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Full Length Research Paper

High *IN VITRO* production of ant-canceric indole alkaloids from periwinkle (*CATHARANTHUS ROSEUS*) tissue culture

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Periwinkle (*CATHARANTHUS ROSEUS*) is one of the most important medical and ornamental plants in the world. In this investigation, periwinkle seeds, after sterilization were cultured on MS medium. Petiole segments of seedlings (4 day old) were subcultured to medium containing various concentrations of NAA accompanied with Kin and subcultured to regenerate the callus and root. Callus and roots were obtained from petioles in some of treatments. The extracts of callus and roots from different treatments were analyzed by spectrophotometer, TLC and HPLC with respect to the indole alkaloids producing capacity. Alkaloids were produced callus and roots from petiole of *C. ROSEUS* in the presence of 0.1, 5, 10 and 20 mg/l Kin and NAA. MS with 0.1 mg/l NAA + 0.1 mg/l Kin had the highest vindoline, catharanthine, vincristine and root organogenesis capacity. But the level of these alkaloids and ajmalicine were very low compared to that in petiole of intact plant, and the level of serpentine was similar. New roots, callus roots, and callus from MS medium containing 0.1 mg/l NAA + 0.1 mg/l Kin were subcultured in hormone-free and 0.1 mg/l NAA + 0.1 mg/l Kin media and for organogenesis and growth. The most alkaloids amount was produced in new roots and callus roots. The indole alkaloid levels of new roots in new media were higher than in petioles of intact plants. In this study, 10-fold catharanthine, 125-fold serpentine, 0.5-fold vindoline and 0.34-fold ajmalicine were produced by new roots. The most interesting result was presentation of two important ant-cancer dimeric alkaloids, vinblastine and vincristine with amounts of 20-fold vinblastine and 6-fold vincristine to compare that in the petioles of intact plants.

Key words: vindoline, vincristin, vinblastine.

INTRODUCTION

The periwinkle, *Catharanthus roseus* L. (G.) Don, produces several commercially valuable secondary metabolites including the anticancer agent, vinblastine, vincristine and the hypertension drugs, ajmalicine and serpentine. Bioengineering efforts to synthesize these indole alkaloids in plant tissue cultures of *C. roseus* have yielded varying responses, with serpentine and ajmalicine being most amenable to *in vitro* manipulations (Moreno et al., 1995; Stern, 2000; Gragg and Newman, 2005). These are dimeric indole alkaloids that are formed *in vivo* by condensation of vindoline and catharanthine. The low yield of dimeric indole alkaloids from the plant (approximately 0.0005%) and their consequent high price have stimulated numerous efforts to develop alter-native strategies for their production (Kurz et al., 1985; Petiard et al., 1985; De Luca and Kurz, 1988, Wijese-kera, 1991; Ebrahimzadeh et al., 1996). The biosynthesis of vinblastine and vincristine in tissue culture systems has been largely elusive, due to the inability to synthesize vindo-

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Abbreviations: NAA, Naphthalen acetic acid; **Kin**, Kinetin; **MS**, Murashige and Skoog; **HPLC**, high performance liquid chromatography; **TLC**, thin layer chromatography.

Table 1. Callus (Ca) and root (R) production in Medium 1 (MS media with different concentration of NAA (mg/l) with kin (mg/l)).

N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
NAA*	0.1	0.1	0.1	0.1	5	5	5	5	10	10	10	10	20	20	20	20
Kin**	0.1	5	10	20	0.1	5	10	20	0.1	5	10	20	0.1	5	10	20
%Ca	15	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
%R	85	60	9	95	100	15	-	-	50	100	-	-	15	100	-	-

*α-Naphthalene acetic acid, **kinetin.

line, one of its precursors (O'Keefe et al., 1997). Despite these intense efforts, attempts to produce antitumor alkaloids from *C. roseus* cell cultures have failed (reviewed by Van der Heijden et al., 1989) and have been attributed to their inability to synthesize the precursor vindoline. Only recently, vindoline has been reported in transformed cell cultures of *C. roseus,* albeit at low levels (O'Keefe et al., 1997). Factors such as tissue differentiation (Constabel et al., 1982; Hirata et al., 1987), light-activated regulation and/or development (De Carolis et al., 1990; De Luca et al., 1988), or both (Hirata et al., 1990; Loyola-Vargas et al., 1992; Tyler et al., 1986; Vasquez-Flota et al., 1997), are considered important to the activity of the biosynthetic pathway to vindoline.

To investigate bottlenecks in the ability of tissue culture systems of *C. roseus* to synthesize vindoline, one approach is to elucidate constitutive levels of precursors, and pathways that divert the desired biosynthetic ability, and then follow responses of the metabolism to perturbbations. Tabersonine, a key precursor of vindoline, has been reported occasionally in tissue cultures (Stockigt and Soll, 1980; Toivonen et al., 1989), and only recently measured extensively in *C. roseus* hairy root cultures (Bhadra and Shanks, 1997). However, the ability of certain cell lines to accumulate high levels of catharanthine and serpentine indicates that they have a high potential for the production of some monoterpenoid indole alkaloids (Kutney et al., 1980a; Stockigt and Soll, 1980; Deus-Neumann and Zenk, 1984). Here, we report that vinblastine and vincristine can increase in new roots from tissue culture of *C. roseus* petioles of intact plants.

MATERIALS AND METHODS

Plant material and induction of callus and roots cultures

The callus and roots cultures were induced from leaves petiole segments of *C. roseus* (Dixon 1987). Seeds of *C. roseus* were obtained from Tehran parks organ green house. Leaves of six week old seedlings, germinated in green house were used, and petiole segments (ca.2 cm) were cut out and used for initiation of tissue cultures. Petiole segments were sterilized by hypochlorite 5% for 45 min and were washed by sterile water. The segments were cultured on MS agar medium (Murashige and Skoog, 1962), supplemented with different concentration of a naphthalene acetic acid (NAA) and kinetin (Kin) treatments (Medium 1: number 1 - 16 of Table 1) and were placed in a growth chamber at dark period for first of two weeks and 24 h light period (fluorescent, cool white light, 7 W/m^2) for second of 4 weeks at 35ºC. The samples of treatment of N.1 (0.1 mg/l NAA + 0.1 mg/l Kin) and N.14 (20 mg/l NAA + 5 mg/l Kin) (Table 1) were sub cultured to MS medium containing 0.1 mg/l of NAA supplemented with 0.1 mg/l Kin, 24 h light period (Medium 2: N. 17, 18 and 19 of Table 6) and hormone-free MS (MS0) (Medium 2: N. 20, 21, 22 and 23 of Table 6). The samples were used for alkaloid assay after 6 weeks.

Alkaloids extraction

New callus, root-callus and roots from 3-5 g fresh weight were homogenized (1 min in 60-100 ml ethanol) and were set in bathroom 60ºC for 10 min. Following filtrated, alkaloids were extracted purified according modified method of Renaudin (1984) as described by Miura et al. (1987). For purification, ethanolic extracts were dried by vacuum evaporator at 60ºC and used. Alkaloids isolation stages were: 1. Acidic phase was isolated by sulphuric acid (5%) and diethelic ether (50/50; v/v) in a decanter. 2. Acidic phase separated and made basic (pH 10) with 10 N NaOH and extracted with 60 - 100 ml chloroform in decanter. 3. Chloroformic phase were concentrated by vacuum evaporator at 60ºC. The resulting alkaloids extracts were dissolved in 1 ml ethanol used for alkaloids assay.

Alkaloids assay

The existence and content of vindoline (vin), catharanthine (cat), vinblastine (vib), vincristine (vcr), ajmalicine (ajc) and serpentine (ser) of callus and roots analyzed by TLC and HPLC at 6 weeks after the initiation of each culture (Table 1). The petiole alkaloids of intact plants (1.5 month old) were also analyzed for comparison.

TLC

Aliquots of 50∝l alkaloids were applied onto thin layer chromatography (TLC) plates. TLC solvent systems routinely used was ethyacetate: ethanol (4:1) and TLC plates were formed by silica gel G60 (Merck). Alkaloids were identified using TLC and the color reaction with Ceric Ammonium Sulfate (CAS) and Ultra violet (UV) detection (λ=254 and 366 nm). The mobility and characters of each of alkaloids analyzed by TLC were compared with alkaloid standards and detected by their RF, colors reaction with CAS and UV detector (λ =254, and 366 nm). The standards were from Fluka and 0.0025 mg from each of six standard alkaloids were dissolved in 1 ml ethanol and used for alkaloids assay.

The standards TLC results showed that ajmalicine (RF= 0.95) color with λ =366 nm and CAS detergent is apple-green; serpentine tartrate (RF= 0.1) color with λ =366, 254 nm was blue and it has no color with CAS detergent; vinblastine (RF= 0.35) and vincristin (RF= 0.25) sulfate color with λ =366 were colorless, λ =254 nm

Table 2. Removing factor (RF) and standards alkaloids color reaction by TLC analysis. Color of alkaloids with UV (λ =366, λ =254) and ceric ammonium sulfate (CAS) reagent.

Alkaloid	RF	λ =366	λ =254	CAS
Serpentine	0.1	Blue	Blue	Non
Vincristine	0.25	Non	Dark	Lavander
Vinblastine	0.35	Non	Dark	Lavander
Cataranthine	0.75	Non	Dark	Blue
Vindoline	0.85	Non	Dark	Dark-Lavander
Ajmalicine	0.95	Apple-green	Dark	Apple-green

Table 3. Retention time and concentration for some standards alkaloids by HPLC analyze.

Alkaloid	Retention time (min)	Concentration (mg)
Serpentine	20.525	2.5×10^{-3}
Vincristine	24.2 5	2.5×10^{-3}
Vinblastine	28.290	2.5×10^{-3}
Cataranthine	32.33	2.5×10^{-3}
Vindoline	35.36	2.5×10^{-3}
Ajmalicine	41.42	2.5×10^{-3}

were dark and they have lavender color with CAS detergent; catharanthine (RF= 0.75) and vindoline (RF= 0.85) color with λ =366 and λ =254 nm were dark and with CAS detergent catharanthine was blue and vindoline was dark-Lavander. Catharanthine and vindoline were separated using 2 dimensional TLC according to modified methods of Morris et al as described by Dixon (1987) (Table 2).

HPLC

HPLC was performed with a Shimatzu LC-6A chromatography system using a programmed isocratic eluents made of acetonitrile and ammonium carbonate buffer pH 7.3 (1:1) on a reversed-phase column was ∝m bondpak C18, and 4.6 ∝m (15 cm × 2.5 mm, I.D) maintained at 30˚C. A guard column with 37-50 ∝m bondapak C18/CO reversed-phase protected the column against contamination from tissue extracts. In HPLC analysis, alkaloids were detected by retention time at 254 nm. Alkaloid color in TLC (with CAS and UV) and retention time of HPLC were compared with six standard alkaloids: retention time of vindoline (vin) 35.36 min, catharanthine (cat) 32.33 min, vinblastine (vib) 28.290 min, vincristin (vcr) 24.25 min, ajmalicine (aic) 41.42 min and serpentine (ser) 20.525 min with 2.5×10^{-3} mg from each (Table 3). In HPLC analyses, for obtaining carefully results, the 20 µl alkaloids were injected three times: without standard alkaloids, mixed with standard alkaloids and standard alkaloids without extracts. Retention times and area under of peaks were compared with a special rate of standards (Table 3).

RESULTS AND DISCUSSION

Callus and root induction

After six week incubation, callus and roots were produced by petiole segments in the presence 0.1, 5, 10 and 20 mg/l of NAA supplemented with 0.1, 5, 10 and 20 mg/l of Kin (Table 1). New roots regenerated in the some media containing: a. 0.1 - 5 mg/l of NAA with 0.1 mg/l of Kin (Figure 1a) and b. 5, 10, 20 mg/l of NAA with 5 mg/l of Kin (Figure 1b). Calluses were produced in all the treatments but in 0.1 mg/l of NAA with 0.1 mg/l of Kin (N.1) was low (15%). Root, callus and root-callus from N.1 and N.14 were subcultured in N.1 and MS0 media; these grew and produced new callus and roots (N. 17 - 23 of Table 6 and Figures 1c, d). Callus and roots were regenerated in N. 18, 19, 20, 21 and 23 of medium 2 but only callus in N.17 and root in N.18 of medium 2 were produced.

Alkaloid assay from petiole, root and callus alkaloids

Result of TLC showed the pattern of vindoline (vin), catharanthine (cat) and ajmalicine (aic) of roots and callus from MS media supplemented with 0.1 mg/l of NAA and 5, 10, and 20 mg/l of Kin, were similar to the pattern of petiole of intact plant (Table 4). HPLC analysis showed that the pattern of mobility and characters of vindoline, catharanthine, ajmalicine, vincristine (vcr) and serpentine (ser) of new roots of petioles in 0.1 mg/l of NAA is similar to the intact plant petioles but with lower levels of each (Table 5).

The result of TLC showed the pattern of vindoline, catharanthine and ajmalicine of roots and callus from the Callus (Ca), Callus-Rooting (CaR), Petiole-Rooting (PR)

Table 4. Results of TLC analysis: Detection of indole alkaloids in the new Organs formed from petiole (Numbers 6-16 contain only serpentine).

Alkaloid	Petiole	N1	N2	N3	N4	N5	N6
Serpentine	+	+	+	+	-	+	+
Vincristine	+	-	-	-	-	-	-
Vinblastine	+	-	-	-	-	-	-
Cataranthine	+	+	+	+	+	+	-
Vindoline	+	+	+	+	+	+	-
Ajmalicine	+	+	+	+	+	+	-

Table 5. Results of HPLC analysis: the relative amount of indole alkaloids in the new organs were formed from petiole (Table1).

Alkaloid	Petiole	N1	N2	N3	N4	N5
Serpentine	0.08	0.09	0.97	0.29	+	+
Vincristine	0.54	+	-	-	-	-
Vinblastine	0.085	+	-	-	-	-
Cataranthine	0.07	0.06	+	+	-	-
Vindoline	1.81	0.05	0.16	-	-	-
Ajmalicine	7.32	0.53	5.86	5.3	5.27	0.32

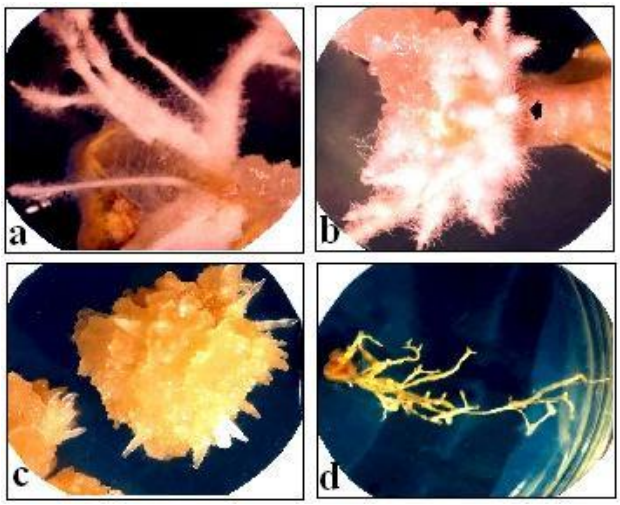

Figure 1. Root organogenesis from petiole on MS medium supplemented with NAA and Kin: A. 0.1 mg/l NAA + 0.1 mg/l kin, B. 5 mg/l NAA + 0.1 mg/l kin, C -callus was subcultured to hormone-free medium, D. roots was subcultured to hormone-free medium.

and Root (R) of N.17- 23 (Table 6) were similar to the pattern of petiole of intact plant. The existence of most usual indole alkaloids in the samples of N. 17 - 23 were confirmed by HPLC analyzes (Table 7). The production of vin and ajc from PR, Ca, R and CaR were low (0.44 to 0.9%) compared to that in petiole (1.8%). The level of vib (1.6%) and vcr (3.4%) of roots (N.22) were higher than that of petiole (0.085 and 0.54%) about 20 and 6 fold but in CaR (N. 23) was very low (Figure 2).

Our results confirmed that vib and vic are formed in new root cultures of *C. roseus* on free hormone MS medium, in light, at the temperature 35°C. Krueger and Carew (1982) and Moreno et al. (1995) reported very little vib and vic from tissue culture but production of these alkaloids by our cultures were greater than that in petiole of parent plant. Although the production of vin was lower, that of cat was high, 10 fold than that in petiole. The level of ser in roots and callus with 1.31 to 16.34%, was much greater than that of petioles with 0.08%. These results suggested that the capability to synthesize vib and vcr as well as that for vin and cat is associated with morphological differentiation, light and high temperature (35°C).

In this investigation, the level of vin (0 - 0.9%) and ajc (0 -5.8%) in all samples were less than that of petiole; vin = 1.81% and ajc = 7.32%. The amount of ser (0.09 to 16.34%) in all samples was much greater than that of petiole (0.08%). *C. roseus* leaves contain the demerit alkaloids such as vincristine and vinblastine in concentrations of 0.0004 to 0.0003% dry weight (d.w) which find use in cancer therapy (Wijesekera et al., 1991).

Our plants petioles contain 0.0005% vinblastine and 0.00324% vincristine in dry weight (d.w) or 0.000085% vinblastine and 0.00054% vincristine in fresh weight (f.w). Vinblastine of petiole was equal with that in Wijesekera et al. (1991) report, but vincristine was about 10 fold more than isolated by the authors. The root and basal stems are rich in monomeric alkaloids such as ajmalicine and serpentine,

Table 6. The growth and organogenesis of organs subculture.

N2	17	18	19	20	21	22	23
Med1	Ca 1	CaR 1	PR 1	Ca 1	R 1	R 1	CaR14
Med2	N.1	N.1	N. 1	MS0	MS0	MS0	MS0
New organs	Ca	CaR	CaR	CaR	CaR	R	CaR
Color	w	w	w	w-g	w-g	w-g	g-y

Med1: Subculturing organs from first media (Table 1)
Med2: Subculturing media
MS0: Hormone-free media (basal MS)
N1: Number 1 of Med1, N2: Numbers of Med2
New organs: new organ from Med2.
w: white, y: yellow, g: green, R: Root, Ca: Callus.

Table 7. Results HPLC analysis: the relative amount of indole alkaloids (vin, cat**,** Vbl, vcr, ajc and ser) in the samples of subculture after 6 weeks (Table 6).

Alkaloid	0	17	18	19	20	21	22	23
Serpentine	0.08	16.34	1.83	1.36	1.301	2.46	12.11	8..95
Vincristine	0.54	+	-	-	-	0.15	3.4	0.156
Vinblastine	0.085	+	0.2	-	0.032	0.26	1.66	0.1
Cataranthine	0.07	-	-	-	-	0.032	1.02	0.052
Vindoline	1.81	-	-	0.44	0.56	+	0..9	0.46
Ajmalicine	7.32	2.46	-	0.1	0.05	-	2.3	0.44

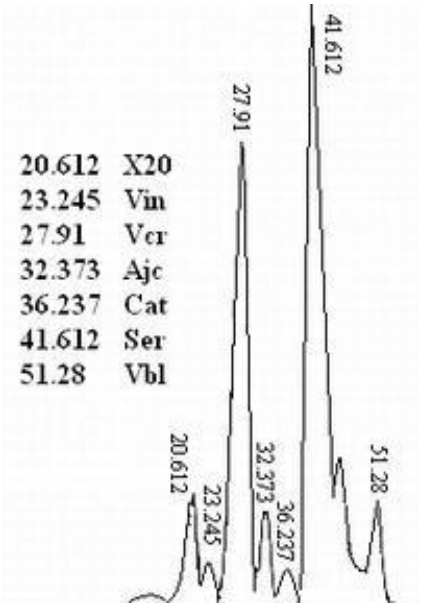

Figure 2. Alkaloid extracts chromatogram of new root (N.22, Table 7) in hormone-free medium; vin (23.245'),vcr (27.91'), ajc (32.373'), cat (36.237'), ser (41.612') and vbl (51.28').

0. 03 to 0.1%, which are employed to reduce high blood pressure (Wijesekera et al., 1991). Ajmalicine, 0.0439% (d.w), serpentine 0.00048% (d.w), ajmalicine, 0.0073% (f.w) and serpentine, 0.00008% (f.w) were obtained from our petiole. In our cultures serpentine was more than in petiole, but ajmalicin was low.

Vindoline, catharantine, vinblastine and vincristine were observed in our culture especially in new roots from petiole in hormone-free medium and light condition (N.22, Table 7) and vib, vcr and ser in this sample were 20, 6, and ,151 fold, respectively, more than from petiole but vin and ajc were low. Endo and Goodbody (1988) induced root and shoot cultures from seedlings of *C. roseus* and reported vinblastine and vindoline in shoots in light condition, with a very low level and Favretto et al., (2001) obtained vincristine and vinblastine in *C. roseus* from somatic embryogenesis. Our results are similar to the report of several authors (Krueger and Carew, 1982; Naaranlahti et al., 1895; Hu et al., 2001; Suk Weon Kim et al., 2004; Schneider, 2002). It has also been reported that vinblastine occur in callus culture with differentiated roots. Dimeric alkaloids occurr in tissue cultures with organogenesis, as well as in roots. The detection of high concentration of dimeric alkaloids in callus and root from petiole cultures indicate the possibility of the development of an efficient production system for vinblastine and vincristine on an industrial process.

In order to produce these useful anticancer drugs much more efficiently, many scientists have tried to apply plant tissue culture technology. In fact, a large number of papers related to this approach have been presented since the first research carried out (Vanisree et al., 2004). However, production of both alkaloids by *de novo*

synthesis using the callus or the suspension cultured cell of *C. roseus* is so far not promising because the productivity of the cultured cells reported so far was very low. But our results showed vindoline, catharantine, vinblastine and vincristine produced in tissue culture especially in new roots from petiole in free hormone media and light condition (N.22, Table 7) in high amount (vib, vcr and ser in this sample were 20, 6, and 151 fold, respectively, more than from petiole) but vin and ajc are low.

REFERENCES

Bhadra R, Shanks JV (1997). Transient studies of nutrient uptake, growth and indole alkaloid accumulation in heterotrophic cultures of hairy roots of *Catharanthus roseus.* Biotechnol. Bioeng. 55: 527-534.

Constabel F, Gaudet-La Prairie P, Kurz WGW, Kutney JP (1982). Alkaloid production in *Catharanthus roseus* cell cultures. XII. Biosynthetic capacity of callus from original explants and regenerated shoots. Plant Cell Rep., 1: 139-142.

De Carolis E, Chan F, Balsevich J, De Luca V (1990). Isolation and characterization of a 2-oxyglutarate dependent dioxygenase involved in the second-to-last step in vindoline biosynthesis. Plant Physiol. 94: 1323-1329.

De Luca V, Fernandez JA, Campbell D, Kurz WGW (1988) Developmental regulation of enzymes of indole alkaloid biosynthesis in *Catharanthus roseus.* Plant Physiol. 86: 447-450.

Dixon R A (1987) Plant cell and tissue culture, IRI Press, Oxford, 1-67

Ebrahimzadeh H, Ataei-Azimi A, Noori-Daloi MR (1996). The distribution of indole alkaloids in different organs of *Catharanthus roseus* G. Don. (*Vinca rosea* L, Daru, J. Sch. Pharm. 6(1&2): 11-24 Persian.

Endo T, Goodbody A (1988). Alkaloid production in root and shoot cultures, Planta. Med, 54: 470-482.

Favretto D, Piovan A, Filippini R, Caniato R (2001). Monitoring the production yields of vincristine and vinblastine in *Catharanthus roseus* from somatic embryogenesis. Semiquantitative determination by flow-injection electrospray ionization mass spectrometry, Rapid Communications in Mass Spectrometry, 15(5): 364-369.

Gragg GM, Newman DJ (2005) Plants as a source of anti-cancer agents, J. Ethnopharmacol, 100(1-2): 72-79.

Hirata K, Yamanaka A, Kurano N, Miyamoto K, Miura Y (1987). Production of indole alkaloids in multiple shoot culture of *Cathranthus roseus* (L). G. Don. Agric. Biol. Chem. 51: 1311-1317.

Krueger RJ, Carew DP (1982) Production of vindolin in root regenration of *Catharanthus roseus*, Planta Med, 45: 56-60.

Loyola-Vargas VM, Mendez-Zeel M, Monforte-Gonzalez M, Miranda-Ham ML (1992). Serpentine accumulation during greening in normal and tumor tissues of *Catharanthus roseus.* J. Plant Physiol, 140: 213-217.

Miura Y, Hirata K, Kurano N (1987). Formation of vinblastine in multiple shoot of *Catharanthus roseus*, Planta Med, 50: 18-20.

Moreno PRH, van der Heijden R, Verpoorte R (1995). Cell and tissue cultures of *Catharanthus roseus:* A literature survey. II. Updating from 1988 to (1993) Plant Cell Tiss. Organ Cult. 42: 1–25.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant 15: 473-497.

Naaranlahti T, Lapinjoki A, Toivonen L (19895). Mass spectral evidence of the occurrence of vindoline in *Catharanthus roseus* cell, planta med 5:155-57

O'Keefe BR, Mahady, GB, Gills JJ, Beecher CWW (1997). Stable vindoline production in transformed cell cultures of C*atharanthus roseus.* J. Natl. Prod. 60: 261-264.

Renaudin JP (1984). Reversed- phase High- performance liquid chromatographic , J. Chromat. 291: 165-174.

Rijhwani SK, Shanks JV (1998). Effects of elicitor dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* hairy root cultures. Biotechnol. Prog. 14: 442-449.

25-Schneider C (2002). First de novo synthesis of the bisindole alkaloid vinblastine, Angewandte Chemie 41(22): 4217-4219.

Stern S (2000). Introductory Plant Biology, 8th ed., McGraw-Hill CompaniesInc, pp. 238-247.

Stockigt J, Soll HJ (1980). Indole alkaloids from cell suspension cultures of *Catharanthus roseus* and *C. ovalis.* Planta Med. 40: 22-30.

Suk Weon Kim S, Su ID, Son Choi P, Liu RJ (2004). Plant Regeneration from Immature Zygotic Embryo-Derived Embryogenic Calluses and Cell Suspension Cultures of *Catharanthus roseus* ,Plant Cell, Tissue Organ Cult. 76(2): 131-135.

Toivonen L, Balsevich J, Kurz WGW (1989). Indole alkaloid production by hairy root cultures of *Catharanthus roseus.* Plant Cell Tissue Organ Cult. 18: 79–83.

Tyler RT, Kurz WGW, Panchuk BD (1986). Photoautotrophic cell suspension cultures of periwinkle (*Catharanthus roseus* (L.) G. Don): Transition from heterotrophic to photoautotrophic growth. Plant Cell Rep., 3: 195-198.

Vanisree M, Yue Lee C, Fung Lo S, Manohar Nalawade S, Yih Lin C, Sheng Tsay H (2004). Studies on the production of some important secondary metabolites, Bot. Bull. Acad. Sin. 45: 1-22

Vasquez-Flota F, De Carolis E, Alarco AM, De Luca V (1997). Molecular cloning and characterization of deacetoxyvindoline-4-hydroxylase, a 2-oxoglutarate dependent dioxygenase involved in the biosynthesis of vindoline in *Catharanthus roseus* (L.) G. Don. Plant Mol. Biol. 34: 935-948.

Wijesekera ROB (1991). The Medicinal plant industry, CRC press, pp. 52-217.