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# Cytotoxic Activity of *Catharanthus roseus* (Apocynaceae) Crude Extracts and Pure Compounds Against Human Colorectal Carcinoma Cell Line

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**Abstract:** *Catharanthus roseus* (Apocynaceae), in Malaysia known as *Kemuning cina*, well known for being rich in alkaloids was investigated for its cytotoxic activity by using MTT assay against Human Colorectal Carcinoma Cell Line (HCT 116). The preliminary cytotoxicity study demonstrated dose independent cytotoxic activity of the methanol extract of *C. roseus* when screened against HCT-116 colorectal carcinoma cell line. n-hexane, chloroform and methanol fractions also showed dose independent cytotoxic activity with chloroform fraction showing the highest activity. Water fraction showed a minor cytotoxic activity, vindoline also showed some cytotoxic activity at 200 μg $mL^{-1}$. Catharanthine showed the most promising activity while dose dependent cytotoxic activity of its $IC_{50}$ value was found to be at 60 μg $mL^{-1}$. Simple and facile method has been developed for the isolation of compounds catharanthine and vindoline from this plant.

**Key words:** *Catharanthus roseus*, catharanthine, vindoline, cytotoxicity, alkaloids

## INTRODUCTION

*Catharanthus roseus* (L.) G. Don (Apocynaceae) a perennial plant is commonly seen in tropical countries. It is more commonly known as Madagascar periwinkle. The local name in Malaysia is Kemuning Cina. This plant produces beautiful flowers with a variety of colours such as purple, pink and white and commonly planted for decorative purposes (Padua *et al*., 1999). Historically, Madagascar periwinkle had been used for various treatments, e.g., diabetes mellitus, high blood pressure and infection (Taylor and Fransworth, 1975). Aerial part of the plant contains about 90 different alkaloids. The isolation and purification of these indole alkaloids have been extensively studied (Sapi and Massiot, 1994). The most abundant ones are the monomers like catharanthine and vindoline. Two of the common anti-cancer drugs which are derived from this plant are vincristine and vinblastine. Vincristine is used in the chemotherapeutic regimen for Hodgkin's lymphoma while vinblastine is used for childhood leukemia (Johnson *et al*., 1963). Various studies suggest the presence of other antineoplastic alkaloids in the plant (El-Sayed *et al*., 1983; El-Sayed and Cordell, 1981). Crude extracts of *C. roseus* using 50 and 100% methanol had significant anti-cancer activity against different cell types *in vitro* (at <15 μg $mL^{-1}$) (Ueda *et al*., 2002). Crude decoction (200 mg and 1 g herb $mL^{-1}$ water) showed moderate anti-angiogenesis effects *in vitro*. Several animal studies have shown that ethanolic extracts of leaves and flowers of *C. roseus* lower blood glucose levels (Ghosh and Gupta, 1980; Chattopadhyay *et al*., 1991, 1992). In another study while aqueous extract could lower blood glucose about 20% in diabetic rats, dichloromethane and methanol extracts lowered blood glucose 49-58%, significantly better than controls (Singh *et al*., 2001). A 70% ethanol extract of leaves in an oral dose of 400 mg $kg^{-1}$ was shown to be 20% as effective as tolbutamide in diabetic rats, though much safer (Chattopadhyay, 1999). Besides alkaloids, other secondary metabolites have been isolated from *C. roseus*, including monoterpenoid glycosides, steroids, phenolics, flavonoids and recently 7-O-methylated anthocyanins isolated from this plant (Williams and Grayer, 2004). Urge for isolation of indole alkaloids for the production of major anti-cancer drugs like vincristine and vinblastine is always in minds of academicians. Recently a simplified procedure for extraction of indole alkaloids from *C. roseus* has been reported (Verma *et al*., 2007).

Natural products have been always a rich source of new chemical entities in the field of medicine and pharmacy. In the field of oncology, significant numbers of commercialized drugs have been obtained from natural sources, either by structural modification or prepare

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semisynthetically. The search for improved cytotoxic agents is important in the discovery of modern anti-cancer drug (Nobili *et al*., 2009). The present study deals with the isolation, structure elucidation and identification of the alkaloids catharanthine and vindoline isolated from chloroform fraction of *C. roseus* leaves in Malaysia and their investigation on the cytotoxic effects of the methanol and sequential extracts along with pure isolated compounds against human colorectal carcinoma cell line HCT 116 using an *in vitro* cytotoxicity MTT assay was performed and hence the present work is reported in this communication.

## MATERIALS AND METHODS

**Preparation of plant extract:** *Catharanthus roseus* leaves cultivated and propagated under controlled conditions with the joint venture of USM-UNIMAP at Titi Tinggi, Perlis, Malaysia. Voucher specimens of the plant materials were deposited at Bilik Herba, School of Pharmaceutical Sciences, Universiti Sains Malaysia. Leaves were collected in second week of June, 2009 and experimental studies were conducted in first week of July, 2009. Fresh leaves of *C. roseus* (1 kg) were ground into a fine powder using a milling machine (Retsch GmbH, Germany). The powdered leaves (400 g) were packed into a Soxhlet (Quick-fit, England) cellulose thimble and covered by cotton wool. The thimble was then placed in a Soxhlet-extractor with a quick-fit water condenser on the top and a five lit quick-fit round bottom distillation flask at the bottom. The distillation was filled in with 3.5 L of the required solvents for extraction. The extraction was carried out sequentially and successively with hexane, chloroform, methanol and water. Each extract was concentrated on a rotary evaporator under vacuum and freeze dried. The lyophilized extracts were then kept in desiccators at room temperature prior to the experiment.

**Chemicals and instruments:** Chemicals and solvents were of analytical grade, which include methanol (Merck), ethanol (Merck), chloroform (Merck), hexane (Merck), Hydrochloric acid, ammonia solution, water (Universiti Sains Malaysia), DMSO (Sigma Aldrich), TLC plates 60 $F_{254}$ (Merck), $CDCl_3$ (Merck) and TMS (Merck),

Bruker 300 MHz NMR spectrometer, Lambda 45 UV/VIS Spectrometer (Perkin Elmer) instruments were used for the study.

**Cell line and cell culture reagents:** Human colorectal carcinoma cell line HCT-116; Catalogue number (CCL-247) was purchased from ATCC, USA. RPMI 1640 cell culture media, Foetal Bovine Serum (FBS) and Penicillin/ Streptomycin solution (PS) were purchased from Gibco, USA; MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] reagent was purchased from Sigma-Aldrich, Germany. Cell culture flasks and plates were purchased from Corning, USA. Cell culture work was done under sterile conditions in Class II bio-safety cabinet (ESCO, USA).

**Preliminary cytotoxicity studies:** The MTT cell proliferation assay was used to study the cytotoxic properties of five extracts and two pure compounds. The assay was performed according to the method developed by Mosmann (1983) with minor modifications. The cells were seeded at $1.5\times10^4$ cells in each well of 96-well plate in 200 µL of fresh culture medium and were allowed to attach for overnight. The stock solutions of the extracts were diluted in cell culture medium to obtain 200, 100 and 50 µg $mL^{-1}$. After 48 h of treatment the medium was aspirated and the cells were washed once with sterile 1x PBS. MTT was prepared at 5 mg $mL^{-1}$ in sterile PBS and was added to each well at 10% v/v. After three hours incubation at 37°C in 5% $CO_2$, the water insoluble formazan salt was solubilized with 200 µL DMSO/well. Absorbance was measured by Multiskan Ascent microplate reader (Thermolab Systems 354, Finland) at primary wave length of 570 nm and a reference wave length of 650 nm. Each plate contained the samples, negative control and blank. The DMSO at less than 0.5% v/v was used as a negative control. The assay was performed in quadricates and the results were presented as a mean percent inhibition to the negative control±SE. The following formula was used to calculate the percent of inhibition:

$$\text{Inhibition (\%)} = (1-(OD_o/OD)) \text{ X } 100$$

Where:
$Od_o$ = Optical Density of the samples
OD = Optical Density of the negative control

**Dose response cytotoxic activity of catharanthine:** In order to study the dose-response relationship of the most interesting compound Catharanthine, MTT assay was performed as mentioned above. Serial dilutions of the stock solution were prepared in RPMI 1640 cell culture medium. Cells were treated for 48 h and quantification of the results was done as mentioned above. The dose response curves were obtained by blotting the percent inhibition versus the concentrations, the regression equations were used to calculate the median inhibitory concentrations ($IC_{50}$ value).

**Statistical analysis:** Data is expressed in Mean±SE. The inhibition concentration ($IC_{50}$) values were analyzed by linear regression equation.

**Isolation of compounds 1 and 2:** Air dried ground leaves of *C. roseus* (400 g) sequentially extracted with n-hexane, chloroform, methanol and finally with water. Each extract were evaporated under reduced pressure and freeze dried. The chloroform extract was used for the isolation of compounds 1 and 2. The crude extract (10 g) was suspended in 10% aqueous hydrochloric acid (250 mL) and extracted with toluene (3×250 mL) to make sure that no traces of alkaloids remains. This toluene extract was further extracted with 10% aqueous hydrochloric acid (3×250 mL), combined the acid phase and extracted the contents with toluene (3×250 mL) to remove the chlorophyll and other colouring agents. Aqueous phase was then basified with ammonia (pH 9) and then extracted with toluene (3×250 mL). Resulting toluene extract can be a source for to enrich the vinblastine and vincristine contents. Again alkaline aqueous phase was further acidified with 20% aqueous tartaric acid (pH 3) and extracted with toluene (3×250 mL). Resulting toluene extract concentrate on a rotary evaporator under vacuum. Column chromatography was performed on an alumina (neutral) with gradient hexane with increasing polarity with ethyl acetate and collected several fractions and finally on crystallization of fraction 2 (55 mg) and 3 (61 mg) yielded two pure compounds 1 and 2. Purity of compounds were confirmed by HPLC (Agilent Technologies) and found to be 95% pure. The identification and characterization of isolated compounds were performed by UV, FTIR, MS and NMR.

## RESULTS

**Characterization and structure elucidation of isolated compounds 1 and 2:** Compound 1 was identified as catharanthine and 2 as vindoline. Further research is in progress to isolate the more compounds from *C. roseus* from Malaysia.

**Compound 1:** Catharanthine (1) A white coloured amorphous solid (25 mg), $R_f$ 0.5 [Silica gel, solvent 5% MeOH: $CHCl_3$]. UV (MeOH) λmax: 226, 284, 297 nm. FTIR (KBr): 3400 $cm^{-1}$ (N-H) and 1740 $cm^{-1}$ (ester C = O). The LC-MS gave M+H at 337. ($^1$HNMR ($CDCl_3$): δ 0.98 (3H, t, J = 7Hz, $CH_2CH_3$), 1.95 (2H, q, J = 6.5Hz, $CH_2CH_3$) 3.78 (3H, s, $OCH_3$), 6.24 (1H, d, J = 5.5Hz, C-15H), 7.02-7.41 (4H, m, Aromatic protons), 10.25(1H, s, NH). Spectral data was consistent with that reported for catharanthine (Gorman *et al*., 1965).

**Compound 2:** Vindoline (2) An off white coloured amorphous solid (34 mg), $R_f$ 0.4[Silica gel, solvent 5% MeOH: $CHCl_3$]. UV (MeOH) λmax: 219, 254, 307 nm. FTIR (Kbr): 3400 $cm^{-1}$ (N-H), 1740 $cm^{-1}$ (ester C = O). The LC-MS gave M+H at 457. ($^1$HNMR ($CDCl_3$): δ 0.36 (3H, t, J = 6.5Hz, $CH_2CH_3$), 1.36 (2H, q, J = 6.5Hz, $CH_2$ $CH_3$), 3.25 (3H, s, N-$CH_3$), 3.46 (3H, s, $COOCH_3$), 1.85(3H, s, OAc), 3.62 (3H, s, $OCH_3$), 5.0 (1H, d, J = 6.3Hz, C-15H), 5.76 (1H, dd, J = 3.5Hz and 10.3Hz, C-14 olefinic protons), 6.12-6.22 (3H, m, Aromatic protons), Spectral data were in accord to literature for Vindoline (Gorman *et al*., 1962). Structure of compound 1 and 2 are shown in Fig. 1.

**Preliminary cytotoxicity studies:** The results of preliminary cytotoxicity screening of five crude extracts and two pure compounds are summarized in Table 1. The assay was conducted in quadricates at three concentrations 200, 100 and 50 µg $mL^{-1}$ and the results are presented as mean percent inhibition±SE.

**Dose response cytotoxic properties catharanthine:** The MTT assay was conducted as mentioned above, serial dilutions of Catharanthine were prepared in RPMI 1640

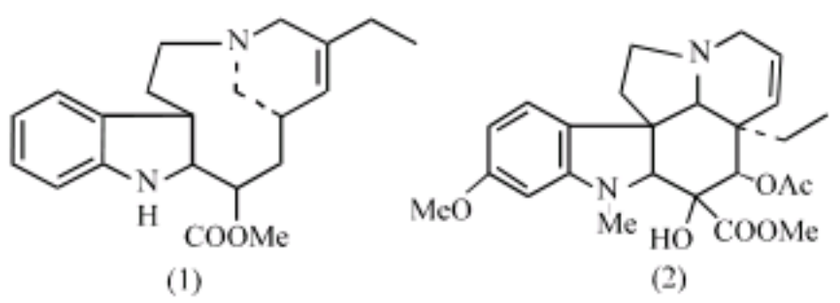


Fig. 1: Chemical structure of compounds isolated from *C. roseus*

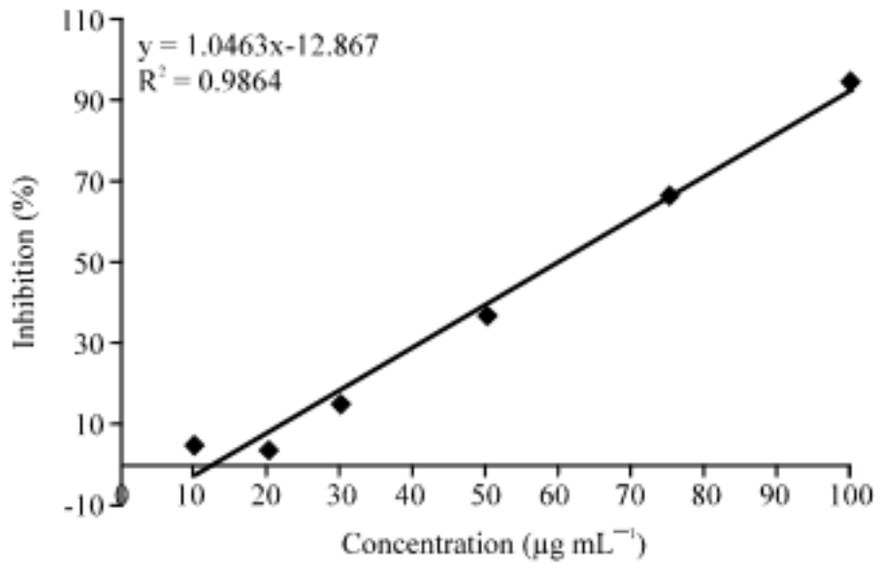


Fig. 2: The dose response curve of Catharanthine on HCT-116. MTT assay was used in which the cells were treated at different concentrations for 48 h and the results are presented as a mean percent inhibition to the negative control±SE. $IC_{50}$ value was calculated from linear regression equation shown in the chart

Table 1: *In vitro* cytotoxic activity of crude extracts along with pure compounds Catharanthine and Vindoline against HCT-116 colon cancer cell line

| Extracts/compounds | 200 | 100 | 50 |
|---|---|---|---|
| | ---------- ($\mu g\ mL^{-1}$) ---------- | | |
| VRPM | 57±2 | 68±3 | 62±3 |
| VRP Hx | 58±2 | 58±2 | 49±4 |
| VRP Ch | 94±2 | 81±3 | 77±1 |
| VRP Me | 61±5 | 59±3 | 58±2 |
| VRP W | 26±3 | 29±2 | 13±5 |
| Catharanthine | 96±1 | 93±2 | 56±2 |
| Vindoline | 59±2 | 34±2 | 25±4 |

cell culture medium and the cells were treated for 48 h and the results are presented as mean percent inhibition to the negative control±SE. Figure 2 shows the dose response curve of Catharanthine on HCT-116 cell line, the cytotoxicity results indicated a dose dependent cytotoxic activity of the compound, $IC_{50}$ value was calculated from the linear regression equation shown in Fig. 2, $IC_{50}$ value was found to be 60 $\mu g\ mL^{-1}$.

## DISCUSSION

The MTT cytotoxicity assay provides a simple method for determination of live cell number in order to assess rate of cell proliferation and to screen cytotoxic agents. MTT assay measures cell viability based on the activity of mitochondria enzymes in living cells that reduce MTT to water-insoluble formazan crystals that can be easily solublized by DMSO. The preliminary cytotoxicity study demonstrated dose independent cytotoxic activity of the methanol extract of *C. roseus* when screened against HCT-116 colorectal carcinoma cell line. n-hexane, chloroform and methanol fractions also showed dose independent cytotoxic activity with chloroform fraction showing the highest activity. Water fraction showed a minor cytotoxic activity. Previous study highlighted the role of vindoline to be examined on cytotoxicity against Eagle's 9KB carcinoma of nasopharynx and the P-388 lymphocytic leukemia test systems *in vitro* (Mukhopadhyay and Cordell, 1981). In another study El-Sayed and Cordell (1981) and El-Sayed *et al*. (1983) examined the cytotoxicity against the highly active alkaloids fractions of *C. roseus*. Likewise in the current study vindoline was examined on HCT-116 colorectal carcinoma cell line and showed some cytotoxic activity at 200 $\mu g\ mL^{-1}$. Catharanthine showed the most promising activity. The dose dependent study was performed only for catharanthine, the compound demonstrated a dose dependent cytotoxic activity, the results showed positive correlation between concentrations and the percent inhibition, $R^2 = 0.99$. Previously a simplified procedure for indole alkaloid extraction from *C. roseus* has been reported (Verma *et al*., 2007) that process deals with the mixture of vindoline and catharanthine and used directly for the production of vinblastine. In the present process indole alkaloids catharanthine and vindoline from *C. roseus* isolated easily by treating the extract with acid base manipulation and can be a good source for commercialization of these alkaloids. Optimization of this process can result in higher yields.

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