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GENETIC DIVERSITY AND ALKALOIDS PROFILE EVALUATION OF *CATHARANTHUS ROSEUS* L. BASED ON RAPD MOLECULAR MARKERS AND RP- HPLC ANALYSIS

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ABSTRACT

Catharanthus roseus is a medicinal plant, which main source of two antitumor terpeniods indole alkaloids (TIAs) vinblastine and vincristine. The aims of this study were to assess genetic diversity and alkaloids profiles using RAPD molecular markers and fast liquid chromatographic C18 column for the separation and identification of TIAs and their precursors for twelve *C. roseus* cultivars grown in Iraq. Genetic similarity between *C. roseus* cultivars ranged between 69% - 100%. Pacifica xp Burgundy Halo and Pacifica xp Cherry Red Halo had lowest genetic distance (0.0) with highest similarity value (100%).

The quantification of Vincristine, Vinblastine and their precursors Vindoline and Catharanthine of the same two cultivars had almost the same alkaloids profile with slight difference in Vindoline percentage. The dendrogram assembled on the basis of RAPD band showed that the Iraqi cultivar was isolated from the rest eleven cultivars. This research was the first attempt linking between genetic diversity and (TIAs) vinblastine and vincristine content.

Keywords: *Catharanthus roseus, RAPD, HPLC,* ***Alkaloids profile****,* TIAs

INTRODUCTION

Catharanthus roseus (L.) G. Don. (Vinca rosea) is one of the most interesting groups of ornamental plants in the world, considerable variation of different colored flowers can be observed. The plant has been put to in folkloric remedies for the treatment of a wide variety of diseases as diabetes (Ahmed *et al*., 2010) malaria(Gathirwa, *et al*., 2007) insect bites diarrhea (Sukumar and Osmani, 1981),skin, eye and throat inflammations, indigestion, toothache, fever and lung congestion (Nejat *et al*., 2015) menstrual disorder (Kumar *et al*., 2013) enhances kidney and liver functions (Adekomi, 2010) vinblastine alkaloids are used in the treatment of various types of lymphoma and leukemia (Lucas *et al*., 2010) Antimicrobial, antitumors, Anti-proliferative activeity of extracts from the plant has been proved (Patil and Ghosh, 2010; Vega-´ Avila, 2012)

For many years phenotypic traits variances among individuals have been used to specify genetic differences, in 20th century electrophoretic techniques, used to separate molecules based on physical and chemical variances the biochemical analysis become as excellent starting point for DNA based studies. Literature confirmed that the assessment of genetic diversity of *Catharanthus* species and cultivars were achieved through secondary metabolites.

Amplified fragment-length polymorphism (*AFLP*) Inter simple sequence repeat (*ISSR*), random-amplified polymorphic DNA (RAPD) and other methods ((Kalpana *et al.*, 2004, Arif, *et al*., 2010; Leal, *et al*., 2010; Chaudhary *et al.*, 2012; El-Domyati *et al*., 2012). They reveal genetic variances, with no effect of stage, physiological conditions, and environments, providing fast results Biochemical genetic markers are expected to play a very important role in characterization of medicinal plant genotypes (Tharachand *et al.*, 2012). (RAPDs) are extensively used to study the genetic diversity of many plants (*C. roseus* among them) (Shaw *et al* 2009; El-Domyati *et al*., 2012; Lal *et al*., 2011) because this method is simple, fast, *and inexpensive* as compared to other types of DNA based techniques. Kim, *et al* (2007) Shaw *et al.*, (2009) Vardhan *et al*., (2012) and Prasad (2014**)** found low- moderate to high genetic variation among *Catharanthus* species and cultivars.

It is one of the important medicinal plants containing more than 130 terpeniods indole alkaloids (TIAs) in different plant parts (Facchini and de Luca, 2008; Zhu *et al*, 2014***) C. roseus*** is the source of two most significant antitumor dimeric TIAs-vinblastine and vincristine and one of their monomeric precursors vindoline (Zhao and Verpoorte, 2007, Liscombe and O'Connor, 2011). Vinblastine and vincristine accumulate in *C. roseus* leaves in trace amounts 0.0003–0.01%, are synthesized by vindoline and catharanthine monomers present in the vacuole of *C. roseus* leaves and stem cells (Liscombe and O'Connor, 2011). Table 1. shows alkaloids contents in *C. roseus*. There are few articles indicating that colored flower is linked to the high level of known alkaloids (Sharma, *et al*., 2012).

Table 1: µg/ g DW content of bisindole and monoindole alkaloids in *Catharanthus roseus* L. leaves modified from different references.

Stage	Vinc	Vinb	Cath	Vino	Ref.
Flowering	1-12	1-48	2-363	142-1254	Gupta *et al*., 2005
Flowering Upper Middle Lower	__	30-70 380 500 200	1050-2030 2200 1500 1400	930-1650 2000 1100 800	Pan 2014
90 days after planting	17	79	___	___	Idrees *et al*., 2010
120 days after planting	449	___	___	___	Muthulakshmi and Pandiyarajan 2013
Flowering	___	300-400	1000-1800	300-700	Xing *et al* 2011

The aims of this study were to assess genetic diversity using RAPD molecular markers for *C. roseus* twelve cultivars grown in Iraq and to evaluate the profiles of TIAs and their precursors in the leaves of the same cultivars.

MATERIALS AND METHODS

Plants Cultivation: *C. roseus* seeds of twelve cultivars numbered as follows (1-Mediterranean xp Rose Halo,2- pacifica xpApricot, 3-pacifica xp Burgundy Halo, 4-pacifica xp Cherry Red Halo ,5-pacifica xpWhite ,6-Titan Icy Red ,7-Titan Icy pink 8-pacifica xp Polka Dot ,9-Pink Cooler,10-Lavender Hue Cooler,11-Iraqi cultivar12-Jams n Jellies Blackberry) were obtained from University of Baghdad Research Station, were used for the entire study. Healthy uniform seeds were selected, socked with distilled water for five days (the water changed several times during the day) to remove germination inhibitors (Gholamhosseinpour, *et al*., 2011). At the first of March, for each cultivar ten seeds were sown in 25cm earthen pots, placed with mixture of river sand and peat moss (1:1) with three replicates. Seedling with four leaves thinned and the best three plants kept. Two weeks later, two plants were removed and one plant per pot was maintained at University of Baghdad Research Station (44° 24'E, 30° 20'N and 34.1 m altitude). The plants irrigated as they needed, fertilizer applied as Gholam hosseinpour, *et al.*, (2011) recommended. For alkaloid content evaluation, plants were harvested at the flowering stage, at the first of July, for maximum alkaloid content in leaves (Idrees, *et al., 2010*).

Fresh foliage was dried on shade to constant weight, and then powdered by a grinder to use for alkaloids analysis.

Isolation of genomic DNA: Upper tender young leaves of the twelve *C. roseus* L. cultivars were collected at the beginning of the flowering stage to isolate genomic DNA, cleaned and stored until used. Around 50 mg of leaf for each cultivar was washed with 70% Ethanol and rinsed three times with distal water. After that, liquid nitrogen was used to grind the samples to fine powder using a mortar and pestle and then it was transferred to a 1.5 ml micro-centrifuge tube. DNA extraction was performed by using the procedure described by DNA Mini Kit protocol (Geneaid, Taipei, Taiwan). Around 100 ng/µL of DNA was extracted and kept in -20°C freezer until use. The analysis was conducted in the laboratory of Genetic Engineering and Biotechnology Institution/ University of Baghdad.

DNA electrophoresis: To check the DNA quality, extracted samples were electrophoresed on 1% agarose gel stained with Ethidium bromide at 200 mA and 70 volts for 60 minutes (Sambrook *et al*., 1989**)**. The gel was carefully removed from the apparatus followed by illuminating under UV trans-illuminator. Photographic record of the DNA samples was made.

RAPD -PCR: Polymerase Chain Reaction (PCR) with 11 Random Amplified Polymorphic DNA (RAPD) markers (table 2). The random decamer oligonucleotide primers used to the DNA amplification were purchased from Bioneer, South Korea. PCR was performed with the AccuPower PCR Premix (Bioneer, Seoul, Korea). The amplification reaction was performed following RAPD technique of (Williams *et al*., 1990). Amplification conditions were as follows: Reaction volume was 20 µL; primer concentration was 10 pmole/ µL; template genomics DNA concentration was 100 ng/µL; one cycle initial denaturation at 95°C for 5 minutes followed by 40 cycles at 95°C denaturation of template DNA for 60 seconds; primer annealing at 40°C for 60 seconds; and primer extension at 72°C for 2 minutes, followed by a 10 minutes final extension at 72°C.

The acquired PCR products were run in 1.5% agarose gel stained with ethidium bromide. Electrophoresis was performed at a constant voltage at 100 V for 1 hour. It was visualized under ultraviolet light and photographed, to verify their size, size was compared with the ladder (Gene Ruler 100 bp ladder plus).

Table 2: The base sequences of DNA primers for RAPD markers analysis

	Primer code	Sequence
1	OP- A03	5´ AGT CAG CCA C 3 `
2	OP- A11	5´CAA TCG CCG T 3`
3	OP-B07	5´ GGT GAC GCA G 3`
4	OP-B09	5´ TGG GGG ACT C 3`
5	OP-B10	5` GTA GAC CCG T 3`
6	OP-C11	5´ AAA GCT GCG G 3 `
7	OP-C12	5´ TGT CAT CCC C 3`
8	OP-AF05	5` CCC GAT CAG A 3`
9	OP-AF15	5` CAC GAA CCT C 3`
10	OP-N15	5` CAG CGA CTG T 3`
11	OP- Z03	5` GGC TGT CCG T 3`

Data analysis: All DNA clear bands (monomerphic and polymorphic) produced by each primer were calculated (Gherardi et al., 1998) and their molecular sizes compared using a DNA ladder from Bioneer, South Korea. Polymorphism was calculated depending on the presence (1) or absence (0) of each DNA bands. Amplified products were analyzed by pairwise comparisons of the cultivars depending on the percentage of DNA fragments, and the genetic distance and similarity for RAPD bands. Dendrogram tree was constructed by applying an un-weighted pair group method of arithmetic averages (UPGMA) cluster analysis (Sneath and Sokal 1973) to show the genetic relationships among the C. roseus cultivars. Calculation was achieved by using statistical software SPSS-10.

Reagents and standards: Standards vincristine, vinblastine, Catharanthine and Vindoline were purchased from Sigma-Aldrich, USA. Solvents used in the extraction were of analytical grade. Methanol, water and acetonitrile of HPLC-grade were purchased from Fisher Scientific and diethylamine was purchased from Sigma-Aldrich.

Extraction Procedures: The procedure proposed by Mu, et al., (2012) has been applied. In brief, 5 g of powdered of *C roseus* leaves were added to flask with 200 ml of 80% ethanol. It was run in an ultrasonic bath (Shimadzu, Kyoto, Japan) at a frequency of 40 kHz with maximum input power of 250W at 45 °C for 30 min. After each extraction, the extraction solutions were filtrated, combined and concentrated on a rotary evaporator instruments (Shimadzu, Japan) at 45 °C, 1ml HPLC-grade methanol was added to the residue obtained from the rotary precipitate, then 50 µL were injected on HPLC according to the optimum separation condition, the quantitative analysis of each were obtained by comparison of the peak area of authentic standard with that of the sample.

Chromatographic Condition: The separation occurred on Liquid chromatography Shimadzu 10AV- LC equipped with binary delivery pump model LC-10A Shimadzu; the eluted peaks were monitored by UV-Vis 10A- SPD. The extract of alkaloids according to enclosed procedure was separated on FLC (Fast Liquid Chromatographic) column, 3µm particle size, phenomenex C18 (50-4.6 mm l.D) column. Mobile phase; solvent A; water: diethylamine 986:14 ml adjusted to pH 7.2 by 0.01M phosphoric acid, solvent B: methanol: acetonitrile (4:1, v/v), Isocratic separation (40 solvent A: 60 solvent B, v/v) detection UV set at 220 nm, flow rate 1.8 ml/min. Injection volume; 50 µl according to the method of (Luo *et al*., 2005) with miner modification.

Quantification of Alkaloids

$$\text{Sample concentration of µg/mL} = \frac{area\ of\ sample}{area\ of\ standard} \times \text{conc. of standard} \times \text{dilution factor}$$

RESULTS AND DISCUSSION

Part of this study was to assess 11 random decamer oligonucleotide primers (RAPD) fingerprinting efficiency for twelve *C. roseus* cultivars. Three of these primers had no amplifications, while eight of them, table 2 had detectable and reproducible banding pattern shown dependable difference of any variation. The amplification results of some primers with the twelve *C. roseus* L cultivars are shown in Fig 1 and the data of the RAPD evaluates shown in Table 3

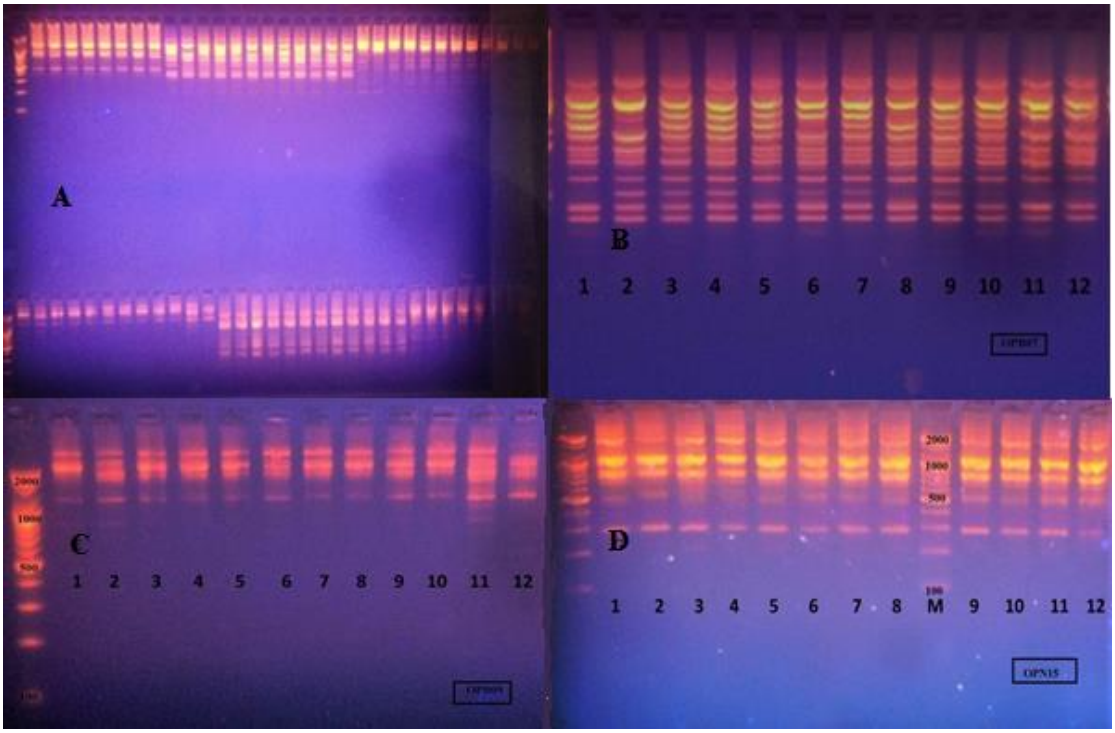


Figure 1: *RAPD fingerprinting of Catharanthus roseus L. twelve cultivars with Marker (2000-100bp) DNA ladder run , on agarose gel (1.5%) and electric voltage (100 V) for (30 min.) Cultivars1-Mediterranean xp Rose Halo,2- pacifica xpApricot, 3-pacifica xp Burgundy Halo, 4-pacifica xp Cherry Red Halo ,5-pacifica xpWhite ,6-Titan Icy Red ,7-Titan Icy pink 8-pacifica xp Polka Dot ,9-Pink Cooler,10-Lavender Hue Cooler,11-Iraqi cultivar12-Jams n Jellies Blackberry (A, B, C and D ???? EXPLIAN PLEASE.*

RAPD fingerprinting: The fingerprinting score was built on the presence or absence of detectable and reproducible banding pattern. The eight primers formed in the amplification of 56 bands, 28 (50%) of them were polymorphic bands present in some cultivars and absent in others' and 28 (50%) were monomorphic bands present in all cultivars Showing adequate degree of genetic variation in the twelve *C. roseus* L cultivars. The number of amplified DNA bands range from 4 to 13 and the mean of amplified bands per primer was eight ranging from 300 bp to 2000 bp length (maybe higher if a leader of 100bp to 3000bp used). Primer OP-B07 produced the highest band number (13 bands) however primer OP-AF15 had the lowest (4 bands). Primer OP-C12 showed the highest Polymorphic percentage (100%), while primer OP-Z03 had the lowest (14%). On the other hand, primer OP-B07 produced two unique bands whereas each of OP-A11and OP- ZO3 had one unique band as shown in table 3.

Table 3. Details of primers and banding pattern of RAPD markers among the 12 C. roseus cultivars

Primer	Nucleotide sequences	Range of Amplicons (bp)	Total Bands	No. of monomorphic bands	No.of polymorphic bands	Polymorphism (%)	No. of exclusive bands
OP-A11	CAATCGCCGT	500-2000	7	1	6	85	1
OP-AF15	CACGAACCTC	300-2000	4	2	2	50	0
OP-B07	GGTGACGCAG	400-2000	13	9	4	30	2
OP-B09	TGGGGGACT	1200-2000	6	2	4	66	0
OP-B10	GTAGACCCGT	300-2000	8	4	4	50	0
OP-C12	TGTCATCCCC	400-1800	6	0	6	100	0
OP-N15	CAGCGACTGT	300-2000	5	4	1	20	0
OP-Z03	GGCTGTCCGT	450-1400	7	6	1	14	1
Total		300-2000	56	28	28		4

Genetic distance and Cluster analysis as revealed by RAPD markers: To determine the genetic similarity among the 12 *C. roseus* L. cultivars, the RAPD data (banding pattern) was used to create similarity matrix and dendrogram, for the cluster construction UPGMA analysis was used as shown in table 4 and fig 2 The dendogram constructed using all the eight primers showed that the maximum genetic distance identified between Iraqi cultivar and Titan Icy pink was (0.309) with least

similarity value (69%). Whereas the minimum genetic distance was observed between pacifica xp Cherry Red Halo and pacifica xp Burgundy Halo (0.0) with highest similarity value (100%).

Table 4. Genetic distance among Catharanthus roseus cultivars

	Mediterranean xp Rose Halo	pacifica xpApricot	pacifica xp Burgundy Halo	pacifica xp Cherry Red Halo	pacifica xpWhite	Titan Icy Red	Titan Icy pink	pacifica xp Polka Dot	Pink Cooler	Lavender Hue Cooler	Iraqi cultivar	Jams n Jellies Blackberry
Mediterranean xp Rose Halo	0	0.115	0.132	0.132	0.058	0.264	0.226	0.115	0.150	0.132	0.236	0.192
pacifica xpApricot	0.115	0	0.132	0.132	0.058	0.16	0.226	0.150	0.150	0.167	0.203	0.157
pacifica xp Burgundy Halo	0.132	0.132	0	0	0.111	0.277	0.207	0.058	0.132	0.148	0.25	0.173
pacifica xp Cherry Red Halo	0.132	0.132	0	0	0.111	0.277	0.207	0.058	0.132	0.148	0.25	0.173
pacifica xpWhite	0.058	0.058	0.113	0.113	0	0.211	0.207	0.096	0.132	0.133	0.218	0.173
Titan Icy Red	0.264	0.16	0.277	0.277	0.211	0	0.127	0.296	0.264	0.245	0.283	0.274
Titan Icy pink	0.226	0.226	0.207	0.207	0.207	0.127	0	0.192	0.192	0.173	0.309	0.235
pacifica xp Polka Dot	0.115	0.150	0.058	0.058	0.096	0.296	0.192	0	0.150	0.096	0.236	0.12
Pink Cooler	0.150	0.150	0.132	0.132	0.132	0.264	0.192	0.150	0	0.132	0.169	0.192
Lavender Hue Cooler	0.132	0.166	0.148	0.148	0.133	0.245	0.173	0.097	0.132	0	0.185	0.137
Iraqi cultivar	0.236	0.203	0.25	0.25	0.218	0.283	0.309	0.236	0.169	0.185	0	0.176
Jams n Jellies Blackberry	0.192	0.157	0.173	0.173	0.173	0.274	0.235	0.12	0.192	0.137	0.176	0

From the dendrogram it can be seen that there were two clear clusters. Cluster one was represented by a single cultivar (Iraqi cultivar) and cluster two was divided into two nodes; one of them had Titan Icy Red and Titan Icy pink cultivars and the other node further sub divided into sub clusters. One sub cluster comprised of pacifica xp Burgundy Halo, pacifica xp Cherry Red Halo and pacifica xp Polka Dot with100% - 94% genetic similarity. The other sub cluster comprised of three cultivars, Mediterranean xp Rose Halo pacifica xpApricot and pacifica xpWhite with similarity value from 89% until 94%. The other three cultivars were outside of both of the two sub clusters, each cultivar stands alone. The analysis of each primer must be had genetic similarity table and dendrograme.

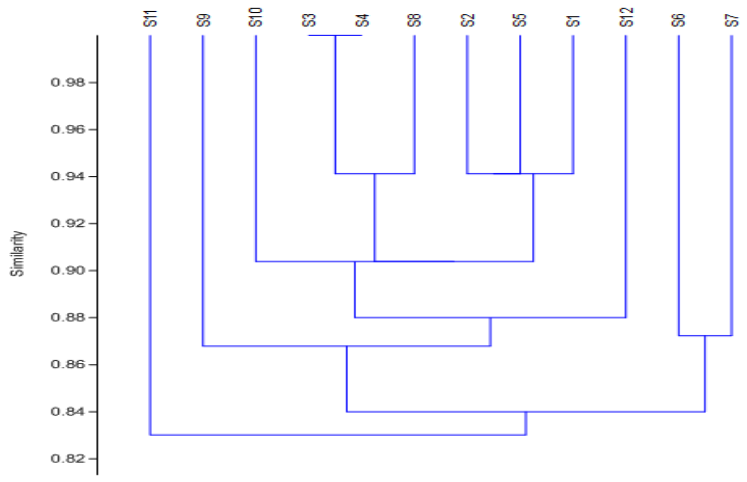


Figure 2: Dendrogram for the 12 Catharanthus roseus cultivars constructed from RAPDs data using Un weighted Pair-group Arithmetic Average (UPGMA) and similarity matrices computed according to coefficients. Cultivars S1-Mediterranean xp Rose Halo, S2- pacifica xpApricot, S3-pacifica xp Burgundy Halo, S4-pacifica xp Cherry Red Halo, S5-pacifica xpWhite, S6-Titan Icy Red, S7-Titan Icy pink S8-pacifica xp Polka Dot, S9-Pink Cooler, S10-Lavender Hue Cooler, S11-Iraqi cultivar and S12-Jams n Jellies Blackberry Each primer must be had genetic similarity table and dendrograme.

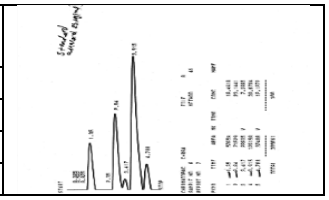
The random amplified polymorphic DNA technique (RAPD) markers are being increasingly used for identification or measure genetic similarity of plant species and cultivars, which are useful for taxonomy and differentiate closely related species. The advantages of this technique are that, the need for small quantity of DNA to carry the test, no need for former knowledge about the plant genetics, the technique is simple, reproducible and with reasonably priced.

The primer determined the differences in banding pattern gained by RAPD markers. Non-amplifications of the three primers could not be clarified. A similar non-amplification of primers was stated by (Shaw *et al*., 2009) were out of 25 primers, only 14 primers had amplifications.

Genetic similarity of *C. roseus* L cultivars studied was over 69% moreover two of the cultivears were identical, Pacifica xp Burgundy Halo and Pacifica xp Cherry Red Halo with 100% similarity. The relatively low polymorphism noticed in *C. roseus* L plant cultivars can be due to the narrow geographical origin of the cultivars studied (most of them were USA cultivars) the RAPD's dendrogram analysis showed two main clusters one cluster was for the Iraqi cultivar and the other was for the USA cultivars, the results of the analysis was fitting with geographical origin that may have narrow genetic basis. A high degree of polymorphism (85% and 100%) was observed with two primers (OP-A11 and OP-C12) proposing that these primers could be used as markers for diversity analysis.

To check whether the same *C. roseus* twelve cultivars could be differentiate according to their alkaloid content, fast liquid reversed phase chromatographic C18 column has been used for separation and identification of four compounds involving, terpenoid indole alkaloids (TIAs) Vincristine, Vinblastine, and their precursors (Vindoline, Catharanthine). Several studies used C18 RP-HPLC columns as the analytical system carefully chosen for an optimal separation and identification of TIAs alkaloids and precursors content in *C. roseus* extracts (Renaudin, 1984; Bhadra *et al*., 1993; Uniyal *et al*., 2001; Zhou *et al*., 2005). The sequences, retention times (min) and quantification of standard compounds (Vindoline, Catharanthine, Vincristine and Vinblastine) is presented in table 5.

Table 5: *HPLC retention time/ min, area of eluted standards and Chromatogram of Catharanthus roseus alkaloid, peaks are indicated as follows 1-Vindoline, 2-Catharanthine 3-Vincristine and 4-Vinblastine, detection was with UV 220 nm length.*

Seq	Compound	Retention time/ min	Area	Concentrations	
1	Vindoline	1.35	57058	25 µg/ ml	
2	Cathranthine	2.84	71529	25 µg/ ml	
3	Vincristine	3.91	120185	25 µg/ ml	
4	Vinblastine	4.70	37480	25 µg/ ml	

The sequence of elution was Vindoline, Catharanthine, Vincristine and Vinblastine, where in Reversed phase HPLC the hydrophilic, Vindoline, Catharanthine (precursors) passed through the column and eluted first, while hydrophobic (TIAs) Vincristine and Vinblastine adsorbed to the hydrophobic packing material. That was in agreement with (Hisiger and Jolicoeur 2007) stated, TIAs are generally more lipophilic than their precursors. Zhou *et al*., (2005) obtained the same elution pattern, On the other hand Singh, *et al*., (2000) Uniyal *et al*., (2001) and Tikhomiroff *et al*, (2002) observed different elution sequence, as small changes of the mobile phase content or column packing can alter the alkaloids structure, and thus influence the elution sequence (Hisiger and Jolicoeur 2007). The results of retention times values disagree with previous reports stating by Singh, *et al*., (2000), Uniyal *et al*., (2001), Tikhomiroff *et al*., (2002); Zhou *et al*., (2005) and many other researchers since retention time depends on type of stationary phase in the column, flow rate and mobile phase.

The identification of TIAs and their precursors from the *C. roseus* twelve cultivars extracts were achieved by comparison of retention time for each peak with those of authentic standards. Fig 3 and Table 6 show the chromatographic profile of TIAs and their precursors, retention time, presence or absence and the percentage of each peak of the twelve *C. roseus* cultivars. It shows a good resolution of the peaks with marginal differences in retention time.

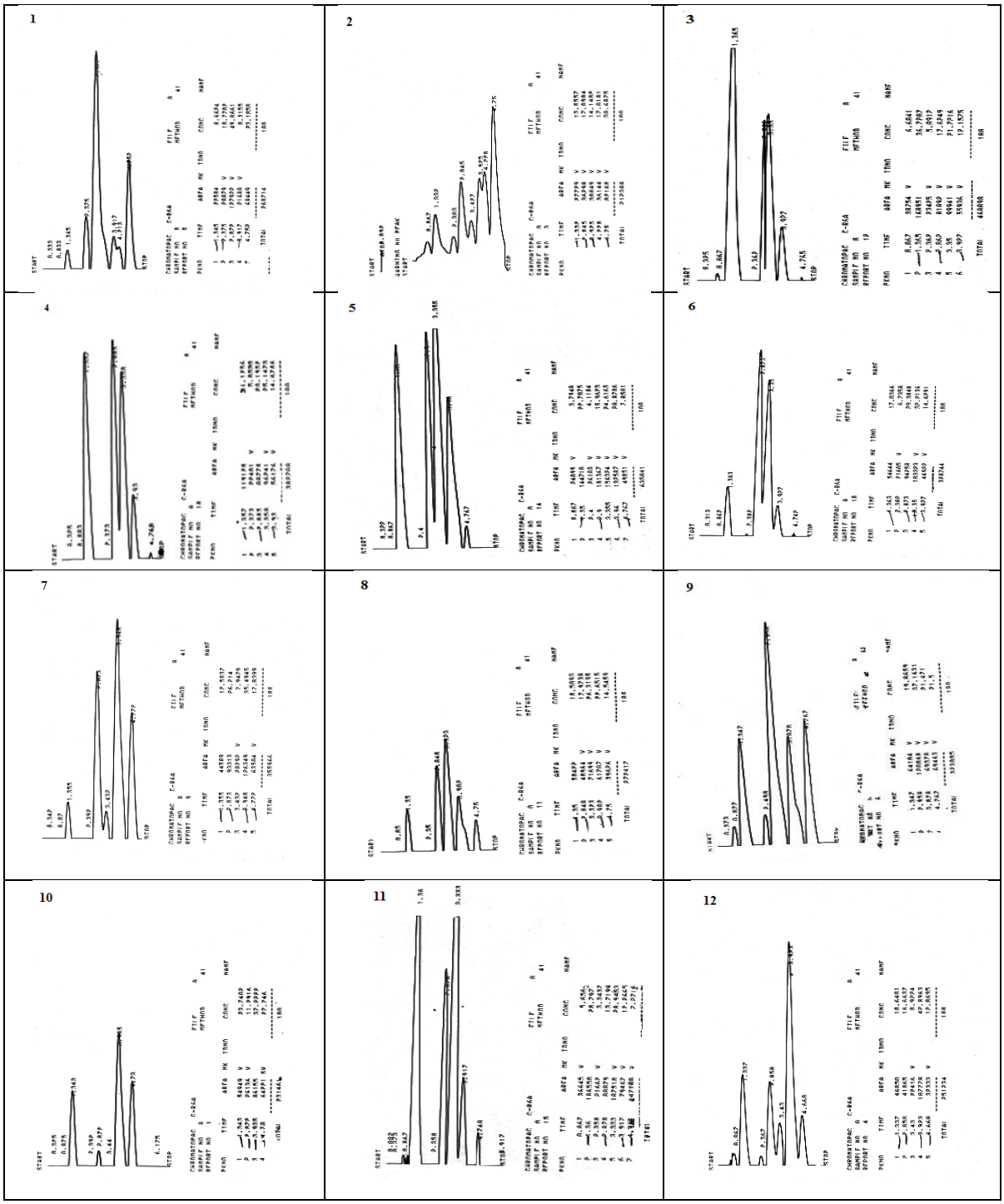


Figure 3. Chromatograms of Vindoline, Catharanthine, Vincristine and Vinblastine of twelve *Catharanthus roseus* cultivars grown in Iraq ,1-Mediterranean xp Rose Halo,2- pacifica xpApricot, 3-pacifica xp Burgundy Halo, 4-pacifica xp Cherry Red Halo ,5-pacifica xpWhite ,6-Titan Icy Red ,7-Titan Icy pink 8-pacifica xp Polka Dot ,9-Pink Cooler,10-Lavender Hue Cooler,11-Iraqi cultivar12-Jams n Jellies Blackberry. Chromatographic conditions: RP-phenomenex C18 column (50 x 4.6 mm I.D) 3 µm particles size. Mobile phase; solvent A; water: diethylamine 986:14 ml adjusted to pH 7.2 by 0.01M phosphoric acid, solvent B: methanol: acetonitrile (4:1, v/v), Isocratic separation (40 solvent A: 60 solvent B, v/v) flow rate 1.8 ml/min, ultraviolet detection with wavelength 220 nm using the method developed by (Luo *et al*., 2005).

Additionally, from the table 6 it can be seen that no two cultivars with same retention time had the same peaks or the same percentage of the peaks, even the cultivar pacifica xp Burgundy Halo and pacifica xp Cherry Red that had minimum genetic distance (0.0) with highest similarity value (100%).

Table 6: Retention time (min), presence or absence and the alkaloids percentage of each peak of the twelve *Catharanthus roseus* cultivars

R-time	**0.333**	**0.867**	**1.332**	**2.383**	**2.845**	**3.40**	**3.91**	**4.228**	**4.70**
1	p	p	8.66	10.77	49.86	-	8.31	p	23.18
2	p	p	13.05	-	17.09	-	14.18	17.01	38.68
3	p	6.68	36.28	5.09	17.62	21.72	12.18	-	P
4	p	p	31.12	5.85	23.19	25.14	14.67	-	P
5	p	3.79	22.78	4.10	15.96	24.61	20.07	-	7.85
6	p	p	17.03	6.73	29.38	32.21	14.62	-	P
7	p	p	12.58	P	26.21	7.94	35.49	-	17.83
8	-	p	18.58	P	17.97	26.31	22.65	-	14.54
9	p	p	19.86	P	37.16	-	21.47	-	21.50
10	p	p	23.74	P	11.29	p	37.22	-	27.74
11	p	5.65	28.79	3.34	13.71	28.94	12.26	-	7.27
12	-	p	18.64	-	16.66	8.92	42.09	-	12.86
Means			20.93%		39.67%		21.26%		14.28 %*
									19.04%**

P = Peak present but not detected * Mean of 12 cultivars ** Mean of 9 cultivars content
1-Mediterranean xp Rose Halo,2- pacifica xpApricot, 3-pacifica xp Burgundy Halo, 4-pacifica xp Cherry Red Halo ,5-pacifica xpWhite ,6-Titan Icy Red ,7-Titan Icy pink 8-pacifica xp Polka Dot ,9-Pink Cooler,10-Lavender Hue Cooler,11-Iraqi cultivar12-Jams n Jellies Blackberry

Quantification of Vincristine, Vinblastine and their precursors Vindoline and Catharanthine was prepared by measuring the peak area at the wavelength 220nm Fig 3 The highest percentage in dry leaves for Vindoline, Catharanthine ,Vincristine and Vinblastine were 0.0653, 0.0283, 0.0310 and 0.0342 of the cultivars: Iraqi cultivar, pacifica xp White, Titan Icy pink and Lavender Hue Cooler respectively, while the means of those alkaloids were 0.0267, 0.0193, 0.0119 and 0.0187% leaves dry weight Table 7. The same table shows that the cultivar pacifica xp Burgundy Halo and pacifica xp Cherry Red with highest similarity value (100%) had almost the same alkaloids profile with slight difference in Vindoline percentage.

Table 7: Vindoline, Catharanthine, Vincristine and Vinblastine content (percent in dry leaves) of twelve *Catharanthus roseus* cultivars grown in Iraq

Cultivars	Vindoline	Catharanthine	Vincristine	Vinblastine
1	0.0049	0.0223	0.0022	0.0201
2	0.0060	0.0063	0.0031	0.0274
3	0.0509	0.0228	0.0093	ND
4	0.0417	0.0248	0.0093	ND
5[w]	0.0507	0.0283	0.0220	0.0266
6	0.0191	0.0263	0.0078	ND
7	0.0156	0.0260	0.0310	0.0338
8[w]	0.0204	0.0136	0.0102	0.0211
9	0.0140	0.0209	0.0072	0.0231
10	0.0192	0.0073	0.0143	0.0342
11	0.0653	0.0248	0.0132	0.0251
12	0.0123	0.0087	0.0134	0.0129
Means	0.0267	0.0193	0.0119	0.0187(0.0249*)

[w]: white flower, ND: not detected, *Mean of nine cultivars that contain Vinblastine.1-Mediterranean xp Rose Halo,2- pacifica xpApricot, 3-pacifica xp Burgundy Halo, 4-pacifica xp Cherry Red Halo ,5-pacifica xpWhite ,6-Titan Icy Red ,7-Titan Icy pink 8-pacifica xp Polka Dot ,9-Pink Cooler,10-Lavender Hue Cooler,11-Iraqi cultivar12-Jams n Jellies Blackberry

The Vindoline percentage of the Iraqi cultivar (wild types) was the highest (0.0653), which may be due to the different growth habit of the cultivar since it takes more time to reach the flowering stage, consequently it may induce more Vinblastine production by the high precursor if it is harvested in full flowering stage, in agreement with those finding of (Naaranlahti , *et al.*, 1991,El-Sayed and Verpoorte, 2005 and Pan, *et al.*, 2014) that the levels of vindoline and

catharanthine increased before flowering and decreased during flowering, whereas bisindole alkaloids (Vincristine and Vinblastine) accumulated at the flowering time as leaves aged so Iraqi cultivar potentially will produced more Vincristine and Vinblastine, the same as with pacifica xp Burgundy Halo, pacifica xp Cherry Red Halo and pacifica xp White the hybrid cultivars that produced high level of vindoline.

The means of Vindoline, Catharanthine, Vincristine and Vinblastine of the twelve cultivars were 0.0267, 0.0193, 0.0119 and 0.0187 respectively. That was in agreement with those finding of (Verpoorte, *et al*., 1993 and Pezzuto, 1997) that Vincristine quantitatively less than Vinblastine in the aerial parts of the plant.

In this study the TIAs alkaloids and precursors production in leaves of the twelve *C. roseus* cultivars showed that the quantitative differences did not relate to flower color and this finding did not confirm previous studies stating that pink color flower had higher alkaloid content in leaves than white color (Idrees, *et al*., 2010; Bhutkar and Bhise,2011). For example, although the genetic distance between pacifica xpWhite and pacifica xp Polka Dot cultivars that had white color flower was very low (0.096) table 4 they vary both qualitatively and quantitatively Table 6 and 7 respectively. Therefore, most importantly to undertake more detailed studies on a large sample size to be representative to determine alkaloid marker compounds that can be considered as specific markers. In this way the genetic diversity between cultivars in relation to the levels of vinblastine and vincristine may be linked to the flower color to breed plants with higher yield of alkaloids.

REFERENCES

Adekomi, D.A., Madagascar periwinkle (*Catharanthus roseus*) enhances kidney and liver functions in Wistar rats, Eur. J. Anat. 14: 111-119 (2010).

Ahmed M.F., S.M. Kazim, S.S. Ghori, S.S. Mehjabeen, S.R. Ahmed, S.M. Ali and M. Ibrahim, Antidiabetic Activity of Vinca rosea Extracts in Alloxan-Induced Diabetic Rats. International Journal of Endocrinology 2010: 1-6 (2010).

Arif, I.A., M.A. Bakir, H.A. Khan and A.H. Al Farhan, A brief review of molecular techniques to assess plant diversity. Int. J. Mol. Sci. 11: 2079-2096 (2010).

Bhadra, R., S. Vani and J.V. Shanks, Production of indole alkaloids by selected hairy root lines of Catharanthus roseus. Biotechnol Bioeng. 41: 581-592 (1993).

Bhutkar, M.A. and S.B. Bhise, Comparative studies on antioxidant properties of *Catharanthus rosea* and *Catharanthus alba*. International Journal of PharmTech Research 3: 1551–1556 (2011).

Chaudhary, A.A., D. Yadav, Hemant, S.S. Jamil and M. Asif, Real time sequence characterized amplified region (RTSCAR) marker: Development and its application for authentication and quantification of *Catharanthus roseus* L. Don. J. Med. Plant. 6:4748-4754 (2012).

El-Domyati, F.M., A.M. Ramadan, N.O. Gadalla, S. Edris, A.M. Shokry, S.M. Hassan, S.E. Hassanien, M.N. Baeshen, N.H. Hajrah, M.A. Al-Kordy, O.A. Abuzinadah, A.S.M. Al-Hajar, C.C. Akoh and A. Bahieldin, Identification of molecular markers for flower characteristics in *Catharanthus roseus* producing anticancer compounds. Life Sci. J. 9: 5949-5960 (2012).

El-Sayed, M. and R. Verpoorte, Methyljasmonate accelerates catabolism of monoterpenoid indole alkaloids in *Catharanthus roseus* during leaf processing. Fitoterapia 76: 83–90 (2005).

Facchini, P.J. and V. de Luca, Opium poppy and Madagascar periwinkle: model non-model systems to investigate alkaloid biosynthesis in plants. The Plant Journal 54: 763-784 (2008).

Gathirwa, J.W., G.M. Rukunga, E.N.M. Njagi et al., In vitro anti-plasmodial and *in vivo* anti-malarial activity of some plants traditionally used for the treatment of malaria by the Meru community in Kenya, Journal of Natural Medicines 61: 261-268 (2007).

Ghérardi, M., B. Mangin, B. Goffinet, D. Bonnet and T. Huguet, A method to measure genetic distance between allogamous populations of alfalfa (Medicago sativa) using RAPD molecular marker. Theoretical Applied Genetics 96: 406-412 (1998).

Gholamhosseinpour, Z., K. Hemati, H. Dorodian and Z. Bashiri-Sadr, Effect of nitrogen fertilizer on yield and amount of alkaloids in periwinkle and determination of vinblastine and vincristine by HPLC and TLC. Plant Sciences Research 3: 4-9 (2011).

Gupta, M.M., D.V. Singh, A.K. Tripathi, R. Pandey, R.K. Verma, S. Singh, A.K. Shasany, and S.P. Khanuja, Simultaneous Determination of Vincristine, Vinblastine, Catharanthine, and Vindoline in Leaves of *Catharanthus roseus* by High-Performance

Liquid Chromatography. Journal of Chromatographic Science 43: 450-453 (2005).

Hisiger, S. and M. Jolicoeur, Analysis of Catharanthus roseus alkaloids by HPLC. Phytochem. Rev. 6: 207-234 (2007).

Idrees, M., M. Naeem and M.M.A. Khan, The superiority of cv 'rosea' over cv 'alba' of periwinkle (*Catharanthus roseus* L.) in alkaloid production and other physiological attributes. Turk. J. Biol. 34: 81-88 (2010).

Kalpana, J., P.C. Warude and P. Bhushan, Molecular markers in herbal drug technology. Curr. Sci. 87: 159-165 (2004).

Kim, S. W., S.H. Ban, S.C. Jeong et al., Genetic discrimination between *Catharanthus roseus* cultivars by metabolic fingerprinting using 1-H NMR spectra of aromatic compounds. Biotechnology and Bioprocess Engineering 12: 646- 652 (2007).

Kumar, O.A., S.S. Tata and K.P. Kumar, Analysis of Several Popular Cultivars of Madagascar Periwinkle (*Catharanthus roseus* (L.) G. Don.) using Biochemical Markers. Not. Sci. Biol. 5: 458-461 (2013).

Lal, S., K.N. Mistry, S.D. Shah, R. Thaker and P.B. Vaidya, Genetic diversity assessment in nine cultivars of *Catharanthus roseus* from Central Gujarat (India) through RAPD, ISSR and SSR markers. Journal of Biological Research 8: 667-675 (2011).

Leal, A.A., C.A. Mangolin, A.T.J. do Amaral, L.S. Goncalves et al., Efficiency of RAPD versus SSR markers for determining genetic diversity among popcorn lines. Genet Mol. Res. 9: 9-18 (2010).

Liscombe, D.K. and S.E. O'Connor, A virus-induced gene silencing approach to understanding alkaloid metabolism in *Catharanthus roseus*. Phytochemistry 72: 1969-1977 (2011).

Lucas, D.M., P.C. Still, L.B. Pérez, M.R. Grever and A.D. Kinghorn. Potential of plant-derived natural products in the treatment of leukemia and lymphoma. Curr. Drug. Targets 11: 812-822 (2010).

Luo, M., Y.J. Fu, Y.G. Zu, Q. Shi, F.S. Mu and Q.Y. Li, Rapid determination of 4 vinca alkaloids by reversed phase high performance liquid chromatography. Chin. J. Anal. Chem. 33: 87-89 (2005).

Mu, F., L. Yang, W. Wang, M. Luo, Y. Fu, X. Guo et al., Negative-Pressure Cavitation Extraction of Four Main Vinca Alkaloids from *Catharanthus roseus* Leaves. Molecules 17: 8742-8752 (2012).

Muthulakshmi, S. and V. Pandiyarajan, Influence of IAA on the vincristine content of *Catharanthus roseus* (L). G. Don. Asian Journal of Plant Science and research 3: 81-87 (2013).

Naaranlahti, T., S. Auriola and S.P. Lapinjoki, Growth-related dimerization of vindoline and catharanthine in *Catharanthus roseus*and effect of wounding on the process. Phytochemistry 30: 1451–1453 (1991).

Nejat, N., A. Valdiani, D. Cahill, Y.H. Tan, M. Maziah and R. Abiri, Ornamental exterior versus therapeutic interior of Madagascar periwinkle (Catharanthus roseus): the two faces of a versatile herb. Sci. World. J. ID: 982412, 19 (2015).

Pan, Q., Metabolomic characteristics of *Catharanthus roseus* plants in time and space. Leiden University- Netherlands dissertation. chapter 3. A simple and rapid HPLC-DAD method for simultaneously monitoring the accumulation of alkaloids and precursors in different parts and different developmental stages of *Catharanthus roseus*plants. Pan,Q. M. Z. Saiman. N. R. Mustafa, R. Verpoorte, Y. H. Choi (2014).

Patil, P.J. and J.S. Ghosh, Antimicrobial activity of *Catharanthus roseus*-A Detailed Study. British Journal of Pharmacology and Toxicology 1: 40-44 (2010).

Pezzuto, J.M., Plant-derived anticancer agents. Biochemical Pharmacology 53: 121-133 (1997).

Prasad, M.P., Detection of genetic diversity of *Catharanthus* species using RAPD PCR. Ind. J. Adv. Plant Res. 1: 24 (2014).

Renaudin, J.P., Reversed-phase high-performance liquid chromatographic characteristics of indole alkaloids from cell suspension cultures of *Catharanthus roseus*. Journal of Chromatography A. 291:165-174 (1984).

Green**,** M.R. and J.Sambrook , Molecular cloning a laboratory manual.4th[ed], Cold Spring Harbour Laboratory Press, New York (2012).

Sharma, V., S. Chaudhary, S. Srivastava, R. Pandey and S. Kumar, Characterization of variation and quantitative trait loci related to terpenoid indole alkaloid yield in a recombinant inbred line mapping population of *Catharanthus roseus*. J. Genet. 91: 49-69 (2012).

Shaw, R.K., L. Acharya and A.K. Mukherjee, Assessment of genetic diversity in a highly valuable medicinal plant *Catharanthus roseus* using molecular markers. Crop. Breed. App. Biotech. 9: 52-59 (2009).

Singh, D.V., A. Maithy, R.K. Verma, M.M. Gupta and S. Kumar, Simultaneous determination of Catharanthus alkaloids using reversed phase high performance liquid chromatography. J Liquid Chromatogr Relat Technol. 23: 601–607 (2000).

Sneath, P. and R. Sokal, Numerical Taxonomy: The Principles and Practice of Numerical Classification, W. H. Freeman and Company, San Francisco, California. (1973).

Sukumar, K. and Z. Osmani, Insect sterilants from *Catharanthus roseus*. Current Science 50: 552-553 (1981).

Tharachand, C., C. Immanuel Selvaraj and M.N. Mythili, Molecular markers in characterization of medicinal plants: An overview. Research in Plant Biology 2: 01-12 (2012).

Tikhomiroff, C., S. Allais, M. Klvana, S. Hisiger and M. Jolicoeur, Continuous selective extraction of secondary metabolites from Catharanthus roseus hairy roots with silicon oil in a two-liquid-phase bioreactor. Biotechnol Progr. 18: 1003-1009 (2002).

Uniyal, G.C., S. Bala, A.K. Mathur and R.N. Kulkarni, Symmetry C18 column: a better choice for the analysis of indole alkaloids of Catharanthus roseus. Phytochem Anal. 12: 206–210 (2001).

Vardhan, S., A.K. Yadav, R.K. Suria and B.B.S.P.Nag, Genetic relatedness and In vitro antimicrobial activities of alkaloids isolated from Indian varieties of *Catharanthus roseus* (L.). Current Botany. 3: 43-48 (2012).

Vega-Avila, E., J.L. Cano-Velasco, F.J. Alarcón-Aguilar, M.D.C. Fajardo Ortíz, J.C. Almanza-Pérez and R. Román-Ramos, Hypoglycemic activity of aqueous extracts from *Catharanthus roseus*, Evidence-Based Complementary and Alternative Medicine 2012: 1- 7 (2012).

Verpoorte, R., R. van der Heijden, J. Schripsema, J.H.C. Hoge and H.J.G. Ten Hoopen, Plant cell biotechnology for the production of alkaloids: present status and prospects. J. Nat. Prod. 56: 186–207 (1993).

Verpoorte, R., R. van der Heijden, J. Schripsema, J.H.C. Hoge and H.J.G. Ten Hoopen, Plant cell biotechnology for the production of alkaloids: present status and prospects. Journal of Natural Products 56: 186-207 (1993).

Williams, J.G., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535 (1990).

Xing, S., Q. Pan, Y. Tian, Q. Wang, P. Liu, J. Zhao, et al., Effect of plant growth regulator combinations on the biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus*. J. Med. Plants Res. 5: 1692-1700 (2011).

Zhao, J. and R. Verpoorte, Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. Phytochem. Rev. 6: 435-457 (2007).

Zhou, H., Y.P. Tai, C.R. Sun and Y.J. Pan, Rapid identification of Vinca alkaloids by direct-injection electrospray ionisation tandem mass spectrometry and confirmation by high-performance liquid chromatography–mass spectrometry. Phytochem. Anal. 16: 328-333 (2005).

Zhu, X., X. Zeng, C. Sun, and S. Chen, Biosynthetic pathway of terpenoid indole alkaloids In *Catharanthus roseus*. Front. Med. 8: 285-293 (2014).