D-xylulose -5-phosphate synthetase	DXPS	176
mevalonate kinase	MK	177
Phosphomevalonate kinase	PMK	178
Proteins involved in secologanin biosynthesis		
geraniol 10-hydroxylase	G10H	124
cytochrome P 450 reductase	CPR	124
secologanin synthase	SCS	179
Proteins involved in the biosynthesis of TIAs		
strictosidine synthase	STR	180
strictosidine β-D-glucosidase	SGD	181
tabersonine 11-hydroxylase	T11H	182

S-adenosyl-L-methionine : 16-hydroxytabersonine *O*-	OMT	182
S-adenosyl-L-methionine: 2,3-dihydro-3-hydroxytabersonine-***N***-methyltransferase	NMT	183
desacetoxyvindoline-17-hydroxylase	D17H	184
Acetyl CoA: 17-*O*-deacetylvindoline 17- *O*-acetyltransferase	DAT	185
Acetyl CoA: minovincinine-*O*-acetyltransferase	MAT	186

METABOLIC AND GENETIC ENGINEERING IN ALKALOIDS BIOSYNTHESIS

In alkaloids biosynthesis the role of several enzymes have been discussed in *Catharanthus roseus*, a few of them have been purified, identified, characterized and their encoding genes were also cloned (Table **4 ,5**).

The alkaloids biosynthesis is a very complex process arise from the precursor's tryptamine and secologanin. These two precursors are derived from two different pathways.

Table 4. Metabolites from cell and tissue culture of *C.roseus*

S.N.	Class	Compound	Reference
1	Tryptophan derivatives	Tryptamine, N, N-dimethyltryptamine, N-acetyltryptamine	187, 188
2	Phenolics	dehydrodiconiferyl glucosides, 2,3-dihydroxybenzoic acid	189, 82, 190, 191
3	Anthocyanins	petunidine malvidin hirsutidin	189
4	Steroids	Campesterol, sitosterol, stigmasterol, cholesterol, isofucosterol, 24-methylene-cholesterol, brassinolide catasterone, α –amyrin, β- amyrin, ursolic acid	192, 193
5	Fatty acids	Palmitic acid, Oleic acid, Linoleic acid	188

Table 5.Enzymes involved in indole alkaloids biosynthesis in *Catharanthus roseus*

Enzymes	Abbreviation	Localization	Reference
Glutamine synthetase	GS	Cytosol, Chloroplast	194
Anthranilate synthetase	AS	-	195
Geraniol 10- hydroxylase	G10H	Provacuolar membranes	196
NADPH-cytochrome P-450 reductase	---	Provacuolar membranes	196, 197
SAM: loganic acid methyltransferase	LAMT	-	198
Trptophan decarboxylase	TDC	Cytosol	121, 199
Strictosidine synthase	SS	Vacuole	200, 201, 122
Strictosidine β- glucosidase	SG	Tonoplast	127
Geissoschizine	--	-	202
Cathenamine	--	-	203
Tetrahydroalastonin e synthase	--	-	130
Peroxidase	--	-	204
SAM: methoxy2,16-dihydro-16- hydrotabersonine-N-methyltransferase	NMT	Vacuole Thylakoid membrance	183
desacetoxyvindoline-4-hydroxylase	OHT	Cytoplasm	205, 206
AcetylCoA: deacetylvindoline, dd 17-0-acetyl- transferase	DAT	Cytoplasm	207

Tryptamine is formed by the enzyme tryptophan decarboxylase (TDC), which has been reviewed earlier by various workers[115,116,117] while the strictosidine synthetase (SSS) helps in the coupling of tryptamine and secologanin to produce strictosidine.[118,119] The other enzymes such as geraniol 10-hydroxylase (G10H), NADPH-cytochrome P-450 reductase, anthranilate synthetase (AS) have the similar TDC activities which are involved in the biosynthesis of indole alkaloids.[120] The TDC enzyme has been purified from cell suspension culture[121] and ultimately the cDNA gene was established.[122] The cytochrome P450 enzyme, geraniol-10-hydroxylase (G10H) and other enzymes have been studied extensively from intact plant of *C. roseus*. By HPLC study[123] and selection of a cell line with high G10H activity;[124] the enzyme was purified to homogeneity.[123] Based on the internal amino acid sequences obtained from the digested protein, gene was cloned and functionally expressed in yeast. The enzyme belongs to the CYP76B subfamily and is designated as CYP76B6. The activity of this enzyme was induced by treating the cells with the cytochrome P450 inducer phenobarbital and decreased after treatment of the inhibitor ketoconazole.[125] Besides, many other enzymes have been identified and characterized that metabolize strictosidine, which after undergoing several rearrangements produced cathenamine and ajmalicine.[126,127] An another important enzyme is desacetoxyvindoline-4-hydroxylase (DAVH), active during vindoline biosynthesis, purified from intact plant of *C. roseus*. The native enzyme is a monomer, has a molecular weight 45KD with three isoforms.[128]

Recently, attention has been paid on the regulation of mevalonate biosynthesis that terminates with the end product strictosidine. The encoding genes and enzymes of different steps of mevalonate pathway have been elucidated.[129] After the formation of strictosidine, first step of alkaloid biosynthesis is the removal of sugar moiety from strictosidine to form an unstable aglycone. Two strictosidine β-glucosidases (SG) were partially purified and characterized from *C.roseus* cell cultures.[130, 127]

Feeding of terpenoids precursors to *C. roseus* cell suspension cultures increased alkaloids production.[131-133] Addition of trytophan (0.5 Mm) to *Catharanthus* cells resulted in high intracellular levels of tryptamine and an increase in STR activity but did not influence ajmalicine accumulation much.[134] As in other feedback inhibitions, product accumulation depend upon the product degradation and this phenomenon has been reported in cell suspension culture of *Catharanthus*. It is now known that the precursor for alkaloids (tryptophan to tryptamide) was located in the cytosol whereas the enzyme SSS has been localized in the vacuole.[135]

COUPLING METHODS FOR ALKALOIDS BIOSYNTHESIS

The bisindoles are derived from the coupling of vindoline and catharanthine. Catharanthine is thought to be derived

from strictosidine via 4,21-dehydrogeissoschizine, stemmadenine and dehydrosecodine while vindoline is derived from strictosidine via stemmadenine and tabersonine. This pathway (transformation of tabersonine to vindoline) has got orderly six reactions.[69,136] The enzyme anhydrovinblastine synthase couples catharanthine and vindoline to yield AVLB which was purified and characterized from *C. roseus* leaves. This heme protein has a molecular weight of 45 KD and shows the peroxidase activity. During this enzymatic coupling both the monomers were incubated at 30°C with cultured *C.roseus* cells as enzymatic source at acidic pH (tris buffer 7.0). Only after 3 h the chemical reaction produced vinblistine and anhydrovinblastine as major products along with other dimeric alkaloids. Vindoline and catharanthine were also nonenzymatically coupled to the dihydropyridinium intermediate (DHPI) under near- UV light irradiation with a peak at 370 nm in the presence of flavin mononucleotide. Subsequently DHPI can be reduced to anhydrovinblastin (AVLB) with an overall yield of 50%, based on initial amount of vindoline. Vinblastine content was further improved up to 50% by using various compounds as stimulants.[137] Similarly, vincristine can be isolated from vinblastine by chemical conversion. Two routes are employed; first route is the isolation of *N*-deformyl-VCR which further converted into vincristine by formylation. The second method involves a formylation of the *C.roseus* extract in which conversion of *N*-deformyl – VLB to VCR takes place, after which the material is oxidized. In both cases vincristine was purified by column chromatography and then sulfated (Lily medicinal information service, Netherland).

It was also reported that $MnCl_2$ and FMN/FAD stimulated coupling process. However, in the absence of *C. roseus* cell suspension enzymes, ferric acid stimulated coupling process. The production of vinblastine through enzymatic coupling pathway is thought to be highly efficient and likely to be used commercially very soon.

Vindoline and bisindole alkaloids are accumulated only in green tissue and not found in root and cell suspension cultures.[138] The developmental regulation of TDS, SSC and the enzymes involved in late steps of vindoline biosynthesis has been studied extensively.[69.139.140] In seedlings of *C.roseus*, *expression* of these enzymes was not under strong developmental control where enzymes activity were modulated by tissue specific or light dependent factors. The concentration of vindoline, catharanthine and 3,'4'-anhydrovinblastine (AVLB) are age dependent.[141] Vinblastine increased as seedlings matured, reaching a steady concentration when the plants become more than three months old. On an average, whole seedlings, young plants and mature plants contained 7, 11.5 and 12-µg/g dry weight VLB, respectively. After induction of shoot formation the VLB contents increased rapidly to similar levels of *in vitro* seedlings.[142]

CELLULAR COMPARTMENTATION

Subcellular compartmentation plays an important role in alkaloids metabolism. This process of metabolism involves the participation of plant cell to separate the enzyme from their substrates and end products. In this, alkaloids biosynthesis requires three celluler compartments, namely vacuole, cytosol, and plastid.[143]

The transformation of tryptophan into tryptamine takes place in cytosol[139,135], SSS in vacuoles.[135,144] SG was tightly bound to the tonoplast boundary.[135] Synthesis of strictosidine takes place inside the vacuole which later transported to the cytoplasm where its glucose moiety detached. Ajmalicine has the potentiality to move freely across the cell membrane and accumulated into the vacuoles here it converted into the serpentine by use of peroxidases[145], produced serpentine stored in vacuole and cannot pass the tonoplast. In cell suspension cultures alkaloid accumulation seems to be restricted to certain cells.[61] Permeability of cell plays a potent role to release plant products. There are several permeabilizing agents like DMSO and Triton X-100 are found to be very effective in *Catharanthus* cell culture. Besides, for the release of secondary products several other agents (chitosan, alginate beads, electroportion and ultra sonication) have been used with or without cell viability in other groups of plants.

The cell membrane with active uptake mechanism has also been noticed in *Catharanthus*. Most of the secondary products are generally accumulated intercellular, however several compounds such as taxol, anthraquinones are identified in the media which filtrate itself through membrane. For this extracellular product secretion, addition of resin XAD-7 enhanced the product adsorption in *Cinchona*[33]. The media provided with amberlite type resin and XAD-7 resin adsorbed ajmalicine and catharanthine effectively in *C. roseus*.

CONCLUSION

Different pharmacological studies and the traditional used proved the high medicinal properties of the *Catharanthus*; which continuously being used in the treatments of numbers diseases. Various important alkaloid, mostly the monomers were successfully identified in culture media with the enhanced yields; however the commercial production is still far away. The main problem is due to the lack of optimization of cultural conditions and several strategies leading with increased accumulation of secondary metabolites. A detail studies are required to know the proper enzyme functions at various levels, product membrane permeability and adsorption for improvements towards achieving a viable economic production methodology. In addition, over-expression of enzymes and the genetic modification could be very useful via organogenesis or somatic embryogenesis for the production of desired levels of secondary metabolite.

ACKNOWLEDGEMENTS

First author (Dr. Junaid Aslam) greatfully acknowledge Professor S.A. Khan for their valuable suggestions during the preparation of the manuscript.

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