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ISOLATION AND CHARACTERIZATION OF ANTINEOPLASTIC ALKALOIDS FROM *CATHARANTHUS ROSEUS* L. DON. CULTIVATED IN EGYPT

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Abstract

Vinblastine and vincristine (the antileukemic agents) were isolated, in a pure form, from *Catharanthus roseus* L. Don., cultivated in Egypt, by several chromatographic techniques. Five modified methods for the preparation of total alkaloids were carried out. All the isolated mixtures were evaluated by HPLC and HPTLC analyses. The antineoplastic alkaloids; vinblastine and vincristine, were isolated by the use of vacuum liquid chromatographic column on silica gel : aluminium oxide (1:1) mixed bed vacuum liquid chromatography (VLC), Charcoal column, and finally purified by centrifugally accelerated radial chromatography (Chromatotrone).

Key words: *Catharanthus roseus* L., Apocyanaceae, Vinblastine, Vincrisitine, Antileuckemic alkaloids, VLC, HPLC, HPTLC.

Introduction

Catharanthus roseus or *Vinca rosea*, is also known as myrtle. It is a perennial, evergreen herb (family: Apocyanaceae). It was native to the Island of Madagascar, and is now growing wildly in most warm regions of the world especially in Egypt (Dobelis, 1989 and Heywood, 1993). *C. roseus* plant produce many pharmaceutically important alkaloids of which the bisindole alkaloids. vinblastine and vincristine. They are antineoplastic medicines and the monoindole alkaloids ajmalicine and serpentine are antihypertension drugs (Zhao and Verpoorte, 2007, Atta-Ur-Alrahman, et al., 1983, 1984, 1985, 1988, Atta-ur-Rahman and Fatima, 1984, Auriola, et al., 1990). Vinblastine sulphate is used commercially for the treatment of neoplasma and is recommended for generalized Hodgkin's disease and resistant choiocarcenoma. The plant has been early used in treatment of diabetes, hypertension, tuberculosis, laryngitis, sore throat, dyspepsia, malaria, and to regulate menstruation (Moreno, et al., 1993; Soo-Young Lee et al., 2003). Fveretto et al., (2001) reported a semi–quantitative determination of vincrisitine and vinblastine by injection electrospray ionization mass spectrometric analysis. Renault et al., (1999) developed a new method for the isolation and purification of indole alkaloids from *C. roseus* by a centrifuged partition chromatography (CPC). Moreover, Scragg, in (1999) determined both ajmalicine and serpentine by HPLC on a Bondapak C_{18} reverse phase column.

The present study aimed to the isolation, purification and evaluation of the antineoplastic alkaloids vincrisitine and viblastine by the use of several chromatographic techniques, *viz* charcoal column, VLC, HPLC, HPTLC and centrifugally accelerated radial chromatography (chromatotrone).

Materials and Methods

Plant Material

Catharanthus roseus L. were obtained from the cultivated sample in the National Research Centre Farm at Giza, Egypt, in June 2006, and kindly authenticated by Prof. Dr. K.H. El Batanouny, Professor of Botany, Faculty of Science, Cairo University, Egypt. A herbarium sheet is kept in the phytochemistry laboratory.

Experimental

TLC Plates, Silica gel Merck 60 F_{254}, (20x20 cm) were used. Trough glass chamber (20x20 cm), with chamber saturated with ethyl acetate : benzene : ethyl alcohol : 25% ammonia solution (100 : 5 : 5 : 3). Samples were applied band wise with CAMAG automatic TLC sampler III, distance from lower edge 8 mm, band length 6 mm, track distance 12 mm, distance from left edge 15mm, 15 applications. The alkaloidal bands were visualized by spraying with Dragendorff's reagent, followed by 10% HCl, CAMAG TLC Scanner with CATS evaluation software.

- Multiwave length scan at λ 254, 289, 366, 400 and 500 nm.
- Scanning speed 20ml/s.
-Multilevel calibration via peak area by linear regression.

- Thin layer chromatographic (TLC) grade silica gel (Merck) and alumina (Neutral Aluminium Oxide 60 GF_{254}, type E, Merck) were used for VLC.
- Centrifugally accelerated radial chromatography (chromatotrone) using silica gel 2mm thickness (type 7749, Merck, Darmstadt, Germany) eluted by chloroform : methanol 95 : 5 (v/v) with flow rate of 6ml/min.
- HPLC (Water's), with 600E delivery system (pump), 486 variable wavelength water's detector, Nova pack C_{18} (water's, 3.9x150mm, results integrated by Millinium 32 chromatography.
- Standard Solutions: 0.5 mg of vincristine (Vc) and 1 mg of vinblastine (Vb) were dissolved; separately; in 1 ml of methylene chloride.

Preparation of the total alkaloids from *C. roseus*

Preparation of total alkaloids was carried out using different methods. The following described were the five most reliable methods.

Method I:

5 kg of dried powdered *C. roseus* were moistened with sufficient amount of tartaric acid (2 %) for one hr, then benzene (9 L) was added. The mixture was shaked for 30 min. and decanted, and benzene was further added. The extraction procedure was repeated twice. The combined benzene extract was concentrated *in vacuo* at 50°C to 150 ml. Tartaric acid 300 ml (2 %) was added to the benzene extract and the remaining benzene was removed *in vacuo* at 50°C. The resulting acidic solution was filtered. The filtrate was extracted with methylene chloride (50 ml x 6). The combined methylene chloride extract was washed with water till free from acidity, dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo* at 50 °C till dryness to give a vindoline rich fraction (A).

After removal of vindoline rich fraction (A), the remaining acidic aqueous solution was adjusted to pH 5.9 with NH_4OH solution (25 %) and the liberated alkaloids were extracted with methylene chloride (500 ml x 4). The alkaloids were extracted with 0.1 M aqueous citric acid (pH 2.5) (750 ml). The pH of the clear acidic solution was adjusted to 4.4 with NH_4OH solution (25 %) and the liberated alkaloids were extracted with methylene chloride (500 ml x 3).

The combined methylene chloride extract was dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo* at 50 °C till dryness to give vinblastine rich fraction (B). The pH of the remaining aqueous layer (pH 4.4) was adjusted to 5.9 with ammonia solution (25 %) and the liberated alkaloids were extracted with methylene chloride to give vincristine rich fraction (C).

Method II:

This method is a modified application for method I where 5 kg dried powdered plant of *C. roseus* were extracted with methanol (80 %, 10 L x 3). The combined methanolic extract was evaporated at 50 °C *in vacuo* till dryness. The residue was dissolved in aqueous tartaric acid solution (2 %), and the method was proceeded as mentioned in method I to give vinblastine rich fraction (D). The pH of the remaining aqueous layer was then adjusted to 5.9 with ammonia solution (25 %) and the liberated alkaloids were extracted in the same way (as mentioned above for pH 4.4 fraction in method I) to give vincristine rich fraction (E).

Method III:

5 kg powdered plant of *C. roseus* was percolated with methanol (95 %, 10 L x 3) for 1 hour each. The methanolic extracts were filtered and concentrated at 50 °C *in vacuo* to 500 ml, diluted with water (200 ml), acidified with 1 N sulfuric acid (pH 2) and extracted with ethyl acetate (500 ml x 3). The EtOAc extract was discarded out. The aqueous solution was adjusted to pH 6.4 using NH_4OH solution (25 %) and then extracted with methylene chloride (500 ml x 3). The combined methylene chloride extract was washed with water till free from acidity, dried over anhydrous sodium sulfate, filtered and evaporated to dryness under reduced pressure to give an alkaloidal fraction (F).

Method IV:

3.5 kg of the air dried powdered plant were percolated in 80 % methanol (3 x 10 L) for 2 days each. The combined methanolic extract was filtered and evaporated *in vacuo* at 50 °C to give a gummy residue. The gummy residue was dissolved in 5 % HCl (750 ml). Left overnight in a refrigerator, filtered and extracted with chloroform (5 x 1 L). The ice-cold acidic solution was basified using ammonia solution (25 %, 200 ml) and extracted with chloroform (5 x 1.5 L). The combined chloroformic extract was dried over anhydrous sodium sulfate, filtered and evaporated under vacuum at 50 °C to give a crude alkaloidal mixture (8.8 g). The total alkaloids (8.8 g) was dissolved in 50 ml chloroform and extracted with (5 x 100 ml) phosphate buffer (pH 2). The chloroform layer was then separated and dried over anhydrous sodium sulfate and finally evaporated *in vacuo* at 50 °C to give 4.4 g of pure total alkaloidal mixture.

The alkaloidal mixture was dissolved again in chloroform (40 ml) and then 6o ml petroleum ether (b.r. 40-60 °C) was added. Precipitation of some alkaloids was observed. The precipitated alkaloids were filtered off. The filtrate was concentrated *in vacuo* at 50 °C to give a gummy residue (0.9 g). The residue was dissolved in ethyl acetate (50 ml) and extracted with 250 ml phosphate buffer (pH 2). The aqueous layer was separated and extracted with chloroform (3 x 200 ml) to afford vindoline and catharanthine rich fraction (G, 0.305 g). The buffer layer was turned alkaline (pH 10) using ammonia solution (25 %) and finally extracted with chloroform (3 x 200 ml) to give vinblastine rich fraction (H, 0.1225 g).

Method V: Adsorption on charcoal column

1 kg of *Catharanthus roseus* powdered plant was percolated overnight with methanol (95 %, 3L x 3). The total methanolic extract was filtered and concentrated at 50 °C *in vacuo* to 500 ml, diluted with water (500 ml), then acidified with 1N sulphuric acid (pH 2). The combined aqueous acidic extract was kept overnight in a refrigerator, and then filtered from resinous bodies. The clear filtrate was allowed to pass through a glass column packed with 500 (g) charcoal (4 cm x 100 cm) previously activated at 120 °C in an oven for 2 hrs. Eluates were taken at different intervals and tested for the presence of alkaloids using Mayer`s reagent to be sure that the alkaloidal mixture was completely adsorbed. The column was first washed with distilled water (2 L), then eluted in a gradient manner starting with 30% methanol, followed by 50 %, 70 % and finally 100 % methanol, respectively using 500 ml for each solvent.

The eluted fractions; separately; were concentrated in *vacuo* at 50 °C till solvent free. The aqueous solution was adjusted to pH 6.4 using NH_4OH solution (25 %) and then extracted with methylene chloride (500 ml x 5). The combined methylene chloride extract was washed with distilled water till alkali free, dried over anhydrous sodium sulfate and then evaporated *in vacuo* to dryness to give an alkaloidal fraction eluted by 70% methanol (I) which was found to be vincristine and vinblastine very rich fraction (fraction I, 1.033 g).

The obtained fractions were separately tested by thin layer chromatography (TLC). The results revealed that the four fractions contain total alkaloid with high purity.

Results and Discussion

Five different methods were carried out for the preparation of the antineoplastic alkaloids from *Catharanthus roseus* powdered plant. Method IV depends on partition between solvents and the fifth one depends on adsorption chromatography. The highest percentage of total alkaloids have been obtained by method no. III, but the purest mixture was obtained from method no V, also the two target alkaloids (*viz.* vinblastine and vincristine) were found together in fraction (I). Table (1) showed that, the maximum quantitative yield of vinblastine rich fraction was obtained from method III. Vincristine was obtained in a maximum quantitative yield from method I. Moreover, a qualitative and quantitative HPLC method was adopted for the determination of vinblastine and vincristine. The different methods applied for the preparation of the alkaloids were examined by HPLC and HPTLC techniques to evaluate the most reliable one.

High performance liquid chromatography HPLC and high performance thin layer chromatography HPTLC studies of different fractions; obtained from the five methods used in the preparation of total alkaloids;

revealed the presence of vinblastine (R_f 0.54) in fractions B, D, F and fraction H while vincristine (R_f 0.21) was found in fractions C and E. whereas fraction I was found to contain both of vinblastine and vincristine as main constituents (Table 1).

All the fractions obtained from the five methods for preparing total alkaloids were subjected to HPLC analyses using isocratic elution with phosphate buffer solution of pH 6.5, acetonitril (55 : 45) as mobile phase,

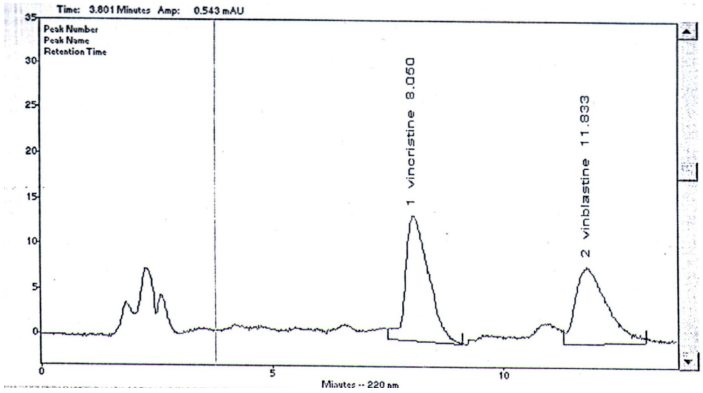

Figure 1: Standard Vb and Vc

Table (1): Quantitative determination of alkaloidal fractions of *C. roseus* (VB & VC rich fractions)

Method	Frations	* % of alkaloidal fractions	*HPLC % of Vb & Vc rich fractions	
			Vb	Vc
I	Vindoline rich fraction (A) Vinblastine rich fraction (B) Vincristin rich fraction (C)	0.748 0.00274 0.0075	- 0.00399 0.00014	- 0.00012 0.00772
II	vinblastine rich fraction (D) Vincristin rich fraction (E)	0.002 0.0015	0.00253 0.0009	0.0007 0.00184
III	vinblastine rich fraction (F)	0.1153	0.1417	0.00017
IV	Vindoline and catharanthine rich fraction(G) Vinblastine rich fraction (H)	0.0087 0.0035	- 0.0041	- 0.0004
V	Vinblastine & Vincristin rich fraction (I)	0.1033	0.0950	0.0110

* Relative to plant dry weight, Mean of duplicate analysis.

according to the conditions mentioned before and the results were shown in Figure 1, these conditions showed difference in retention times of the target authentic alkaloids *viz.* vinblastine and vincristine of 3.771 mins.

From HPLC and HPTLC analyses of the total alkaloids prepared by the five methods, it was illustrated that fraction eluted from charcoal column by 70% methanol (K) gave mixture relatively pure fraction containing the target alkaloids vinblastine (Vb) and vincristine (Vc) with only six other alkaloids, these fraction was subjected to VLC (5g on mixed bed column of equi portions of silica gel g for TLC and neutral Alumina (250 g packed in glass column 120 x 5 cm, i.d.) eluted with chloroform containing increasing proportions of methanol with an increament of 2.5 %, resulted in ten fractions. Fraction eluted by chloroform /methanol 85 : 15 (v/v) was found to contain the target alkaloids with only two more contaminants. The collected fractions containing the target alkaloids (750 mg) were subjected to further purification using the centrifugally accelerated radial chromatography (chromatotrone) using silica gel 2mm thickness (type 7749, Merck, Darmstadt, Germany) eluted by chloroform : methanol 95 : 5 (v/v) with flow rate of 6ml/min. to give pure Vb and Vc. Their identity and purity were established by co- chromatography by TLC, HPTLC and HPLC with authentic samples.

In conclusion: charcoal column is very simple, easy, cheap, perfect and reliable for isolation of highly purified form of the total alkaloids especially Vb and Vc and proved to be very reproducible particularly for the

production scale. Also, the results obtained from HPTLC analysis were promising and in the range of those obtained from HPLC.

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