

## ANTI-INVASIVE EFFECT OF *Catharanthus roseus* EXTRACT ON HIGHLY METASTATIC HUMAN BREAST CANCER MDA-MB-231 CELLS

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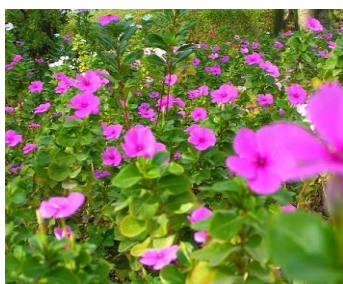
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### Graphical abstract



### Abstract

*Catharanthus roseus* (*C. roseus*) is an important medicinal plant contains a lot of useful alkaloids. The objective of this study is to determine the effect of *Catharanthus roseus* leaves extract on the MDA-MB-231 cancer cell invasion potential and matrix metalloproteinases activity (MMP-2 and MMP-9) in breast cancer cell lines, MDA-MB-231 and MCF7. MTS cytotoxicity assay was used to evaluate the cytotoxic effect of the extract against the two tumour cell lines. Transwell invasion assay was used to investigate the invasive activity of MDA-MB-231 cancer cell line and gelatin zymography was used to determine the effect of *C. roseus* extract on the activity of MMP-2 and MMP-9 on the two tumour cell lines. The result showed that *C. roseus* extract was found to be not cytotoxic for both tumor cell line, MDA-MB-231 and MCF-7, with  $IC_{50}$  values more than 200 $\mu$ g/ml. However the extract showed significant inhibitory effect on MDA-MB-231 invasion ability ( $p < 0.001$ ), and suppressed the MMP-2 and MMP-9 activity on both tumour cell lines. In conclusion, our findings show *C. roseus* as a promising therapeutic candidate that inhibits invasive ability of breast cancer through modulating MMP-2 and MMP-9 activity and could potentially suppress the progression of breast cancer.

Keywords: *Catharanthus roseus*, MTS, zymography, MMP -2, MMP-9, MDA-MB-231.

### Abstrak

*Catharanthus roseus* (*C. roseus*) merupakan herba perubatan penting yang mengandungi alkaloid pelbagai kegunaan. Objektif kajian ini ialah untuk menentukan kesan ekstrak daun *C. roseus* terhadap potensi invasi sel kanser MDA-MB-231 dan aktiviti matrix metalloproteinase (MMP-2 dan MMP-9) dalam fittisan sel kanser payudara, MDA-MB-231 dan MCF7. Assai sitotoksiti MTS telah digunakan untuk menilai kesan ekstrak terhadap kedua-dua sel tumor. Assai invasi transwell digunakan untuk mengkaji aktiviti invasi sel kanser MDA-MB-231 dan zimografi gelatin telah digunakan untuk menentukan kesan ekstrak *C. roseus* terhadap aktiviti MMP-2 dan MMP-9 atas kedua-dua sel tumor. Keputusan menunjukkan bahawa ekstrak *C. roseus* tidak sitotoksik terhadap kedua-dua sel tumor, MDA-MB-231 dan MCF-7, dengan nilai-nilai  $IC_{50}$  melebihi 200  $\mu$ g/ml. Namun begitu ekstrak menunjukkan kesan perencatan pada keupayaan invasi MDA-MB-231 ( $p < 0.001$ ), dengan merencat aktiviti MMP-2 dan MMP-9 dalam kedua-dua sel tumor tersebut. Kesimpulannya, penemuan kami telah menunjukkan *C. roseus* dianggap sebagai calon terapeutik yang berpotensi untuk merencat perkembangan kanser payudara melalui modulasi aktiviti MMP-2 dan MMP-9 dan berpotensi untuk merencat perkembangan kanser payudara.

Kata kunci: *Catharanthus roseus*, MTS, zimografi, MMP-2, MMP-9

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## 1.0 INTRODUCTION

Breast cancer is the most common type of cancer and the main cause of cancer mortality among women worldwide [1]. The primary cause of the majority of deaths from breast cancer is due to breast cancer metastasis from the original tumour to other sites in the patient [2]. Metastatic cells have to undergo multiple steps. The metastasis process starts with the invasion of cancer cells from the original tumour in the neighboring tissues. The invasive cells continue and intravasate into the blood or lymphatic vessels. The blood stream or the lymphatic vessels disseminate the tumor cells to other sites [3, 4]. Then the cell cycle of tumour cells is arrested until they adhere to capillary beds within the target organ and extravasating into the organ parenchyma. Once they are settled down on the new organ they start proliferating and trigger angiogenesis [3]. During the metastasis process, tumour cells must escape from the immune response and apoptosis signals [3, 4]. Degradation of cell-to-cell adhesion and cell adhesion to the extracellular matrix (ECM) are crucial for the invasive tumour cells. Cell-to-cell adhesion is mediated by cadherin family [5], while the adherence of tumor cells to the ECM is mediated through integrins [6].

The invasive tumour cells must degrade the ECM surrounds the primary tumour in order to invade tissue boundaries. This degradation is carried out mainly through metalloproteinases (MMPs) and the urokinase plasminogen activator (uPA) [7, 8]. Among all MMPs, MMP-2 and MMP-9 was shown to be associated with breast cancer metastasis [9]. Besides the direct proteolytic actions of MMP-2 on the ECM, its activity in cancer cells resulted in suppression of tumor growth through reducing angiogenesis [10]. MMP-9 can activate various matrix proteins that involved in the process of invasion and angiogenesis, such as urokinase plasminogen activator (uPA) and serpin protease nexin-1 (PN-1) [11]. Therefore, regulating the activity of MMPs enzymes could be considered as a promising therapeutic target for breast cancer metastasis and angiogenesis.

Studies have shown that plant secondary metabolites are able to modulate multiple pathways used by cancer cells, such as cell proliferation, survival, angiogenesis, invasion, and metastasis [12]. Blocking of one or more of these pathways provides an effective way for therapeutic intervention of breast cancer. Various phytochemicals have been reported to effectively suppress the invasive and metastatic ability of different cancer cell lines.

*Catharanthus roseus* (*C. roseus*) is an important medicinal plant of the family Apocynaceae, which contains many useful alkaloids. It is a popular ornamental plant found in tropical and subtropical parts of the world. In Malaysia, commonly known as kemunting Cina or periwinkle [13], the plant is used traditionally for treating diabetes, reduce blood pressure, insomnia and cancer [14]. *C. roseus* also

known as “an anticancerous drug yielding plant” contains enormous phytochemical components of various therapeutic properties [15]. The anticancer alkaloids vinblastine and vincristine, which are derived from the plant have a growth inhibition effect on various human tumours [15]. *C. roseus* is considered as an important agent in cancer chemotherapy, however the anti-invasive effect of this important medicinal plant has not been studied yet.

The main objective of this study is to investigate whether *C. roseus* extract has the ability to inhibit cancer cell invasion and matrix metalloproteinases (MMP-2 and MMP-9) activity. Prior to that, cytotoxic effect of *C. roseus* on the cancer cells was screened in order to choose a non-toxic effective dose.

## 2.0 METHODOLOGY

### 2.1 Materials

Dulbecco's modified eagle medium (DMEM), penicillin/streptomycin and trypsin-EDTA were purchased from Gibco (Bio-Diagnostic Sdn Bhd, Petaling Jaya, Selangor, Malaysia). CellTiter 96@Aqueous non-radioactive cell proliferation assay kit was purchased from Promega (Madison, WI, USA).

### 2.2 Plant Extract

*C. roseus* plant was cultivated and propagated under controlled conditions at Titi Tinggi, Perlis, Malaysia. The leaves were collected, air dried and pulverized into a fine powder using a milling machine (Retsch GmbH, Germany). The leaves powder was extracted with methanol using soxhlet extractor for 12 h. The extract was concentrated on a rotary evaporator under vacuum and dried in oven at 50 °C. The plant extract was prepared by dissolving 20 mg in 2 ml of DMSO (Final concentration 10mg/ml).

### 2.3 Cell Culture

Human breast cancer MDA-MB-231 and MCF7 cells were originally obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C under a humidified atmosphere containing 5% CO<sub>2</sub>. The cells were subcultured when nearly confluent using 0.25% trypsin-EDTA.

### 2.4 MTS Cytotoxicity Assay

The effect of the extract on cell viability was assessed using CellTiter 96@Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, USA) according to manufacturer's instructions. Briefly, MDA MB-231 and MCF-7 cells were seeded into a 96-well plate at a density of 10 x10<sup>4</sup> and 8x10<sup>3</sup> cells/well respectively,

and incubated for 24h. The cells were then treated with plant extract at different concentrations (6.25 to 200 $\mu$ g/ml) and incubated for 48 hours. Following incubation, 20  $\mu$ l of the MTS reagent in combination with the electron coupling agent solution was added into each well and incubated for 5 h. The absorbance was then measured using Bio-Rad micro-fiter plate reader at a wavelength of 490nm with a reference wavelength 630nm. Percentage of viability of treated cells is expressed as a percentage compared to control (<0.1% DMSO). Data are expressed as mean values  $\pm$  SD and obtained from three different experiments against each cell line

### 2.5 Transwell Invasion Assay

The *in vitro* invasion assay was performed using Transwell system (24 wells, 8 $\mu$ m pore size with polycarbonate membrane; Corning coster, Lowell, MA, USA). The polycarbonate membranes were coated with 100 $\mu$ l matrigel (500 $\mu$ l matrigel and 1ml serum-free media) and incubated at 37°C for 4 hours to form a reconstituted basement membrane. MDA-MB-231 (70x10<sup>3</sup>cells/ml) cells were placed in 0.5 ml of serum-free media containing vehicle or 10, 100  $\mu$ g/ml from the extract. 100  $\mu$ l of the prepared cells was added to the upper chamber in quadruplicate and 500  $\mu$ l of medium containing a 10% FBS were added to the lower chamber. After incubation for 48 hours, cells that have invaded through the membrane were collected and 500  $\mu$ l of trypsin/EDTA were added to the lower chamber to detached cells that still attached to the membrane. Cells were then counted using an automated cell counter (CASY Model TTC).

### 2.6 Gelatin Zymography

Gelatin zymography was performed to measure the enzymatic activity of pro-MMP-2 and pro-MMP-9. Human foreskin fibroblasts (HFF-1) were grown in 6-well plate in DMEM supplemented with 10% FBS at a density of 1.3x10<sup>5</sup> cells/ml for 24 hours. MDA MB-231 or MCF-7 cells at 1x10<sup>5</sup> cell/ml density were co-cultured with the seeded HFF-1 and incubated for 24 hours. The co-culture cells were treated with DMSO or different concentrations of the plant extract in serum free media. After 24 hours, cell supernatant was harvested and loaded onto a 10% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, gels were washed with distilled water and were incubated with 2.5% triton-X for 1 hour to remove SDS. Then, gels were incubated overnight at 37°C in developing buffer (50 mM Tris-HCl pH 7.4, 200 mM NaCl, 5 mM CaCl<sub>2</sub>, and 0.02% Brij-35). Bands corresponding to enzyme activity were visualised by staining using 0.1% Coomassie brilliant blue for 10h, and then destained with 30% methanol and 10% acetic acid solution. Quantification of MMP was done by densitometry analysis using Alphamager HP software.

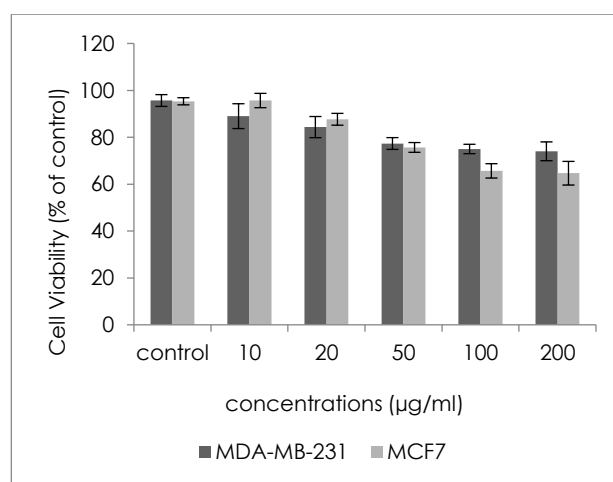
### 2.7 Statistical Analysis

The results were expressed as Mean  $\pm$  SD of triplicates from three independent experiments. One way analysis of variance (ANOVA) and Dunnet's Multiple Comparison Test were used to evaluate the significant difference. Values at p<0.05 were considered as significant.

## 3.0 RESULTS AND DISCUSSION:

In this study, the effect of *C. roseus* extract on the invasive potential of MDA-MB-231 cell line and gelatinases (MMP-2 and MMP-9) activity in MDA-MB-231 and MCF7 cell lines was investigated. The cytotoxic effect of the extract on the two selected cancer cell lines was tested in order to select non-cytotoxic concentrations.

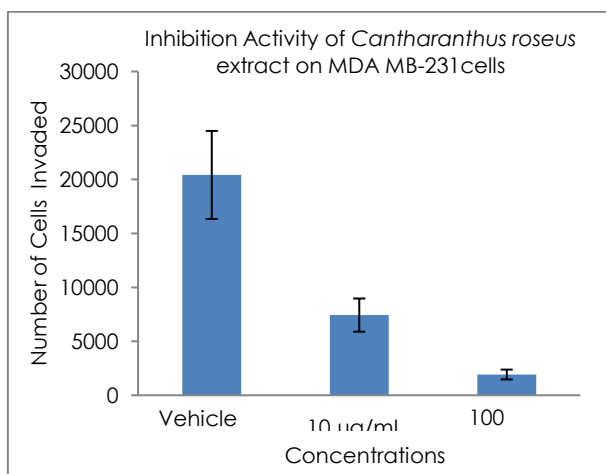
The effect of the extract (0–200  $\mu$ g/ml) on cell viability was detected by MTS assay. The result showed that, the extract was not cytotoxic for both cancer cell lines. As shown in Figure 1, cell viability was not significantly affected in extract-treated groups after incubation for 48 h. This finding is in agreement with a previous study that has been done by Wang and his colleagues. The methanol extract of *C. roseus* leaves did not inhibit the growth of MCF7 and MDA-MB-231 breast cancer cell lines [16]. *C. roseus* is well known for its medicinal alkaloids, vinblastine and vincristine, which are used to treat different types of cancer such as Hodgkin's disease, acute leukemia and some other solide tumours [17]. From what stated above, we can infer that the anticancer activity of *C. roseus* may be specific to certain types of cancer.



**Figure 1** Effect of *C. roseus* extract on cell viability in MDA-MB-231 and MCF7 cells. The cells were treated with different concentrations and DMSO as a control for 48 h and the cell viability was tested using MTS assay.

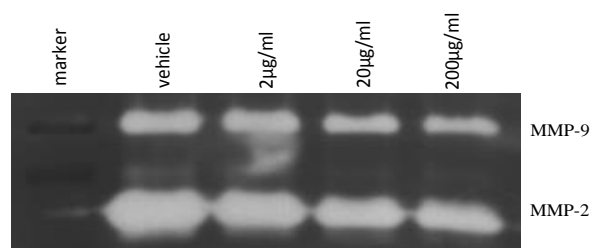
We selected MDA-MB-231 cell line to further investigate the anti-invasive ability of *C. roseus* extract. MDA-MB-231 is a well-established model to investigate the anti-invasive effect of the anti-cancer compounds and to elucidate the mechanism of cancer invasion and metastasis [18–19]. This cell line lacks the estrogen receptor, progesterone receptor and HER2 and classified as basal-like subtype of breast cancer [20]. The MDA-MB-231 cell line is originated from the most aggressive and highly metastatic breast tumours, thus it is the most suitable in vitro model to investigate the potential of compounds to suppress the invasion and metastasis process [18, 19]. On the other hand, ER-positive MCF7 cell has been widely considered as poorly invasive breast cancer cell line [21] and thus it is not a good model to investigate the invasive potential of the extract.

The ability of the extract to inhibit invasive activity of MDA-MB-231 cells was assessed by Transwell invasion assay. Two different non-cytotoxic concentrations (10 and 100  $\mu\text{g/ml}$ ) were selected to determine the anti-invasive potential of the extract. The results showed that *C. roseus* extract was able to inhibit the invasiveness of the MDA-MB-231 cells at very low concentration, 10  $\mu\text{g/ml}$  ( $p < 0.01$ ). Figure 2 shows that the number of treated cells that has invaded through the Matrigel coated membrane was markedly reduced in a dose-dependent manner compared to untreated cells. At 100  $\mu\text{g/ml}$  concentration, the extract significantly inhibited the number of invading MDA-MB-231 cells ( $p < 0.001$ ). This result suggests that, *C. roesus* has potent anti-invasive potential against the highly metastatic breast cancer cell line, MDA-MB-231. Increasing evidence shows that some medicinal herbs inhibit the invasiveness of different cancer cells including breast cancer [22]. Common cancer treatment aims to kill the cancer, inhibit cell cycle progression, suppress tumour migration and invasion or to block the angiogenesis. Several natural compounds can treat cancer through inhibiting one of these events [23, 24].

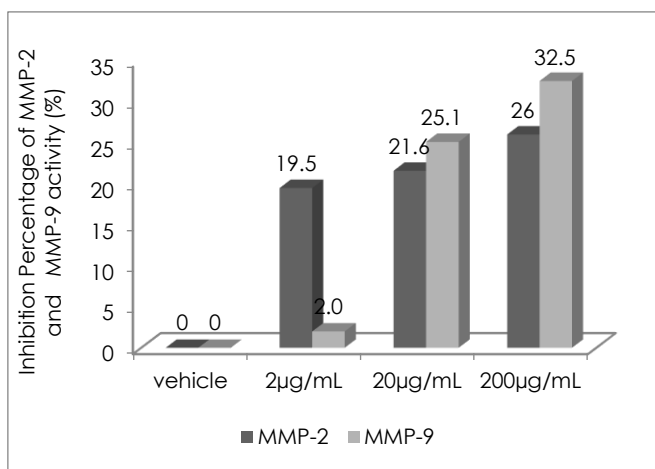


**Figure 2** *C. roseus* inhibited the invasive activity of MDA-MB-231 cells. The cells treated by two different concentrations (10 and 100  $\mu\text{g/ml}$ ).

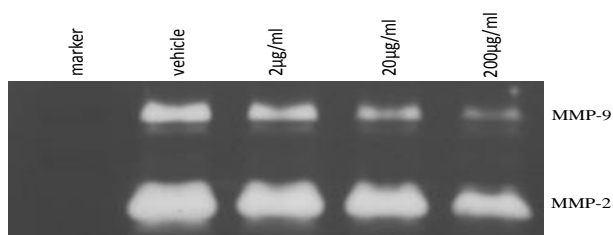
The effect of *C. roseus* extract on MMP-2 and MMP-9 activity was investigated in the two cancer cell lines, MDA-MB-231 and MCF7 using gelatin zymography. At low concentrations (1, 10 and 100  $\mu\text{g/ml}$ ), no significant effect was observed (data not shown). However, when the concentrations were increased to 2, 20 and 200  $\mu\text{g/ml}$ , the extract exhibited a significant dose dependent inhibitory effect. Figure 3 A and 4 A show the effect of the extract on MMP-2 and MMP-9 activity on the two cancer cell lines. The effect of the extract was more potent on the highly invasive breast cancer cell line than that in the breast cancer cell line with poor invasive potential. Moreover, the MMP-9 activity was more affected by the extract than MMP-2 activity in MDA-MB-231 cell line. Densitometric analysis of the bands demonstrates the inhibition percentage of the extract on MMP-2 and MMP-9 activity on the two cancer cell lines ( $p < 0.001$ ) (Figure 3 B and 4 B). At 200  $\mu\text{g/ml}$  concentration, *C. roseus* extract inhibited 87.9 and 45.7% of MMP-9 and MMP-2 activity on MDA-MB-231 cell line, respectively. The inhibitory effect was increased with the increase of the extract concentration. These findings conclude that, *C. roseus* has the ability to inhibit MMP-2 and MMP-9 activity in the two breast cancer cell lines, MDA-MB-231 and MCF7. MMP-2 and MMP-9 are known to play an important role in breast cancer invasion and metastasis. In advanced breast cancer with poor prognosis, proteolytic enzymes such as MMP-2 and MMP-9 are overexpressed [25] and they play a very important role in ECM and basement membrane degradation that is required for invasion and metastasis [26]. However, the role of MMP-2 and MMP-9 is not limited to extracellular matrix remodeling and metastasis facilitating, but also regulate the function of various proteins in the tumour microenvironment and stimulate tumour growth [27].



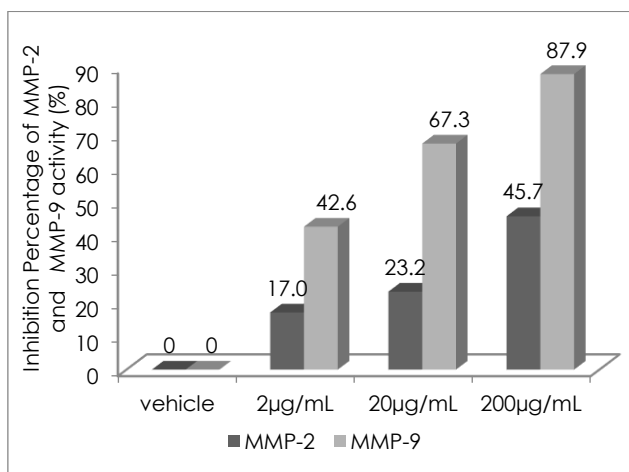
**Figure 3 A** Effect of *C. roseus* extract on the activity of MMP-2 and MMP-9 on MCF7 cells. Cells were treated with 2, 20 and 200  $\mu\text{g/ml}$  *C. roseus* extract.



**Figure 3 B** Densitometric analysis of MMP-2 and MMP-9 activity inhibition percentage on MCF7 cells treated with different concentrations (2, 20 and 200 µg/ml) of *C. roseus* extract.



**Figure 4 A** Effect of *C. roseus* extract on the activity of MMP-2 and MMP-9 on MDA-MB-231 cells. Cells were treated with 2, 20 and 200 µg/ml *C. roseus* extract.



**Figure 4 B** Densitometric analysis of MMP-2 and MMP-9 activity inhibition percentage on MDA-MB-231 treated with different concentrations (2, 20 and 200 µg/ml) of *C. roseus* extract.

Therefore, regulating the activity of these MMPs by *C. roseus* extract could suppress the metastasis as well as cell growth and progression of breast cancer cells. Previous studies have revealed that some natural products can inhibit cancer metastasis by ECM

degradation through inhibition of proteolytic enzymes [9, 19, 28].

The significant inhibition of MMP-9 activity on MDA-MB-231 cells by the extract could be the reason beyond the anti-invasive effect of the extract. Previous reports have demonstrated that certain cell signaling mechanisms have been implicated in the ability of a cancer cell to invade the surrounding tissues. The nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) signaling pathways have been reported to be involved in the initiation of the invasion process of tumor cells [29, 30]. In this study the anti-invasive effect of the extract was associated with significant MMP-9 inhibition, which could be due to blocking of one of these pathways. Elucidation of the molecular mechanisms responsible for the anti-invasive and MMPs inhibition effects of *C. roseus* requires further studies.

*C. roseus* possesses many alkaloids with various biological properties which are used as pharmaceuticals. Vinblastine and vincristine alkaloids are well known for their potent anticancer property [31]. In this study, the anti-invasive *C. roseus* ability and MMP-2 and MMP-9 activity inhibition could be due to the presence of one of these two important therapeutic alkaloids or others. Further studies are needed to isolate the active compounds and elucidate the mechanisms of action.

## 4.0 CONCLUSION

In conclusion, *C. roseus* exhibits anti-invasive property against the highly invasive human MDA-MB-231 breast cancer cell line. The extract also modulates the activity of MMP-2 and MMP-9 metalloproteinases in dose dependent fashion in two breast cancer cell lines with different invasive potential and significant disparity in general properties, MDA-MB- 231 and MCF7.

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