Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of Indole Alkaloids Isolated from *Catharanthus roseus* (L.) G. Don Cultivated Conventionally and Derived from *In vitro* Cultures

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Abstract

*Catharanthus roseus* (periwinkle) is a medicinal plant commonly known for its wide biological activity. In many countries different parts of this plant are used for the treatment of diabetes, hypertension and for menstrual regulation. Due to the ability of production of alkaloids, which can be applied in cancer therapy, is still extensively investigated. Two, the most valuable alkaloids (vincristine and vinblastine) are present in *C. roseus* in very low concentrations. Micropropagation is promising technique used to enhance the level of important secondary metabolites. The main objective of present study was alkaloids extraction from plants cultivated conventionally and derived from *in vitro* cultures. In this order the aerial parts of periwinkle were extracted with 96% ethanol at room temperature (method I) and heated with 96% ethanol at 55 °C for 90 minutes (method II). The obtained mixtures of different indole alkaloids were analyzed by gas chromatography – mass spectrometry (GC-MS). Analysis revealed the presence of 15 alkaloids, among which vindoline, vindorosine, isovindolinine and ajmalicine were the most abundant. The obtained results indicated that the propagation method had a significant effect on the percentage content of alkaloids in *C. roseus* herb. Plants derived from *in vitro* cultures were richer in vindorosine and vindoline, while conventionally cultivated – in tetrahydroalstonine and ajmalicine. Moreover, in case of isovindolinine, vindoline and ajmalicine, extraction at 55 °C was more effective, while for pericyclivine – maceration at room temperature. Interestingly, the pericyclivine was not detected in the mixture of alkaloids obtained from periwinkle herb by the extraction at 55 °C.

Keywords: bioactivity, extraction method, Madagascar periwinkle, *Vinca* alkaloids, tissue cultures

Introduction

*Catharanthus roseus* (L.) G. Don, known as *Vinca rosea* or Madagascar periwinkle, can be found as an ornamental subshrub in gardens throughout the world (Larbie and Abboah-Offei, 2014). In addition to its pharmaceutical value, periwinkle has long been cultivated as a herbal medicine. It has been used for centuries to treat diabetes, high blood pressure, fever, malaria and chest complaints (Aslam et al., 2010; Gajalakshmi et al., 2013). In India, juice from the periwinkle flowers has been used to treat skin problems, dermatitis, eczema and acne, while juice from the leaves has been applied for wasp stings (El-Sayed and Cordell, 1981). The antibacterial, antifungal, antiviral and antioxidant properties of this herb have also been well documented (Carev and Patterson, 1970; Garg, 2010). *C. roseus* is also a rich source of monomeric and dimeric indole alkaloids, which possess antihypertensive and anticancer activities (Kulkarni et al., 1999; Mishra et al., 2001). Anticancer drugs, vincristine and vinblastine, are produced in *vivo* by the condensation of vindoline and catharanthine, two monomeric precursors (Noble, 1990; Laflamme et al., 2001). These two dimeric alkaloids may be used as single agents or in combination therapy for the treatment of acute leukemia and Hodgkin’s disease as well as for a wide variety of neoplasms including breast, bladder and lung cancers (Noble, 1990; Ramirez et al., 1997; Tabakovic et al., 1997). Other alkaloids, such as ajmalicine and serpentine are used as antihypertensive and anti-
neuro-inflammatory agents, while vindoline, tetrahydroalstonine, catharanthine, and vindoline exhibit hypoglycemic activity (Sveboda, 1969; Marles and Farnsworth, 1995; Almagro et al., 2015).

The alkaloid content varies considerably in the individual parts of C. roseus. In the leaves ranges from 0.32 to 1.16%, in the stems from 0.074 to 0.48%, in the flowers from 0.005 to 0.84%, while in roots – between 0.14 and 1.34% (Joy et al., 1998).

Periwinkle is still intensively studied because of its valuable properties. Researchers are trying to increase the synthesis of alkaloids through the technique of tissue cultures. Also analytical methods for the rapid identification and quantitative extraction of alkaloids are continuously improved.

The aim of this research was to compare the alkaloid composition of Catharanthus roseus cultivated conventionally and derived from in vitro cultures using GC-MS method. Moreover, two different extraction methods were applied in order to show the influence of temperature on identified alkaloids.

Materials and Methods

Plant material

The research material consisted of herb (leafy flowered stems) of periwinkle (Catharanthus roseus (L.) G. Don ‘Mediterranean Lilac’ (Thompson & Morgan, England).

The field experiment was carried out at the experimental station which belongs to the Department of Horticulture of the West Pomeranian University of Technology Szczecin (North-Western Poland). The experiment was established in randomized blocks with four replicates. The experimental plot area was 2.75 m² (40 plants per plot). The field was prepared according to agrotechnique proper for periwinkle cultivation (Thomas et al., 2012). Mineral fertilization, in the form of NPK, was applied during the field preparation, in amounts: 20:30:30 kg ha⁻¹, according to Khode et al. (2000).

There were two methods of plant propagation compared in this study: generative (conventional method of cultivation from seedlings) and in vitro propagation.

In the conventional method, the seedlings of periwinkle were produced in the greenhouse. Seeds were sown in trays with peat moss, on 26th February. The trays were kept in the greenhouse at the temperature 22±2 °C and relative humidity 60±5%. Seedlings were transferred to multiplates containing peat moss on 24th April and then transplanted into the open field on 28th June, at a density of 25×20 cm.

In the in vitro propagation seeds of Catharanthus roseus were immersed in 70% ethanol for 30 seconds followed by surface disinfection in 7.5% NaClO for 15 minutes. Seeds were transferred onto initiation media containing macro- and microelements according to MS medium (Murashige and Skoog, 1962). Seedlings, initiated for growth, were proliferated through the technique of tissue cultures. Also analytical methods for the rapid identification and quantitative extraction of alkaloids are continuously improved.

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Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the alkaloid mixture

The qualitative GC-MS analysis of the obtained alkaloid mixture was carried out using an HP 6890 gas chromatograph, equipped with a HP-5MS capillary column (30 m x 0.25 mm; film thickness 0.25 μm) and coupled with HP 5973 Mass Selective Detector.

The flow rate of carrier gas (He) was 1 mL/min. Samples of 4 μL were injected in the split mode at a ratio of 5:1. The injector and the transfer line were kept at 280 °C. The ion source temperature was 230 °C.

The initial temperature of the column was 40 °C for 5 min, then increased to 60 °C at a rate of 30 °C min⁻¹, next to 230 °C at a rate of 6 °C min⁻¹ (kept constant for 10 min), and then increased to a final temperature of 280 °C at a rate of 30 °C min⁻¹. The oven was held at this temperature for 30 min. Mass spectra were taken at 70 eV. Mass range was from 40 to 550 m/z. Solvent delay was 6 min. The total running time for a sample was about 76 min.

The identification of the alkaloids was confirmed by comparing the mass spectral data with those of authentic compounds and with data obtained from the literature (Djerassi et al., 1962; Gilbert, 1965; Kohl et al., 1981; Zoeller et al., 2005; Zhao et al., 2013; Akhgari et al., 2015).

Further identification was made by comparison of their mass spectra with those stored in Wiley NBS75K, NIST 2005 and NIST 2008 mass spectral libraries (isovindolinine, vindolinine, ajmalicine, tetrahydroalstonine, pleiocarpamine).

Retention indices (RI) values were measured on HP-5MS column for all identified alkaloids. For RI calculation, a reference substance as well as the lack of mass spectra libraries. According to Hisiger and Jolicoeur (2007), about eight indole alkaloids are commercially available and only few (about 11) of the large group of these valuable compounds produced by periwinkle are frequently analyzed. Moreover, GC is unsuitable to the analysis of bisindole alkaloids as they have high melting point (Chen et al., 2013).

Statistical analysis

The results of the study were subjected to an analysis of variance which was performed with AWAR software, made by Department of Applied Informatics, Institute of Soil Science and Plant Cultivation in Puławy, Poland. The three experimental factors (I – alkaloid, II – propagation method, III – extraction method) were tested in a split-split-plot design and the means were separated using the Tukey’s Studentized Range Test at P = 0.05.

Results and Discussion

In the current research the bioactive compounds of C. roseus extracts were analyzed by gas chromatography-mass spectrometry (GC-MS). A total of 15 different alkaloids have been identified (Table 1), among which vindoline (28.695-34.255%), vindorosine (10.665-18.890%) and ajmalicine (6.785-12.660%) were the most abundant. Some alkaloids remained unidentified due to the lack of reference substances as well as the lack of mass spectra libraries. According to Hisiger and Jolicoeur (2007), about eight indole alkaloids are commercially available and only few (about 11) of the large group of these valuable compounds produced by periwinkle are frequently analyzed. Moreover, GC is unsuitable to the analysis of bisindole alkaloids as they have high melting point (Chen et al., 2013).

GC-MS chromatograms of ethanol extracts of C. roseus are presented in Fig. 1 and Fig. 2, while Fig. 3 shows the chemical structures of identified alkaloids.

Vindoline (30.865%) was the main alkaloid of periwinkle herb (Table 1). Vindorosine (14.740%) was the second one. High concentrations were also noted for ajmalicine (9.866%), isovindolinine (9.239%), vindolinine (8.209%) and tetrahydroalstonine (7.504%). The amounts of the other alkaloids ranged between 2.206 and 0.218%.

There was also a significant interaction recorded between alkaloid and propagation method. Significant differences were noted in case of the four of alkaloids. Higher amounts of vindorosine and vindoline were detected according to the in vitro plant propagation, while for tetrahydroalstonine and ajmalicine – conventional method of propagation. Moreover, an interaction between alkaloid and extraction method was also statistically significant for some of the compounds. In the case of isovindolinine, vindolinine and ajmalicine, extraction at 55 °C was more effective, while for pericyclivine – maceration at room temperature. When using extraction at 55 °C pericyclivine was not detected.

Mass spectrometry of C. roseus extracts indicated that isovindolinine, vindolinine, 19-epivindoline, 20-epivindoline and tabersonine have the same molecular ion at m/z 336 (Table 2).
The vindolinine series. This group is dominated by vindolinine, 19-piperidine portion of the molecule and occurs in all mass spectra of 1), very close to each other. Moreover, 19-epivindolinine, 19-Epivindolinine, 19-Epivindolinine, 19-Epivindolinine, and tabersonine showed similar fragmentation patterns and retention times (Table I), very close to each other.

Deperss et al. (1962) reported that peaks at m/z 93, 107, 120, 121, 122 as well as at m/z 134, 135 are associated with the piperidine portion of the molecule and occurs in all mass spectra of the vindolinine series. This group is dominated by vindolinine, 19-epivindolinine and their N-oxides.

Vindoline and tabersonine with [M+] at 336 m/z are considered as catharanthine isomers (Ferreres et al., 2010).

Interestingly, the base peak at m/z 336 (100%) in vincoline and tabersonine [M+] at 336 m/z are considered as catharanthine isomers (Ferreres et al., 2010). The presence of peaks at m/z 93, 107, 120, 121, 122 and 134, 135 confirms the identification of vindoline and tabersonine.
Pleiocarpamine shows prominent signals at m/z 322 (M⁺), m/z 263 (M⁺-COxCH₃) and m/z 180 (quinolinium ion). Our results are in agreement with published data for pleiocarpamine (Hesse et al., 1964; Akhgari et al., 2015).

Comparing the mass spectrum of coronaridine with literature data (Zoccoler et al., 2005), we were observing only small differences in the relative ion intensities.

The mass spectrum of percyclivine was characterized by the ions at m/z 322, 321, 291, 263 and 169. Similar fragmentation patterns were noted by Vieira et al. (2008).

The spectra of ajmalicine and tetrahydroalstonine exhibited fragments of the β-carboline skeleton at m/z 184, 170, 169 and 156, which is in accordance with the results published by Hesse (1974). However, both compounds were identified according to standards.

Vindorosine (molecular weight, 426) showed difference of 30 m/z in mass as compared to vindoline (molecular weight, 456). This may suggest that vindorosine is derivative of vindoline. Moreover, vindoline and vindorosine have the same molecular ion at m/z 426. Slight differences were noted only in their intensity. Detailed fragmentation patterns of the mass spectra of these compounds can be found in Table 2. The major peaks observed in the mass spectrum of vindorosine were the same as those reported by Zhao et al. (2013).

Our results indicated that the GC-MS method can be successfully applied for the analysis of the mixture of periwinkle alkaloids without necessity of derivatization. Kreh et al. (1995), who applied for the first time GC-MS in the analysis of underivatized Amaryllidaceae alkaloids from *Narcissus pseudonarcissus* have demonstrated its advantages over the analysis of silylated samples. Tram et al. (2002) reported the results of GC-MS analysis of underivatized alkaloids from leaves of *Grunia latifolia*, while Tosun and Tamer (2004) used this method for determination of pyrrolizidine alkaloids in seeds of *Heliotropium europaeum*.

According to Subbaian et al. (2014), gas chromatography coupled to mass spectrometry is the best for identification of long chain hydrocarbons, alcohols, acids, esters, alkaloids, steroids and nitrogen compounds.

*Catharanthus roseus* is one of the main source of terpenoid indole alkaloids used for the preparation of diabetic drugs, cardiac drugs, in hypertension and in anticancer drugs formation by the pharmaceutical industries (Misra et al., 2009). Moreover, some of these therapeutic molecules are obtained by semi-synthesis using natural precursors extracted from *C. roseus* leaves.

The low content of dimeric alkaloids in the plant as well as their high market price encouraged the researchers to improve the production of these valuable metabolites through the technique of tissue cultures. The influence of concentration of different growth regulators on the production of callus and the amount of synthesized alkaloids have been investigated (Misawa, 1994; Junaid et al., 2008; Kalidass et al., 2010; Verma et al., 2012; Soleimani et al., 2013).

Soleimani et al. (2013) reported stimulatory effect of 2,4-D (2,4-dichlorophenoxyacetic acid) on callus formation as well as on the level of vinblastine and vincristine. They also found that increasing the concentration of kinetin resulted in a significant decrease in callogenesis and alkaloid production. Kalidass et al. (2010) demonstrated that the use of kinetin in a concentration above 1 mgdm⁻³ had less effect of vinblastine production. Moreover, increase of its concentration was associated with decrease of vinblastine synthesis.

Verma et al. (2012) revealed that half strength basal MS medium supplemented with 2,4-D and BAP (0.5 mgdm⁻³ and 1.0 mgdm⁻³, respectively), and with 6% sucrose was the best for callus biomass production along with high alkaloid content.

Generally, the auxins were approval as the best for callus proliferations and growth. However, the combination of auxins with cytokinins were found to be better for leaf callus growth and enhancement in alkaloids content (Misawa, 1994; Junaid et al., 2008).

An antagonistic effect of gibberellins and cytokinins on the biosynthesis of monoterpenoid indole alkaloids in *C. roseus* was reported by Amini et al. (2009). According to them, the inhibitory effect of gibberellin was correlated with the silencing of two genes, encoding enzymes of the alkaloids biosynthetic pathway. Normally this process is regulated by cytokinins. In case of exogenously applied cytokinin they observed considerably increase of synthesis of ajmalicine and serpentine in untransformed cotyledon callus. The use of 2,4-D decreased the accumulation of these metabolites.

Garnier et al. (1996) found that BAP (6-benzylaminopurine) and in some cases NAA (naphtaleneacetic acid) stimulated the accumulation of vindoline and catharanthine in periwinkle.

In general, MS method is most widely used in tissue cultures, but the number of inorganic and organic salts and their levels are usually different. The ammonium and nitrate ions inhibit alkaloids production (Van Gulik et al., 1993). These salts are added to the media for growth promotion and differentiation.

The maximum production of alkaloids occurs at low levels of phosphate, nitrate and ammonia in the medium (Schlatmann et al., 2015).
Vitamins and organic components had slight effect on the alkaloids production. Only in case of glucose positive effect on ajmalicine production in hairy-root culture was noted by Moreno et al. (1992). On the other hand, Zhao et al. (2001) showed that the addition of succinic acid, tryptamine and tryptophan to the culture medium caused significant growth of ajmalicine and catharanthine levels.

The present study indicated that the MS basal medium supplemented with kinetin (2.0 mg dm⁻³), IBA (1.0 mg dm⁻³), NAA (0.2 mg dm⁻³) and sucrose (30 mg dm⁻³) caused the increase of the production of vindorosine and vindoline in C. roseus tissue cultures. However, increasing the levels of important periwinkle alkaloids is extremely difficult and dependent on many factors. This explains why the role of different plant growth regulators in regulating monoterpene indole alkaloids (MIAs) biosynthetic pathway is still being extensively investigated.

Conclusion

The obtained results showed that the plants cultivated conventionally had higher amounts of ajmalicine and tetrahydropalmatine, while these from in vitro propagation – higher amounts of vindorosine and vindoline. Moreover, the temperature of extraction also affected the percentage content of isolated alkaloids. In case of isovindolinine, vindolinine and catharanthine the more effective was extraction at 55 °C, while for periclyclinium – maceration at room temperature. Our study may find wide applications in the optimization of production and isolation of Vinca alkaloids.

References


