Optimized Thin Layer Chromatographic Method for Screening Pharmaceutically Valuable Alkaloids of *Catharanthus roseus* (Madagascar Periwinkle)

N.L. Magagula , V. Mohanlall and B. Odhav

Department of Biotechnology and Food Technology, Durban University of Technology,

P.O. Box 1334, Durban 4001, South Africa

Corresponding author. E-mail: vireshm@dut.ac.za

Contact: (cell) +2782 308 3904/ (work) +2731 373 5321

Abstract

The aerial parts of *Catharanthus roseus (C. roseus)* plant have been known to contain many important alkaloids such as Vincristine, Vinblastine, Vindoline and Ajmalicine which are of interest to this study. Ajmalicine has also been found in abundance in the roots of this medicinal plant. The main objective of this study was to screen a methanolic extract of *C. roseus* for the presence of pharmaceutically valuable alkaloids using previously optimized conditions established in our laboratory set up. This methanolic extract was further purified for specific alkaloids present using modified Shams *et al.* (2009) method I extraction procedure. Extracts of *C. roseus* have been subjected to optimum conditions before and after Thin layer Chromatography which was performed in a trough chamber of 10 cm x 10 cm area saturated with Ethyl acetate: Benzene: Methanol: 25% Ammonia solution (100:5:5:3) as a developing solvent and sprayed with Cerium Ammonium Sulphate (CAS) reagent specific for the detection of alkaloids. Results have shown that the alkaloids of interest; Vincristine, Vinblastine, Vindoline and Ajmalicine could be chromatographically identified in extracts of *C. roseus* intact plant after individual alkaloid purification procedure. This has confirmed the anti-neoplastic and anti-hypertensive properties these alkaloids have in making the plant effective in healing cancer and high blood pressure as it was originally used by traditional people.

Key words: *Catharanthus roseus*, Vincristine, Vinblastine, Vindoline, Ajmalicine, Thin Layer Chromatography, Cerium Ammonium Sulphate reagent.

Introduction

This preliminary investigation has reported the presence of alkaloids of interest in the intact plants of *C. roseus*. These alkaloids have been found by previous researchers to be responsible for some of the properties such as anti-neoplastic characteristics which have been owed to the presence of **Vincristine** and **Vinblastine**. The anti-hypertensive characteristics have been due to the presence of **Ajmalicine** which has been found to act as an α_1-adrenergic receptor antagonist (also known as alpha blocker) with preferential actions over α_2-adrenergic receptors, underlying its hypotensive rather than hypertensive effects (Wink and Roberts (1998); Roquebert and Demichel (1984). Ajmalicine has also been found to combat diseases such as heart arrhythmias and also used to improve blood circulation in the brain (Van Der Heijden, Jacobs et al. (2004); Wink and Roberts (1998). **Vindoline** is a plumeran moiety or a precursor in the biosynthetic pathway of Vincristine and Vinblastine. Its absence in the plant will therefore determine that the two bisindole alkaloids could not be formed (Schmelzer (2011). The structures of few known alkaloids present in the upper shoots of the plant and which are of interest in this study are shown in figure 1 (a-d).

The purpose of this study was to screen for the presence of alkaloids in the intact plant of *C. roseus* and see if they could be semi-quantitatively analyzed using a simplified, optimized TLC methods and results previously established for alkaloid standards (Aniszewski (2007). TLC being a less sensitive method of detection, it has been discovered that *C. roseus* alkaloid, Ajmalicine could be detected from as low as 0.0007% at 10 ul volume by TLC. Vincristine could be detected from as low as 0.0055% at 10 ul volume whereas Vinblastine and Vindoline were not that sensitive, could both be only detected from as high as 0.05% concentrations at 10 ul volume, Table 1.1 (Magagula, Mohanlall et al. (2012/3).

Cerium Ammonium Sulphate (CAS) reagent has been a specific chromogenic reagent used in enhancing detection of alkaloids chromatographically since it has been known to react with substance analyte to produce visible coloured spots on TLC plate. Most colour reactions could be viewed before heat actvation at concentrations as high as 0.05%. Temperature also has played an important role in increasing intensity of viewing samples on TLC plates over time. Individual sample colour reactions were different before and after heat activation and when viewed under UV light, this has been shown in Table 1.2 and 1.3. Different alkaloids have shown different colour reactions in the following manner: TLC plate white background 1) before heat; 2) after heat; and 3) under UV. These have been as follows for Ajmalicine 1) clear to yellowish green; 2) blackish green and 3) blue to blue around yellow. Vindoline: 1) pink to pink around yellow; 2) purple/bluish black and 3) grayish black. Vincristine: 1) clear to brownish; 2) yellow pink/purplish and 3) yellow to blue around yellow. Vinblastine: 1) brownish pink; 2) purple and 3) pink/orange to yellow around orange, respectively.

Table 1.3 has also shown that colour reaction on individual alkaloids was dependent on sample concentration. For example Ajmalicine appeared blue at low concentration when viewed under UV light, at high concentration the blue colour developed around yellow colour. Vincristine had the same colour reaction as Ajmalicine at high concentration whereas at low concentration it gave a yellow colour reaction under UV light.

Vindoline appeared pink at concentration as low as 0.1 % on TLC plate white background before heat treatment, at high concentration greater than 0.2% the pink colour started to develop around yellow colour.

A most popular way of detecting sample components on TLC plate was through measurement of retention factor (RF) which was defined as the distance travelled by the sample over distance travelled by the solvent. RF values could be kept constant by keeping chromatography conditions constant. Such conditions include solvent system, adsorbent, thickness of the adsorbent, amount of the sample spotted as well as temperature. Some of the previously established RF values of *C. roseus* alkaloids under optimized conditions have been illustrated on Table 1.4. RF values together with the differences in CAS colour reactions could simplify detection for closely related complex structured alkaloids which might show more or less the same characteristics in some properties.

Materials and Methods

Standard Preparation

When sensitivity of TLC was investigated for the purpose of detecting alkaloids of interest in our laboratory. We found that most alkaloid standards were detectable at 0.05% and thus, standards were prepared accordingly by dissolving 5 mg in 10 ml methanol.

Plant Material

Some aerial parts of *C. roseus* intact plant were provided by Prof Baijanath (Department of Biotechnology and Food Technology, Durban University of Technology) and these were sterilized using 70% methanol, rinsed three times with distilled water, then dried at 35 ^{o}C for 8 - 16 days which was found to be the best optimum period for drying. The dried plant material was then milled into a powdered form using a blender.

Optimization of extraction method

In our previous laboratory experiment we established that for every 10 gm powdered sample extracted for TLC analysis, 3 ml methanol added to every final extract dried in vacuo, was sufficient to concentrate alkaloids to a detectable levels for analysis.

Extraction method

Powder of 250 gm of dried aerial parts of plant material was added to 1.56 L of 80% methanol and kept in a room temperature in a shaker at 160 rpm for 48 hours. The extract was then filtered and dried in vacuo at 50 ^{o}C till dryness. Sufficient methanol was added to chromatographically screen the methanol extract for the presence of alkaloids. The remainder of the methanolic extract was then dried at 50 ^{o}C. A small volume of 15.63 ml 2% tartaric acid was used to acidify the dried extract. Equal volume of dichloromethane was added into the acidified extract and this was used to extract chlorophyll, neutral alkaloids and other neutral compounds. Separation of extraction solvents was achieved by centrifugation at 4000 rpm for 10 minutes at 23 ^{o}C. The tartaric acid layer was removed and adjusted to pH 5.9 with 25% NH_4OH. Most neutral alkaloids remained in the organic phase while most other alkaloids were isolated after basification. The two separated liquid phases were further extracted to obtain individual alkaloid rich extracts using modified method 1 according to Shams *et al.* (2009) as follows:

Vindoline - rich extraction:

The remaining dichloromethane layer was washed three times with distilled water. Water layer was decanted and the organic layer was dried over anhydrous Sodium Sulphate. This was then filtered and concentrated in vacuo at 50 ^{o}C till dryness. The extract was then dissolved in 75 ml methanol for chromatographic analysis.

Vinblastine - rich extraction:

The alkaloids in the adjusted pH 5.9 layer were further extracted with equal volume of dichloromethane. Dichloromethane layer was removed and adjusted to pH 2.5 with 0.1M aqueous citric acid. The citric acid layer was removed, adjusted to pH 4.4 with 25% NH_4OH and the liberated alkaloids were extracted with equal volume of dichloromethane. Citric acid layer (pH 4.4) was separated from Vinblastine rich organic layer which was evaporated in vacuo and the resulted extract was dissolved in 75ml methanol. The liberated alkaloids from the acid layer were further extracted with equal volume of dichloromethane. The organic layer was removed from citric acid layer which was kept for further extraction. The organic layer was rich in Vinblastine and therefore, dried over anhydrous Sodium Sulphate, filtered, evaporated at 50 ^{o}C till dryness and then dissolved in 75 ml methanol. The two resulted Vinblastine methanolic extracts were combined for analysis.

Vincristine – rich extraction:

The citric acid (pH 4.4) layer kept was adjusted to pH 5.9 and the liberated alkaloids were extracted with dicloromethane. The Vincristine - rich organic layer was removed from the citric acid layer, dried and dissolved in the same volume of methanol as mentioned previously, for further analysis with TLC.

Optimization of TLC method

The spotting volume for both standards and sample extracts were worked out to be 25 microliters using 5 ul adjusted finn pipette to allow alkaloid detection in our sample extracts. For standards, we considered that the concentration will increase as the spotting volume is increased. For instance 5 ul will equal to 0.05% known concentration, 10 ul will increase to 0.1%, 15 ul will increase to 0.15%, 20 ul to 0.2% and 25 ul will increase concentration to 0.25%, Table 1.3. The optimum drying of the TLC plate was achieved by heat activation at 100 ^{o}C for 30 minutes.

TLC method

Analytical and preparative thin layer chromatography was performed according to the methods illustrated by Shams, Nazif et al. (2009) on silica gel plates Merck 60 F 254 with Ethyl acetate: Benzene: Methanol: 25% Ammonia solution (100:5:5:3) as solvent system. This method was slightly modified in that we used methanol instead of ethanol and preparative plant extract procedures were also different. Chromatograms after air drying were sprayed with Cerium Ammonium Sulphate (CAS) chromogenic reagent. CAS reagent reacted externally with most TLC plate sample analytes that were greater or equal to 25 ul spotting volume and at 0.05% concentration. 1% Cerium Ammonium Sulphate was prepared in 85% phosphoric acid (Baerheim-Svendsen and Verpoorte (1983). TLC plates were then activated by heat at 100 ^{o}C for 30 minutes and then visualized under ultra violet light at 366 nm.

Results

CAS reagent reaction on TLC plate

Cerium Ammonium Sulphate chromogenic reagent has shown differences in colour reaction of alkaloids on TLC plate white background before heat activation, after heat activation as well as under 366 nm UV light. This was illustrated on Table 2.1.

Migration of unpurified methanolic extracts on TLC plate after activation with CAS reagent

Approximately 25 ul alkaloid standards were spotted on TLC plate and their migration were compared to 25 ul migrated spots of samples of unpurified methanolic extract after spraying with CAS reagent. These results were shown on Figure 2.1.

Migration of purified methanolic extracts on TLC plate before heat activation

Approximately 25 ul Alkaloids standards were spotted on TLC plate and their migration were compared to 25 ul migrated spots of *C. roseus* samples of purified methanolic extract before they were exposed to heat for activation and this was illustrated on Figure 2.2.

Migration of purified methanolic extracts on TLC plate after heat activation

CAS staining required some heat to develop fully. Figure 2.3 has shown a different colour reaction after activation with heat compared to prior heating and these were confirmed by viewing under UV light at 366 nm, Figure 2.4.

RF Values

In addition to different colour reactions mentioned, the retention factor (Rf) which has been a measure for separation of sample components has been defined as the distance travelled by the compound divided by the distance travelled by the solvent (Ataei-Azimi, Hashemloian et al. (2008). The RF values for alkaloid standards were calculated and then compared with those calculated for sample extracts and these were illustrated on Table 2.2.

The presence of alkaloids of interest in each alkaloid specific extract after purification

Vinblastine rich extract has shown the presence of all four alkaloids of interest. Vincristine rich extract has shown the presence of other three alkaloids except for Vinblastine. Vindoline rich extract has show the presence of both Vindoline and Ajmalicine only, Table 2.3.

Discussion

Methanol extract obtained from aerial parts of *C. roseus* was carried out to thin layer chromatography in order to establish the purity and composition of materials. Figure 2.1 (A – B) was a representation of methanolic *C. roseus* sample extracts before purification whereas Figure 2.2 – 2.4 represented methanolic extracts after purification procedure. Composition of materials became more visible and clearer after purification. Lane 1 of Figure 2.2 – 2.4 was a mixture of four standards before heat activation, after heat activation, and when viewed under UV light. Only the colour reactions for Vinblastine and Vindoline could be detected before heat activation. Heat activation for 30 minutes at 100 ^{o}C has shown the presence of all alkaloids of interest in the purified extract of *C. roseus* intact plant, Figure 2.3.

Though Ajmalicine was still not quite clear even after treatment, its presence was confirmed by viewing the heat activated plate under UV light, Figure 2.4. When comparing lane 4 of purified Vinblastine rich extract with that of standards, we noted that all alkaloids of interest could be detected though the extract was meant to isolate Vinblastine. This was also illustrated on Table 2.3. Vinblastine could not be detected in lane 5 and 6. Lane 5 of purified Vincristine rich methanolic extract, has confirmed the presence of the other three alkaloids of interest. Vindoline rich extract on the other hand only confirmed the presence of Vindoline and Ajmalicine. Vindoline was highly detected lane 6 than in any other lane and this was also confirmed by CAS colour reaction which formed a pink colour around yellow before heat activation, to confirm its highest concentration in the extract (refer to Table 2.1).

RF values for these compounds were calculated to be: Vincristine was observed at RF = 0.45; Vinblastine was observed at RF = 0.63; Vindoline was observed at RF = 0.77 and Ajmalicine was observed at RF = 0.92. The mobile phase used was a good separating solvent since it could separate Vincristine from Vinblastine which usually have a tendency to co-elute due to their similarities in structures.

The previous researchers have reported more than 130 alkaloids present in *C. roseus* plant. The alkaloids present in the aerial parts of *C. roseus* were well extracted using Shams *et al.* (2009) modified method**.** During separation for each specific alkaloid rich extract, it was observed that not only the alkaloids of interest were present per specific alkaloid rich extract. For instance, both Vinblastine rich extract and Vindoline rich extract resulted in separation of 13 bands including alkaloids of interest. Vincristine rich extract resulted In separation of 8 bands.

The alkaloids have been separated successfully by this optimized thin layer chromatography technique. The results have proven the technique to be reproducible since they were more or less the same with those obtained in our previous work. This technique was able to detect Vincristine and Vinblastine at its lowesr intact plant

concentration. These optimized extraction and separation methods can therefore, be useful in optimizing screening of Vincristine, Vinblastine, Vindoline and Ajmalicine that are present in *C. roseus* intact plant. The optimized methods may also be useful with screening of other alkaloids provided that their specific methods of extraction are known.

References

Aniszewski, T. (2007). Alkaloids--secrets of Life: Alkaloid Chemistry, Biological Significance, Applications and Ecological Role. Netherlands, Elsevier**:** 316.

Ataei-Azimi, A., B. D. Hashemloian, et al. (2008). "High in vitro production of ant-canceric indole alkaloids from periwinkle (Catharanthus roseus) tissue culture." African Journal of Biotechnology **7**(16): 2834-2839.

Baerheim-Svendsen, A. and R. Verpoorte (1983). Chromatography of alkaloids Part A: Thin Layer Chromatography. Amsterdam, Elsevier. **23**.

Magagula, N. L., V. Mohanlall, et al. (2012/3). Optimizing production of alkaloids in cell suspension culture, callus culture, and *Agrobacterium rhizogen* transformed hairy root culture of *Catharanthus roseus* South Africa, Durban University of Technology.

Roquebert, J. and P. Demichel (1984). "Inhibition of the alpha 1 and alpha 2-adrenoceptor-mediated pressor response in pithed rats by raubasine, tetrahydroalstonine and akuammigine." Eur J Pharmacol. **106**(1): 3.

Schmelzer, G. H. (2011). Plant Resources of Tropical Africa. African ornamentals. Proposals and examples. *Catharanthus roseus* (L) G. Don. PROTA. Wageningen, Netherlands, PROTA foundation**:** 56.

Shams, K. A., N. M. Nazif, et al. (2009). "Isolation and characterization of antineoplastic alkaloids from *Catharanthus roseus* L. Don. Cultivated in Egypt." Afr. J. Traditional, Complementary and Alternative Medicines **6**(2): 118 - 122.

Van Der Heijden, R., D. Jacobs, et al. (2004). "The Catharanthus alkaloids: pharmacognosy and biotechnology." Current Medicinal Chemistry **11**(5): 607-628.

Wink, M. and M. F. Roberts (1998). Alkaloids: biochemistry, ecology, and medicinal applications. Alkaloids: biochemistry, ecology, and medicinal applications. New York, Plenum Press**:** 479.

Legend for Figures

Figure 1: Structure of indole alkaloids: a) Ajmalicine, b) Vindoline, c) Vincristine and d) Vinblastine.

Figure 2: Vindoline reaction with CAS reagent before heat activation: Lane 1 – 0.1% at 10 ul; Lane 2 – 0.1% at 20 ul; Lane 3 = 0.1% at 30 ul; Lane 4 = 0.1% at 40 ul; Lane 5 = 0.1% at 50 ul; Lane 6 = 0.05% at 10 ul.

Figure 2.1: Comparison of migration of alkaloid standards with alkaloids of unpurified methanolic extracts on white background of TLC plate after activation with CAS reagent; A) before heat activation B) After heat activation under 366 nm UV light. Lane 1 = Vincristine standard; Lane 2 = Vinblastine standard; Lane 3 = Vindoline standard; Lane 4 = Ajmalicine standard; Lane 5 = 8 days unpurified methanolic extract; Lane 6 = 16 days unpurified methanolic extract.

Figure 2.2: Comparison of migration of alkaloid standards with alkaloids of methanolic extracts after purification before they were exposed to heat for activation. Lane 1= Mixture of 4 standards; Lane 2 = Vincristine; Lane 3 = Vinblastine; Lane 4 = Vinblastine rich extract; Lane 5 = Vincristine rich extract; Lane 6 = Vindoline rich extract.

Figure 2.3: Migration of alkaloid standards and purified methanolic extracts after heat activation. Lane 1= Mixture of 4 standards; Lane 2 = Vincristine; Lane 3 = Vinblastine; Lane 4 = Vinblastine rich extract; Lane 5 = Vincristine rich extract; Lane 6 = Vindoline rich extract.

Figure 2.4: Migration of alkaloid standards and purified methanolic extracts under 366 nm UV light. Lane 1= Mixture of 4 standards; Lane 2 = Vincristine; Lane 3 = Vinblastine; Lane 4 = Vinblastine rich extract; Lane 5 = Vincristine rich extract; Lane 6 = Vindoline rich extract.

Legend of Tables

Table 1.1: Previous development of optimized screening of alkaloid standards show TLC sensitivity based on different standards concentration and standards sample volume. TLC Plates, Silica gel Merck 60 F254, (10cm x 10 cm) were used. (+/- = Slightly positive results; + = positive results; - = negative results).

Table 1.2: Previous development of optimized screening of alkaloids show colour reaction visible on the white background of TLC plate after spraying with CAS reagent before and after heat activation.

Table 1.3: Previous development of optimized screening of alkaloid standards show different colour reactions viewed on TLC plate under 366 nm UV light, (+/- = slightly, - = negative).

Table 1.4: Previous development of optimized screening of alkaloid standards show RF values on TLC plate, 10cm x 10cm Slilica gel Merck 60 F254.

Table 2.1: Illustration of Cerium ammonium colour reactions on TLC plate before and after heat activation as well as under 366 nm UV light.

Table 2.2: Comparison of calculated RF values of Vc = Vincristine, Vb = Vinblastine, Vd = Vindoline and Ajm = Ajmalicine standards with that of sample extract.

Table 2.3: Representation of alkaloids present in each alkaloid specific extract: Vb rich = Vinblastine rich extract; Vc rich = Vincristine rich extract; Vd rich = Vindoline rich extract, + = present; - = absent.

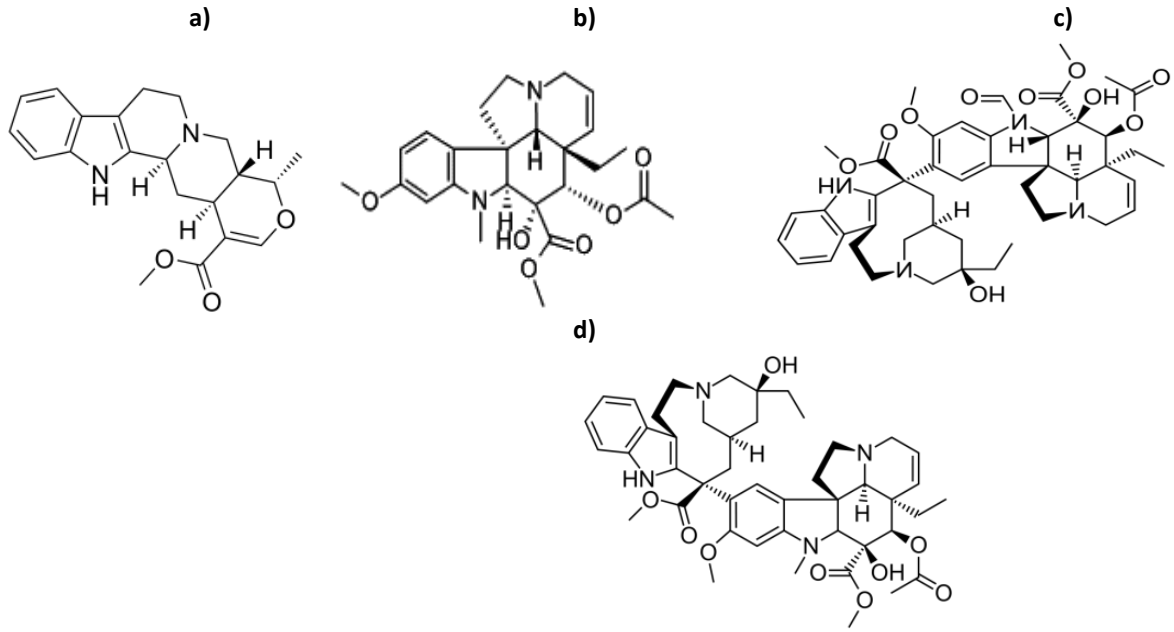
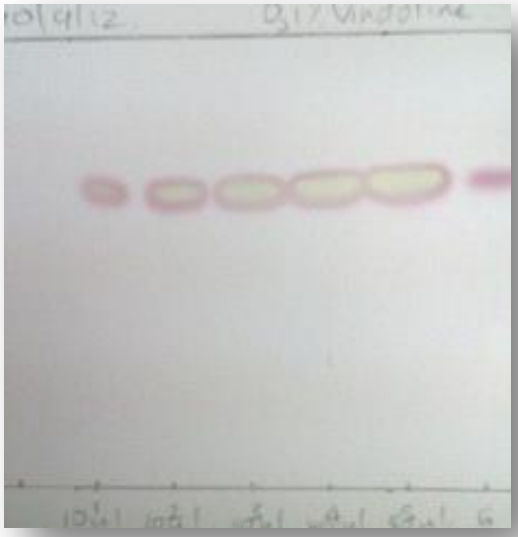


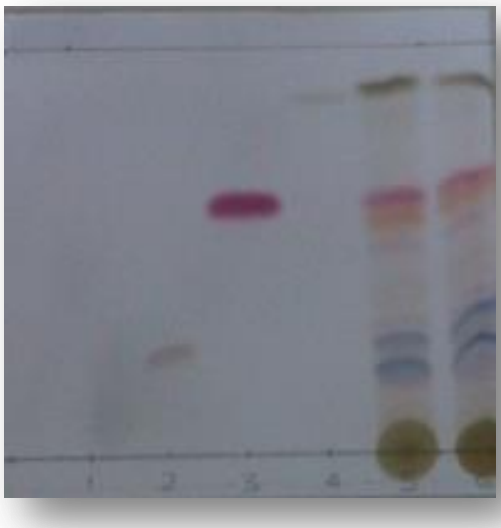
Figure 1: Structure of indole alkaloids a) Ajmalicine, b) Vindoline, c) Vincristine and d) Vinblastine



1 2 3 4 5 6

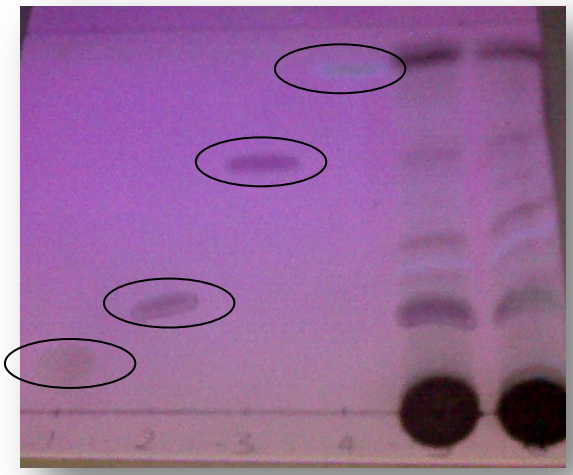
Figure 2: Vindoline reaction with CAS reagent before heat activation: Lane 1 – 0.1% at 10 ul; Lane 2 – 0.1% at 20 ul; Lane 3 = 0.1% at 30 ul; Lane 4 = 0.1% at 40 ul; Lane 5 = 0.1% at 50 ul; Lane 6 = 0.05% at 10 ul

A)



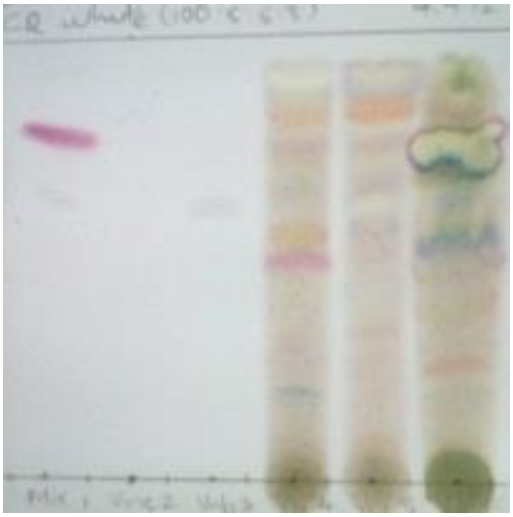
1 2 3 4 5 6

B)



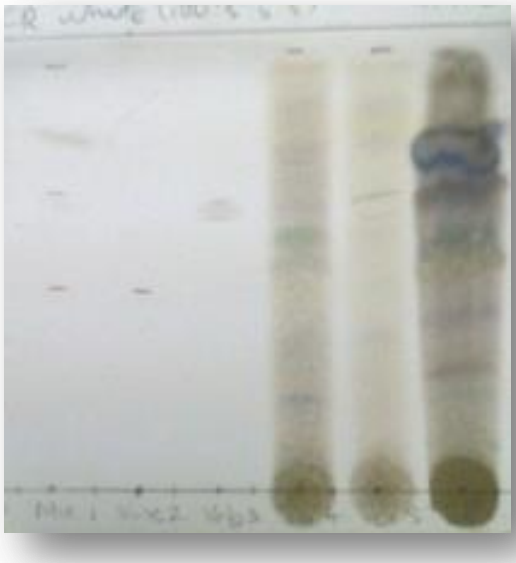
1 2 3 4 5 6

Figure 2.1: Comparison of migration of alkaloid standards with alkaloids of unpurified methanolic extracts on white background of TLC plate after activation with CAS reagent; A) before heat activation B) After heat activation under 366 nm UV light. Lane 1 = Vincristine standard; Lane 2 = Vinblastine standard; Lane 3 = Vindoline standard; Lane 4 = Ajmalicine standard; Lane 5 = 8 days unpurified methanolic extract; Lane 6 = 16 days unpurified methanolic extract.



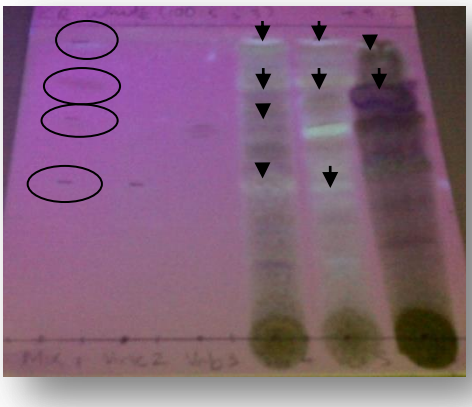
1 2 3 4 5 6

Figure 2.2: Comparison of migration of alkaloid standards with alkaloids of methanolic extracts after purification before they were exposed to heat for activation. Lane 1= Mixture of 4 standards; Lane 2 = Vincristine; Lane 3 = Vinblastine; Lane 4 = Vinblastine rich extract; Lane 5 = Vincristine rich extract; Lane 6 = Vindoline rich extract.



1 2 3 4 5 6

Figure 2.3: Migration of alkaloid standards and purified methanolic extracts after heat activation. Lane 1= Mixture of 4 standards; Lane 2 = Vincristine; Lane 3 = Vinblastine; Lane 4 = Vinblastine rich extract; Lane 5 = Vincristine rich extract; Lane 6 = Vindoline rich extract.



1 2 3 4 5 6

Figure 2.4: Migration of alkaloid standards and purified methanolic extracts under 366 nm UV light. Lane 1= Mixture of 4 standards (from bottom: Vincristine, Vinblastine, Vindoline, Ajmalicine); Lane 2 = Vincristine; Lane 3 = Vinblastine; Lane 4 = Vinblastine rich extract; Lane 5 = Vincristine rich extract; Lane 6 = Vindoline rich extract.

Standard	Volume (ul)	Concentration (%)						
		0.0003	0.0007	0.001	0.003	0.0055	0.05	0.1
Vincristine	10	–	–	–	–	–/+	+	+
	20	–	–	–	–	+	+	+
	30	–	–	–	–	+	+	+
	40	–	–	–	–	+	+	+
	50	–	–	–	–	+	+	+
Vinblastine	10	–	–	–	–	–	+	+
	20	–	–	–	–	–	+	+
	30	–	–	–	–	–	+	+
	40	–	–	–	–	–	+	+
	50	–	–	–	–	–	+	+
Vindoline	10	–	–	–	–	–	+	+
	20	–	–	–	–	–	+	+
	30	–	–	–	–	–	+	+
	40	–	–	–	–	–	+	+
	50	–	–	–	–	–	+	+
Ajmalicine	10	–	–/+	+	+	+	+	+
	20	–	+	+	+	+	+	+
	30	–	+	+	+	+	+	+

	40	–	+	+	+	+	+	+
	50	–	+	+	+	+	+	+

Table 1.1: Previous development of optimized screening of alkaloid standards show TLC sensitivity based on different standards concentration and standards sample volume. TLC Plates, Silica gel Merck 60 F254, (10cm x 10 cm) were used. (+/- = Slightly positive results; + = positive results; - = negative results)

Alkaloid standard	Colour reaction with CAS reagent	
	Before heat activation	After heat activation
Vincristine	Pink/yellowish	Pink/purplish
Vinblastine	Brownish pink	Purple
Vindoline	Pink/pink around yellow	Purple/bluish black
Ajmalicine	Yellowish green	Blackish green

Table 1.2: Previous development of optimized screening of alkaloids show colour reaction visible on the white background of TLC plate after spraying with CAS reagent before and after heat activation.

Standard	Volume (ul)	Concentration (%)					
		0.0007	0.001	0.003	0.0055	0.05	0.1
Vincristine	10	-	-	-	Yellow	Blue/yellow	Blue around yellow
	20	-	-	-	Yellow	Blue around yellow	Blue around yellow
	30	-	-	-	Yellow	Blue around yellow	Blue around yellow
	40	-	-	-	Yellow	Blue around yellow	Blue around yellow

	50	-	-	-	Yellow	Blue around yellow	Blue around yellow
Vinblastine	10	-	-	-	-	Pink/orange	Yellow around orange
	20	-	-	-	-	Yellow around Orange	Yellow around orange
	30	-	-	-	-	Yellow around orange	Yellow around orange
	40	-	-	-	-	Yellow around orange	Yellow around orange
	50	-	-	-	-	Yellow around orange	Yellow around orange
Vindoline	10	-	-	-	-	Grey black	Grey black
	20	-	-	-	-	Grey black	Grey black
	30	-	-	-	-	Grey black	Grey black
	40	-	-	-	-	Grey black	Grey black
	50	-	-	-	-	Grey black	Grey black
Ajmalicine	10	+/-	Blue	Blue	Blue	Blue	Blue around yellow
	20	Blue	Blue	Blue	Blue	Blue around yellow	Blue around yellow
	30	Blue	Blue	Blue	Blue	Blue around yellow	Blue around yellow
	40	Blue	Blue	Blue	Blue	Blue around yellow	Blue around yellow
	50	Blue	Blue	Blue	Blue	Blue around yellow	Blue around yellow

Table 1.3: Previous development of optimized screening of alkaloid standards show different colour reactions viewed on TLC plate under 366 nm UV light, (+/- = slightly, - = negative).

	Vincristine	Vinblastine	Vindoline	Ajmalicine
RF value	0.44	0.63	0.81	0.91

Table 1.4: Previous development of optimized screening of alkaloid standards show RF values on TLC plate, 10cm x 10cm Slilica gel Merck 60 F254.

Standard	Colour reaction on TLC plate		
	Before heat	After heat	UV (366 nm)
Vincristine	Brownish but not clear	Pink-purple	Yellow
Vinblastine	Brownish pink	Purple	Pinkish orange
Vindoline	Pink around yellow	Blue/black	Grey black
Ajmalicine	Clear	Yellow green	Blue

Table 2.1: Illustration of Cerium ammonium colour reactions on TLC plate before and after heat activation as well as under 366 nm UV light

	Vc	Vb	Vd	Ajm
Standard	0.44	0.63	0.76	0.9
Sample	0.45	0.63	0.77	0.92

Table 2.2: Comparison of calculated RF values of Vc = Vincristine, Vb = Vinblastine, Vd = Vindoline and Ajm = Ajmalicine standards with that of sample extract

Standard	Vb rich	Vc rich	Vd rich
Vincristine	+	+	-
Vinblastine	+	-	-
Vindoline	+	+	++
Ajmalicine	+	+	+

Table 2.1: Representation of alkaloids present in each alkaloid specific extract: Vb rich = Vinblastine rich extract; Vc rich = Vincristine rich extract; Vd rich = Vindoline rich extract, + = present; - = absent