Biologia (2021) 76:1017–1032
https://doi.org/10.2478/s11756-020-00641-5

ORIGINAL ARTICLE

Catharanthus roseus L. extract downregulates the expression profile of motility-related genes in highly invasive human breast cancer cell line MDA-MB-231

Nagla Mustafa Eltayeb[1,2] · Mohammad Al-Amin[1] · Aiman Mohammed Yousif[1] · Venugopal Balakrishnan[3] · Salizawati Muhamad Salhimi[1]

Received: 12 August 2020 / Accepted: 23 October 2020 / Published online: 19 November 2020

Abstract

This study aimed to investigate the effect of *Catharanthus roseus* L. (*C. roseus*) leaf extract on the migration and invasion of MDA-MB-231 cell line and elucidate the molecular mechanisms of action. Effect of the extract on cell viability was evaluated by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl2H-tetrazoliumbromide) assay. Anti-migratory and anti-invasive effects were evaluated using scratch and Transwell assays. Effect on the levels and activities of matrix metalloproteinase (MMP)-2 and MMP-9 was determined using ELISA and gelatin zymography. Furthermore, changes in the expression of 84 genes commonly involved in cell motility were assessed by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and cell motility RT^2 profiler PCR array. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using DAVID. Using STRING and Cytoscape software, hub genes were determined. The extract significantly ($p < 0.001$) inhibited the migration and invasion of MDA-MB-231 cells at non-cytotoxic concentrations. The activities and levels of MMP-2 and MMP-9 were decreased in a dose-dependent manner following *C. roseus* exposure. At 4 μg/mL, the extract significantly downregulated the expression of 52 genes involved in extracellular matrix degradation, cytoskeleton reorganisation, focal adhesions and invadopodia formation. GO and KEGG pathway analysis revealed that the downregulated genes were significantly enriched in biological processes and pathways closely related to cell motility. Our findings showed that *C. roseus* inhibited the migration and invasion of MDA-MB-231 cells through altering the expression of various motility-related genes. This study provided data about the potential of *C. roseus* phytochemicals as promising therapeutic agents against breast cancer metastasis, especially at gene level.

Keywords *Catharanthus roseus* · MDA-MB-231 cell line · Anti-migratory effect · Anti-invasive effect · Gene expression analysis

Abbreviations

C. roseus	*Catharanthus roseus* L.
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl2H-tetrazoliumbromide
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
GO	Gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
DAVID	Database for Annotation, Visualization and Integrated Discovery
STRING	Search Tool for Retrieval of Interacting Genes / Proteins
PPI	Protein-protein interaction
TNBC	Triple-negative breast cancer
ECM	Extracellular matrix
MIAs	Monoterpenoid indole alkaloids
HER-2	Human epidermal growth factor receptor-2

✉ Salizawati Muhamad Salhimi
saliza@usm.my

[1] Discipline of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 USM Penang, Malaysia

[2] Department of Immunology and Biotechnology, Tropical Medicine Research Institute (TMRI), National Centre for Research, Khartoum, Sudan

[3] Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800 USM Penang, Malaysia

Introduction

Breast cancer is the most common type of malignancy and the leading cause of mortality amongst females worldwide. Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer with poor prognosis. It accounts for approximately 15–20% of all breast cancer cases (Yao et al. 2019). The treatment of patients with TNBC is highly challenging due to the lack of therapeutic molecular targets, oestrogen receptor, progesterone receptor and HER2 gene overexpression (Denkert et al. 2017). Patients with TNBC have a high risk of developing metastasis (Yao et al. 2019), which is the main cause of deaths amongst patients with breast cancer (Redig and McAllister 2013). Metastatic breast cancer is incurable, and current treatment strategies aim to limit metastasis-associated consequences, delay cancer growth and enhance the quality of life for patients (Tauro and Lynch 2018). Knowledge on the mechanisms that facilitate breast cancer dissemination reveals opportunities of therapeutic interventions to limit or prevent the disease. The cascade paradigm of metastasis illustrates that migration and invasion into surrounding areas are essential processes for the dissemination of cancer cells. Migration and invasion are orchestrated by various cellular events and molecular pathways within the cells and tumour microenvironment (McSherry et al. 2007). The most important events include chemotaxis, extracellular matrix (ECM) degradation, reorganisation of actin cytoskeleton and formation of membrane protrusions (Fife et al. 2014; Tauro and Lynch 2018; Yamaguchi and Condeelis 2007). Targeting the genes implicated in these processes and pathways is an important strategy to disrupt such an organised cascade and prevent or delay the invasion and metastasis of breast cancer. The suppression of such complicated interconnected events requires the action of many substances that work simultaneously against various targets. Nowadays, a combination of inhibitors specific for several targets important for tumour cell progression and metastasis is a promising concept for therapeutics (Pezzani et al. 2019; Zhou et al. 2016). Recently, the use of MMP-9 inhibitors in combination with cytotoxic drugs increased the overall response of patients with HER2-negative gastric and gastroesophageal junction adenocarcinoma in phase I clinical trial (Fields 2019). Therefore, looking for specific inhibitors for proteins implicated in migration and invasion of cancer cells, such as MMPs and RHO GTPases family is of great value and may lead to design new targeted therapies for invasive cancers.

Catharanthus roseus L. (*C. roseus*), also known as Madagascar periwinkle, is a remarkable source of useful medicinal substances with a wide range of important pharmaceutical properties. The plant mainly produces monoterpenoid indole alkaloids (MIAs), which are biologically active constituents with a variety of pharmaceutical features (Sharma et al. 2019). Some of these alkaloids, along with their semisynthetic and synthetic derivatives, are essential anticancer substances, such as vinblastine, vincristine, vinorelbine and vindesine (Choudhari et al. 2020). The plant gains much attention in the field of cancer therapy because it represents the only source of the essential oncologic agents, vincristine and vinblastine (Sharma et al. 2019). Although, *C. roseus* has been extensively studied for its cytotoxicity, there are very few data regarding other anti-cancer activities such as anti-migration, anti-invasion and anti-metastasis. The anti-invasive effect of *C. roseus* against highly invasive TNBC cell line MDA-MB-231 was reported previously (Eltayeb et al. 2016). However, studies on the molecular mechanisms of anti-migratory and anti-invasive properties of *C. roseus* on invasive breast cancer cells are lacking. Therefore, this study aimed to examine the anti-migratory and anti-invasive properties of *C. roseus* extract and elucidate its molecular mechanisms of action on MDA-MB-231 cell line. The effect of the crude extract on the expression profile of 84 motility related genes was investigated to identify the main molecular targets of the extract on MDA-MB-231 cell line.

Materials and methods

Chemicals and reagents

Human breast adenocarcinoma cell line MDA-MB-231 (cat. no. HTB-26) was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin-EDTA and phosphate buffered saline (PBS) were purchased from Thermo Fisher Scientific (USA). Matrigel was obtained from BD Biosciences (USA) and 3-(4,5-dimethyl-2-thaizol)-2,5-diphenyltetrazolium bromide (MTT) powder was purchased from iDNA (South USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (UK) and Coomassie brilliant blue R-250 from Sigma-Aldrich (USA). Human MMP-2 and MMP-9 ELISA kits were purchased from Elabscience (USA). Human cell motility RT^2 profiler PCR array, RNeasy mini kit, RNase-free DNase set, RT^2 first strand kit and RT^2 SYBR green mastermix were purchased from Qiagen (USA).

Plant material collection and extraction

C. roseus leaves were collected from Balik Pulau, Penang, Malaysia and authentication of the plant was performed by the botanist Dr. Rahmad Zakaria, Department of Botany, School of Biological Sciences, USM and a voucher specimen (Accession number. 11,780) was deposited at the herbarium of the school. The leaves were thoroughly washed with tap water, rinsed with distilled water and dried in the oven at 50 °C for three days. The dried leaves were grounded into fine

powder and the powder (35 g) was extracted according to the method described by Siddiqui et al. (2011), using methanol solvent and soxhlet extractor. The plant extract was stored at -20 °C until use.

Cell culture and cell line

MDA-MB-231 cells were grown in DMEM supplemented with 10% FBS and incubated in a humidified incubator at 37 °C under an atmosphere containing 5% CO_2. The cells were sub-cultured when nearly confluent using 0.25% trypsin-EDTA.

Cell viability assay (MTT assay)

The effect of the crude extract on the viability and proliferation of MDA-MB-231 cells was assessed by MTT assay. In brief, MDA-MB-231 cells were seeded in a 96-well plate at density of 10×10^3 cell / well and incubated at 37 °C for 24 h. At the end of incubation time, the cells were treated with different concentrations (1–32 µg/mL) of *C. roseus* extract for 24, 48 and 72 h. The untreated cells that received ≤ 0.1% DMSO (v/v) in medium were used as vehicle controls. After incubation periods, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and the plate was incubated for 3 h at 37 °C. Then, the medium was removed and 200 µL of DMSO was added to the wells. After 15 minutes of incubation, the optical density was measured at 570 nm (630 nm was used as a reference) using ELISA microplate reader (TECAN, sunrise).

Scratch (Wound healing) assay

The scratch assay was used to determine the effect of *C. roseus* extract on the collective migration of MDA-MB-231 cells. Briefly, the cells were seeded in 6-well plate and grown to 80% confluence at 37 °C. The monolayer of the cells was scratched using sterile 200 µL pipette tip to make straight line and free-floating cells were removed by washing with PBS. Then, the cells were exposed to 1, 2 and 4 µg/mL *C. roseus* extract in serum-free DMEM and incubated for 48 h. The wound closure was monitored at 0, 24 and 48 h. Three random photographs were taken per sample at each time point using digital camera attached to an inverted microscope (Zeiss Primovert). The area of the wound was measured using Image J software (National Institutes of Health (NIH) 2020) and the percentage of wound closure was calculated using the following equation:

$$\text{Wound Closure } \% = \left(\frac{A_{t=0h} - A_{t=xh}}{A_{t=0h}}\right) \times 100\ \%$$

$A_t = 0\ h$ is the area of the wound measured immediately after scratching ($t = 0\ h$).
$A_t = xh$ is the area of the wound measured after 24 or 48 h after scratching (t = 24 or 48 h).

Transwell migration assay

Transwell migration assay was used to assess the effect of *C. roseus* extract on the directional migration of MDA-MB-231 cells toward a chemo-attractant (FBS) using 24-well Transwell plates with 8.0 µm pores polycarbonate membrane inserts (Corning Costar, USA). MDA-MB-231 cells were suspended in serum-free medium and treated with 1, 2 and 4 µg/mL of *C. roseus* extract for 30 min. MDA-MB-231 cells (5×10^4 cell) were added to the upper chambers of the inserts and 500 µL complete media (10% FBS) was added to the lower chambers and the plate was incubated for 20 h at 37 °C. At the end of the incubation period, the non-migrated cells that were remained on the upper side of the inserts were removed using cotton swabs. The cells that migrated to the lower side of the inserts were fixed with 70% ethanol and stained with 0.5% crystal violet. The migrated cells were counted under inverted microscope in five random fields per insert and averages were taken. The migrated cells were photographed using digital camera at magnification of 200 X.

Transwell invasion assay

The *in vitro* invasion assay was conducted using Matrigel (BD Biosciences) and 24-well Transwell plate. The steps of the method were identical to Transwell migration assay described previously expect that the membranes of the inserts were coated with 100 µL of Matrigel before cell seeding.

Measurement of MMP-2 and MMP-9 Levels by ELISA

MDA-MB-231 cells were seeded in 6-well plates and incubated overnight at 37 °C. The cells were then treated with *C. roseus* extract at doses of 1, 2 and 4 µg/mL for 24 h. The conditioned media were collected and concentrated by ultrafiltration using 10 kDa MWCO concentrator (GE Healthcare). The levels of secreted gelatinases were determined using MMP-2 and MMP-9 immunoassay kits (Elabscience, USA) according to the manufacturer's protocol.

Gelatin zymography

Gelatin zymography was performed to determine the effect of *C. roseus* extract on the activities of gelatinases. MDA-MB-231 cells were incubated in serum-free DMEM for 24 h in the presence of vehicle control or *C. roseus* extract doses (1, 2, 4 and 6 µg/mL). Following incubation, the conditioned media

were collected and concentrated using 10 kDa MWCO concentrator. Then 20 µL of concentrated samples was separated in 8% polyacrylamide gel containing 0.1% gelatin. After separation, the gel was rinsed in 2.5% (v/v) Triton X-100 to remove SDS and then incubated overnight at 37 °C in reaction buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM $ZnCl_2$ and 10 mM $CaCl_2$) to allow proteolysis of the gelatin. Gel was stained with 0.5% Coomassie brilliant blue R-250 and de-stained with mixture of 40% methanol and 10% acetic acid. The gel was photographed using gel imager (Fujifilm, Las-3000) and the densities of the bands were analyzed using ImageJ.

Real-time RT-PCR and human cell motility RT^2 profiler PCR array

MDA-MB-231 cells were exposed to 4 µg/mL *C. roseus* extract and DMSO (< 0.1%) as vehicle control for 24 h. Total RNA was extracted from treated and control cells by RNeasy mini kit (Qiagen, USA) according to manufacturer's protocol. The total RNA was further purified using RNase-free DNase treatment. Total RNA concentration and purity were determined using NanoPhotometer™ (Implen GmbH). The integrity of the extracted RNA was checked using 1% agarose gel electrophoresis. RT^2 first strand kit (Qiagen, USA) was used to synthesize cDNA following the manufacturer's protocol. Firstly, 10 µL of genomic DNA elimination mix was prepared for each sample (control and treated) in PCR tubes by adding 2 µL of buffer GE to 2000 ng RNA and the volume was completed to 10 µL by RNase-free water. Secondly, 10 µL of reverse transcription mix was prepared by mixing 3 µL of RNase-free water, 4 µL of 5 x buffer BC3, 2 µL RE3 reverse transcriptase mix and 1 µL of control P2. The 10 µL reverse-transcription mix was then added to the genomic DNA elimination mix and incubated at 42 °C for 15 min. Finally, the reaction was terminated by incubation at 95 °C for 5 min. Afterwards, 91 µL RNase-free water was added to each reaction and the resulting cDNA was stored at -20 °C until used. The gene expression of 84 genes related to cell motility was analyzed using human cell motility RT^2 profiler PCR array (Qiagen, USA). The 96-well plate array contains 84 motility-related genes, 5 housekeeping genes, 1 genomic DNA control, 3 reverse-transcription controls and 3 positive PCR controls. PCR components mix was prepared by mixing 1350 µL 2 x RT^2 SYBR green mastermix, 102 µL cDNA synthesis reaction and 1248 µL RNase-free water. A volume of 25 µL from the mixture was pipetted into each well of 96-well PCR array plates. Human cell motility RT^2 profiler PCR array plates were run on StepOnePlus™ (Applied Biosystems) using the following program: 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Cycle thresholds from RT-PCR were exported to an excel file and analyzed using web-based PCR array data analysis software available at https://geneglobe.qiagen.com/my/analyze/. Relative gene expression was determined by comparing ΔΔCt for each gene in *C. roseus*-treated array to the control array. A fold change ≤ 2 was considered significant.

Gene functional annotation

Gene annotation analysis was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resource (https://david.ncifcrf.gov). The official gene symbols of the downregulated genes were submitted to the website and the analysis was performed. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed to classify genes according to their participation in various biological processes and pathways.

Protein-protein interactions network

The Search Tool for Retrieval of Interacting Genes / Proteins (STRING) bioinformatics resource version 10.5 (Szklarczyk et al. 2015) was used to construct the protein-protein interaction (PPI) map (https://string-db.org). The map was constructed with a combined score > 0.7 (cut-off point). Cytoscape software (Cytoscape, 3.8.0) was used to visualize the molecular interaction between the regulated genes. Genes with high correlation in the network (Hub genes) were identified using Cytohubba with Maximal Clique Centrality (MCC) (Wang et al. 2020).

Statistical analysis

The results were expressed as means ± SEM of triplicates of three independent experiments. SPSS software (Version 23) was used for statistical analysis. One-way analysis of variance (ANOVA) followed by post-hoc test (Dunnet's test) was performed to evaluate the significant difference. *P* value less than 0.05 was considered statistically significant.

Results

Effect of *C. roseus* extract on the viability of MDA-MB-231 cells

The effect of *C. roseus* extract on the viability of MDA-MB-231 cells was assessed by MTT assay. MDA-MB-231 cells were exposed to different concentrations of *C. roseus* (1–32 µg/mL) for 24, 48 and 72 h. The extract did not show cytotoxic effects on MDA-MB-231 cells within the range of

1–16 μg/mL in all time intervals. However, the extract significantly decreased the viability of the cells at high dose of 32 μg/mL (Fig. 1). Non-cytotoxic concentrations (1, 2 and 4 μg/mL) were selected for the following experiments.

C. roseus extract inhibits the migration of MDA-MB-231 cells

The ability of the *C. roseus* extract to inhibit the migration of MDA-MB-231 cells was firstly assessed by a scratch assay. The extract affected the natural capability of the cells to migrate and cover the created wound in a dose-dependent manner (Fig. 2a). In response to 2 and 4 μg/mL *C. roseus* treatment for 24 h, the wound closure percentages of the cells were decreased to 41.24% ± 2.24% and 21.31% ± 1.61%, respectively, compared with the control (58.03% ± 1.8%). Similarly, after incubation for 48 h, the percentages of wound closure were 73.04% ± 1.23% and 51.61% ± 2.84% at 2 and 4 μg/mL *C. roseus*, respectively compared to almost 100% for control. Even at a very low concentration of 1 μg/mL, the wound did not completely close after 48 h. The wound closure data of MDA-MB-231 cells treated with *C. roseus* from three independent experiments are shown in column statistics (Fig. 2b).

The anti-migratory effect of the *C. roseus* extract on MDA-MB-231 cells was further confirmed by Transwell migration assay. The assay assessed the inhibitory effect of the extract on the ability of MDA-MB-231 cells to migrate through a porous membrane towards a chemoattractant (10% FBS). The results revealed that, *C. roseus* extract decreased the directional migration of MDA-MB-231 cells in a dose-dependent manner. At 2 and 4 μg/mL *C. roseus* extract, the number of migrated cells was reduced signifcantly ($p < 0.001$) relative to that of the control. Figure 3a shows the number of migrated cells following *C. roseus* exposure. The numbers of migrated MDA-MB-231 cells of three independent experiments are shown in column statistics (Fig. 3b).

C. roseus extract inhibits the invasion of MDA-MB-231 cells

The effect of *C. roseus* extract on the invasiveness of MDA-MB-231 cells was assessed by Transwell invasion assay. The treated cells were seeded on the top of membrane-covered Matrigel to mimic the ECM and then incubated for 20 h. *C. roseus* extract exerted a dose-dependent anti-invasive effect on the highly invasive breast cancer cell line. Figure 4a shows that the number of invading cells was decreased by the increased treatment concentrations, indicating a dose-dependent effect. *C. roseus* extract at 2 and 4 μg/mL significantly ($p < 0.001$) decreased the number of invading cells compared with the control. Figure 4b shows the numbers of invading MDA-MB-231 cells of three independent experiments compared with control.

C. roseus extract decreases MMP-2 and MMP-9 secretion levels

The levels of MMP-2 and MMP-9 in the conditioned media of MDA-MB-231 cells treated with different concentrations of *C. roseus* were analysed by ELISA by using anti-human MMP-2 and MMP-9 antibodies. The results showed that at 2 and 4 μg/mL *C. roseus* extract the secreted levels of both MMPs were significantly ($p < 0.001$) decreased relative to the control. The effect of the extract on MMP-9 level was more potent than that on MMP-2 at 2 and 4 μg/mL. The secretion level of MMP-2 was 75.17% ± 1.39% and 59.43% ± 4.19% that of control at 2 and 4 μg/mL, respectively. However, MMP-9 secretion levels were 59.76% ± 3.15% and 41.86% ± 4.19% that of the control at 2 and 4 μg/mL, respectively. By contrast, at 1 μg/mL *C. roseus*, the secretion percentage of MMP-2 was significantly ($p < 0.05$) decreased to 80.8% ± 2.2%, whereas MMP-9 secretion was 93.9% ± 1.4% compared with that of control cells. A statistical analysis of MMP-2 and MMP-9 secretion levels (%) compared with control cells is shown in Fig. 5.

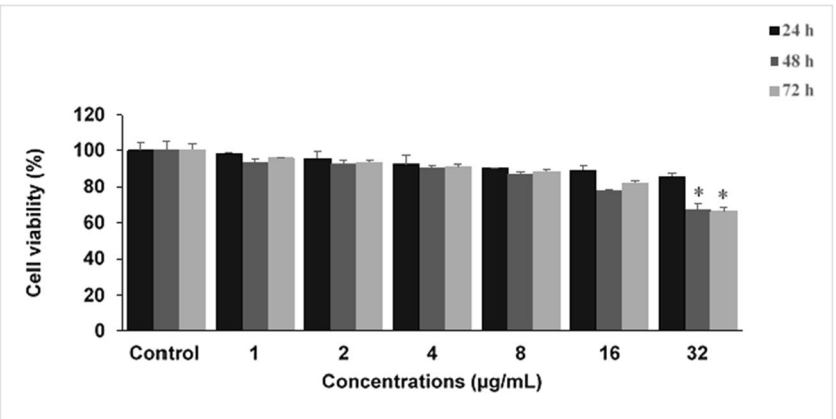

Fig. 1 Effect of *C. roseus* extract on MDA-MB-231 cells viability by MTT assay. The *C. roseus* extract did not show cytotoxicity at a concentration range of 1–16 μg/mL for all examined time intervals. Data are the mean ± SEM of three independent experiments. (*$p < 0.05$)

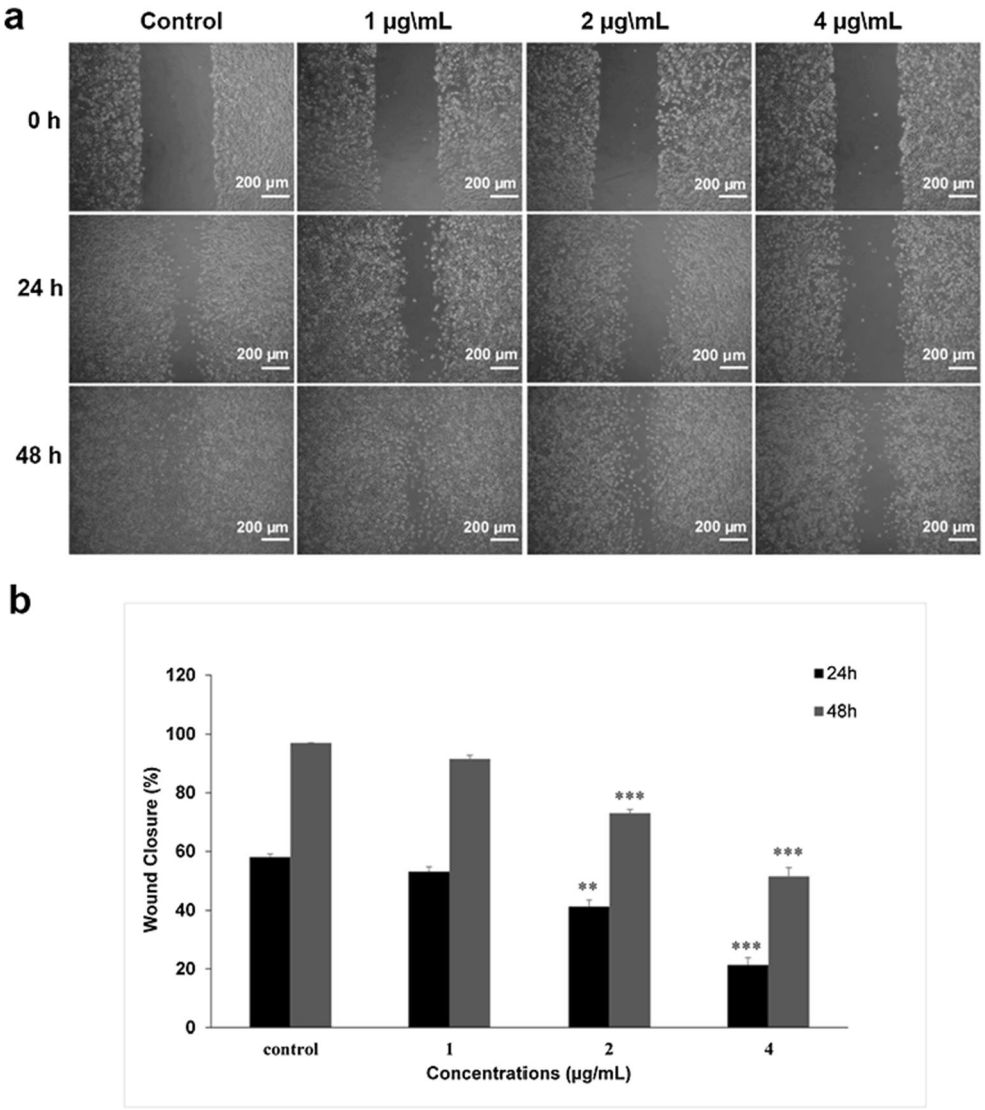

Fig. 2 Effect of *C. roseus* extract on the migration of MDA-MB-231 cells in scratch assay. **a** Cells were exposed to 1, 2 and 4 µg/mL *C. roseus* and photographed at 0, 24 and 48 h. **b** Statistical analysis of wound closure (%) of MDA-MB-231 cells treated with *C. roseus*. Data are the mean ± SEM of three independent experiments. (**$p < 0.01$ and ***$p < 0.001$)

C. roseus extract suppresses MMP-2 and MMP-9 activities

The effect of the extract on the gelatinolytic activity of secreted MMP-2 and MMP-9 in the media of MDA-MB-231 cells was investigated by gelatin zymography. Four different concentrations (1, 2, 4 and 6 µg/mL) were used to assess the inhibitory effect of the crude extract. As shown in Fig. 6a, the extract showed noticeable dose-dependent inhibition for both MMPs. Densitometric analysis was performed using Image J software. As shown in Fig. 6b, at *C. roseus* extract concentrations of 2, 4 and 6 µg/mL, the activities of MMP-2 were reduced to 80.2% ± 1.7%, 72.5% ± 2.2% and 63.3% ± 2.5%, respectively, compared with the control cells. Similarly, the activities of MMP-9 decreased to 77.4% ± 3.1%, 60.6% ± 1.2% and 57.5% ± 2.9% following exposure to 2, 4 and 6 µg/mL *C. roseus* extract, respectively (Fig. 6b).

C. roseus extract alters the gene expression profile of motility-related genes in MDA-MB-231 cell line

To elucidate the effect of *C. roseus* extract on MDA-MB-231 cell motility at the genomic level, we performed pathway-focused gene expression analysis by using RT-PCR and human cell motility RT^2 profiler PCR array. The array contains 84 genes, which are grouped based on the functions of their proteins to nine main groups as follows: chemotaxis, receptors, growth factors, Rho family GTPase, cell adhesion molecules (CAMs), integrin signaling, cellular projections, cell polarity and proteases & protease inhibitors. Rho family GTPases, CAMs and cellular projection groups were further classified into subgroups (Online Resource 1). Amongst the 84 genes examined, 52 (62%) were significantly ($p < 0.05$) downregulated in MDA-MB-231 cells following exposure to 4 µg/mL *C. roseus* for 24 h (Table 1). We selected this concentration based on our anti-proliferative,

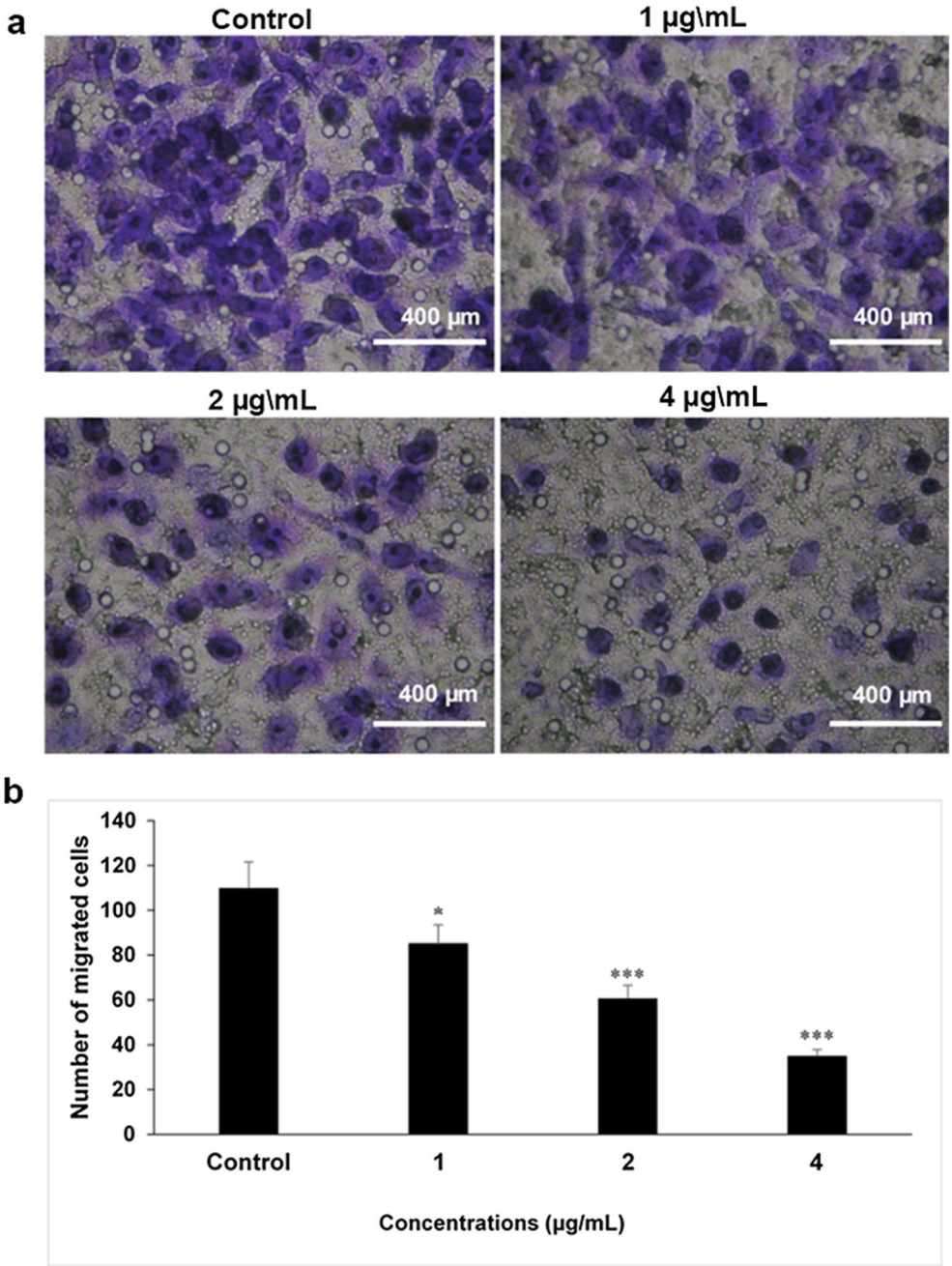

Fig. 3 Effect of *C. roseus* extract on the migration of MDA-MB-231 cells. **a** Cells were exposed to 1, 2 and 4 µg/mL *C. roseus* extract for 20 h. **b** Number of migrated MDA-MB-231 cells treated with different concentrations of *C. roseus*. Data are the mean ± SEM of three independent experiments. (*$p < 0.05$, ***$p < 0.001$)

anti-migratory and anti-invasive studies as it was non-cytotoxic and showed significant anti-migratory and anti-invasive effect on MDA-MB-231 cells. The list of the down-regulated genes based on their participations in the various functional categories and sub-categories as classified by Qiagen is given as Online Resource 1. The downregulated genes are mainly under the following categories: Rho family GTPases, focal adhesions, invadopodia formation and proteases, which are key regulators required for cellular functions associated with cell motility, such as actin cytoskeleton reorganisation, cell adhesion, ECM degradation and invasion.

Gene functional annotation

In order to determine the cellular processes and biological pathways that were regulated following *C. roseus* exposure, we used DAVID bioinformatics resource. DAVID utilises a complete set of functional annotation tools to classify genes depending on their participation in cellular functions and signaling pathways. Gene ontology (GO) and KEGG pathway analyses revealed that the downregulated genes participated in several biological processes and pathways important for cell motility. Tables 2 and 3 show the most enriched biological processes and pathways, respectively.

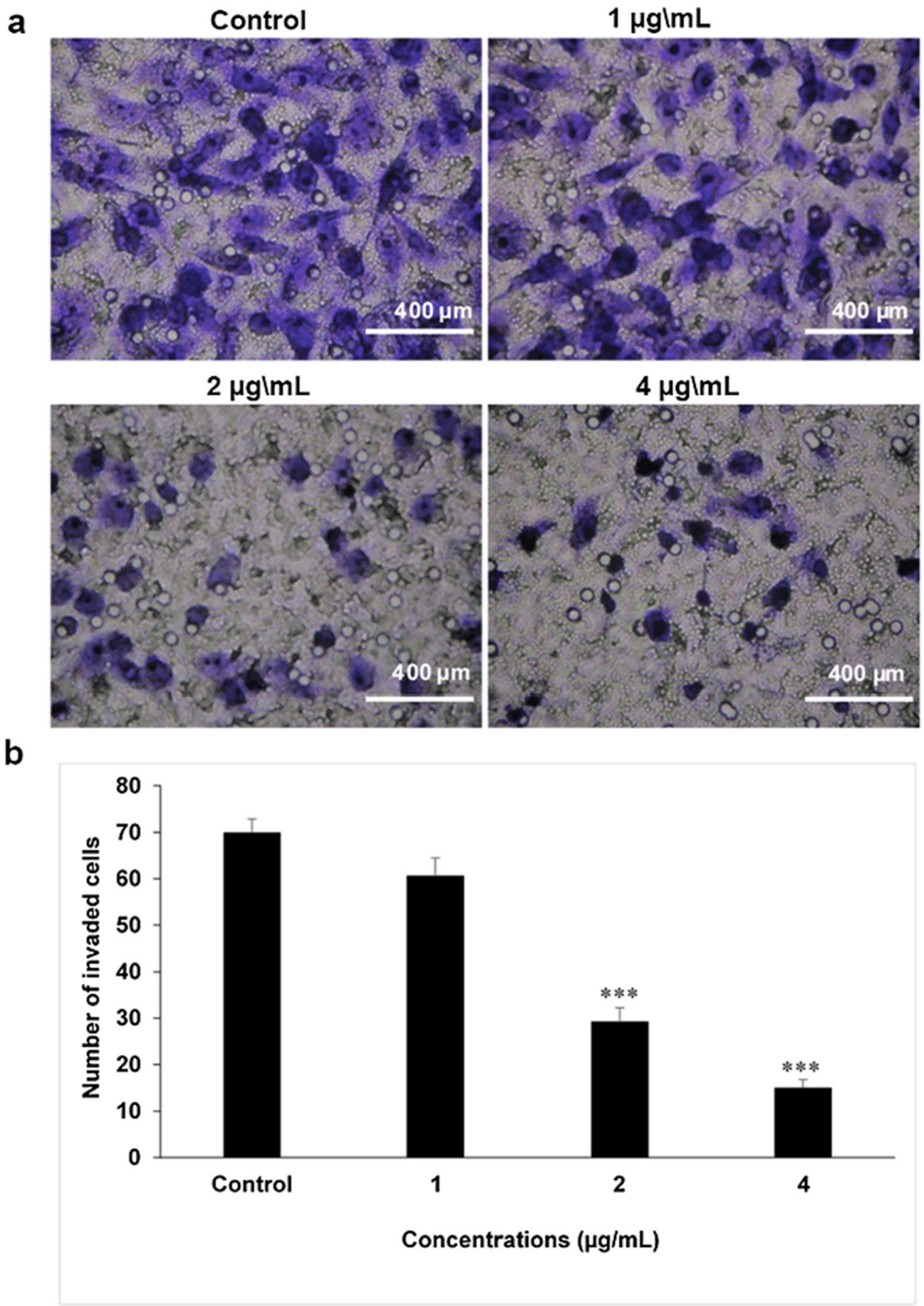

Fig. 4 Effect of *C. roseus* extract on the invasion of MDA-MB-231 cells. **a** Cells were exposed to 1, 2 and 4 µg/mL *C. roseus* extract for 20 h. **b** Number of invading MDA-MB-231 cells treated with different concentrations of *C. roseus*. Data are the mean ± SEM of three independent experiments. (***$p < 0.001$)

Protein-protein interaction network and hub genes identification

The interaction between the regulated genes was illustrated in a PPI network constructed by STRING database and visualised with Cytoscape software. As shown in Fig. 7, the constructed map showed significant interactions between the downregulated genes with PPI enrichment p-value of < 1.0e-16. The expected number of edges for the 52 genes was 29, however, the network showed 205 edges (Average node = 7.88, local clustering coefficient = 0.515). All the regulated genes and edges of the constructed PPI network were analysed and predicted by Cytohubba software using the MCC ranking method to identify the essential genes. The top ten genes which may represent the key (hub) genes amongst the current PPI map were identified and shown in orange colour in the constructed map (Fig. 7).

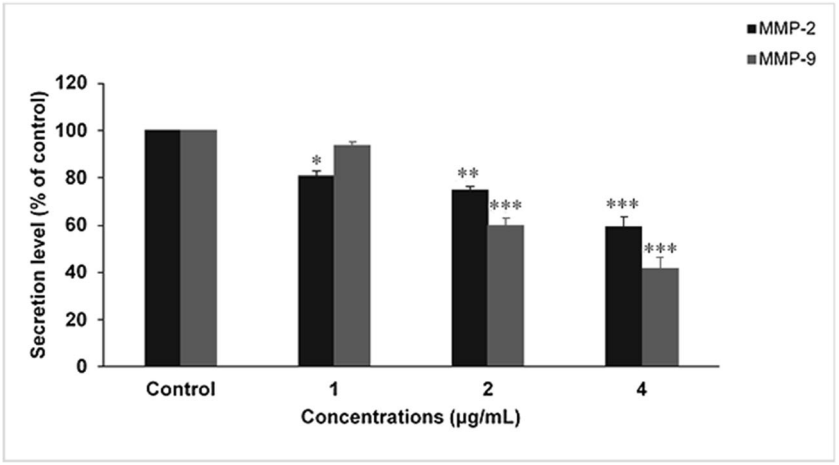

Fig. 5 Effect of *C. roseus* on MMP-2 and MMP-9 secretions in the conditioned medium of treated MDA-MB-231 cells. The levels of secreted MMP-2 and MMP-9 were determined using ELISA kits as described in Materials and Methods. Three independent experiments were performed, and the results are presented as the mean ± SEM. (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$)

Discussion

Cellular migration and invasion represent fundamental steps in cancer metastasis and are commonly achieved by various coordinated cellular processes. The inhibition of these processes may provide opportunities to prevent or delay cancer progression and dissemination. Studies have shown that, several plants and plant-derived substances exert anti-cancer activities on tumour cells through inhibiting various motility-related processes (Kapinova et al. 2018; Shin et al. 2018).

C. roseus has been widely used in traditional practice in many countries for the treatment of diabetes and cancer diseases. Several *in vivo* studies have been conducted to evaluate its toxic effects. Oral administration of *C. roseus* extracts to rats was found to be safe up to 5000 mg kg -1. Higher doses produce signs of toxicities in liver, kidney and heart of rats (Vutukuri et al. 2017; Ajuru et al. 2019). Vinblastine and vincristine, the cytotoxic agents isolated from *C. roseus*, are also known to have severe adverse side effects in patients. The main adverse side effects associated with vinblastine and vincristine that determine their doses are neurotoxicity and myelosuppression. Additionally, both cytotoxic agents showed poor water solubility and low bioavailability which limit their efficacy (Bates and Eastman 2017; Chagas and Alisaraie 2019). The synergistic interaction between phytochemicals can improve the therapeutic effect of anticancer agents. The use of many compounds together not only provide different mechanisms of action but also can improve the pharmacokinetics of compounds. Moreover, combination therapy can reduce the adverse side effects of cytotoxic agents (Zhou et al. 2016). Previously, the synergistic effect of indole alkaloids in *C. roseus* extract was reported. The indole alkaloid-

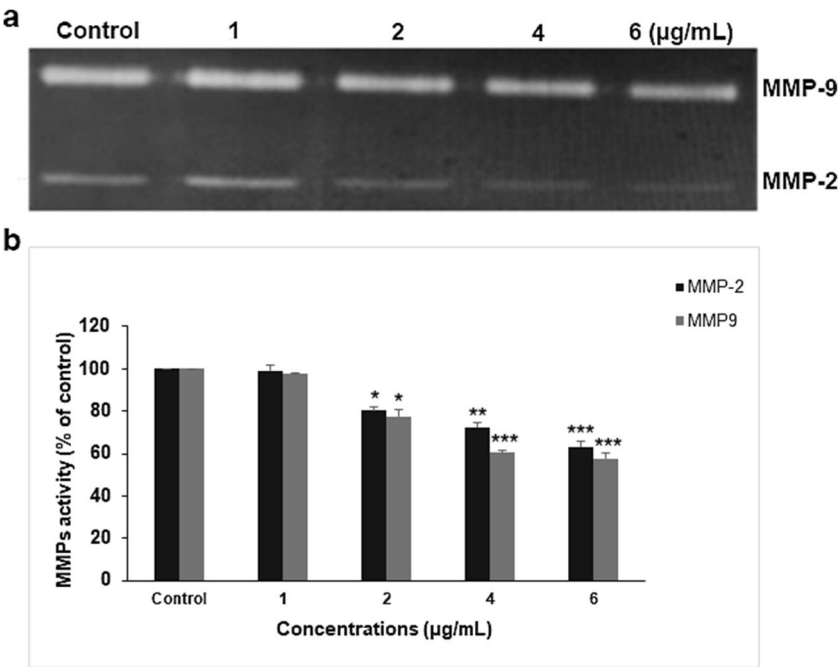

Fig. 6 Effects of *C. roseus* on MMP-2 and MMP-9 activity in the conditioned medium of MDA-MB-231 cells. **a** The cells were exposed to 1, 2, 4 and 6 µg/mL *C. roseus* for 24 h and the MMP-2 and MMP-9 activities were assessed using gelatin zymography. **b** Densitometric analysis of the bands obtained by gelatin zymography using ImageJ software. Three independent experiments were performed, and the results are presented as the mean ± SEM. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

Table 1 The downregulated genes (52) in MDA-MB-231 cells treated with *C. roseus* extract (≥2-fold)

No	Gene symbol	Gene Definition	Fold regulation
1	PAK1	P21 protein (Cdc42/Rac)-activated kinase 1	-5.43
2	PLD1	Phospholipase D1, phosphatidylcholine-specific	-4.86
3	MYLK	Myosin light chain kinase	-4.75
4	PRKCA	Protein kinase C, alpha	4.27
5	RHO	Rhodopsin	-4.19
6	MMP14	Matrix metallopeptidase 14 (membrane-inserted)	-4.15
7	ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	-4.15
8	SH3PXD2A	SH3 and PX domains 2A	-4.14
9	WIPF1	WAS/WASL interacting protein family, member 1	-3.95
10	CTTN	Cortactin	-3.85
11	RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	-3.81
12	RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	-3.79
13	CAPN2	Calpain 2, (m/II) large subunit	-3.69
14	CDC42	Cell division cycle 42 (GTP binding protein, 25 kDa)	-3.56
15	CAPN1	Calpain 1, (mu/I) large subunit	-3.54
16	ACTN3	Actinin, alpha 3	-3.53
17	ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7	-3.50
18	RASA1	RAS p21 protein activator (GTPase activating protein) 1	-3.45
19	CSF1	Colony stimulating factor 1 (macrophage)	-3.41
20	MYH10	Myosin, heavy chain 10, non-muscle	-3.27
21	PLCG1	Phospholipase C, gamma 1	-3.27
22	WASL	Wiskott-Aldrich syndrome-like	-3.24
23	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	-3.18
24	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	-3.16
25	LIMK1	LIM domain kinase 1	-3.14
26	HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)	-3.11
27	MET	Met proto-oncogene (hepatocyte growth factor receptor)	-3.07
28	ILK	Integrin-linked kinase	-3.05
29	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	-3.02
30	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	-3.01
31	MSN	Moesin	-2.97
32	PAK4	P21 protein (Cdc42/Rac)-activated kinase 4	-2.95
33	TLN1	Talin 1	-2.90
34	VCL	Vinculin	-2.89
35	EGF	Epidermal growth factor	-2.87
36	PFN1	Profilin 1	-2.85
37	PTPN1	Protein tyrosine phosphatase, non-receptor type 1	-2.83
38	PXN	Paxillin	-2.82
39	FGF2	Fibroblast growth factor 2 (basic)	-2.82
40	DIAPH1	Diaphanous homolog 1 (Drosophila)	-2.79
41	ACTN4	Actinin, alpha 4	-2.79
42	EGFR	Epidermal growth factor receptor	-2.74
43	ACTR2	ARP2 actin-related protein 2 homolog (yeast)	-2.73
44	ENAH	Enabled homolog (Drosophila)	-2.67
45	PLAUR	Plasminogen activator, urokinase receptor	-2.66
46	IGF1R	Insulin-like growth factor 1 receptor	-2.59
47	EZR	Ezrin	-2.46
48	BCAR1	Breast cancer anti-estrogen resistance 1	-2.44
49	FAP	Fibroblast activation protein, alpha	-2.32

Table 1 (continued)

No	Gene symbol	Gene Definition	Fold regulation
50	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	-2.32
51	DPP4	Dipeptidyl-peptidase 4	-2.14
52	RND3	Rho family GTPase 3	-2.08

enriched *C. roseus* extract showed more cytotoxicity on leukemia cell lines compared to the effect of the alkaloids separately which indicates the synergistic action of *C. roseus* compounds (Fernández-Pérez et al. 2013). The use of *C. roseus* alkaloids at low doses and in combination may give better therapeutic effect, reduce the cytotoxicity and enhance the bioavailability of the bioactive compounds.

In the current study, the effect of low doses of *C. roseus* extract on migration, invasion, gelatinase secretion and activities of MDA-MB-231 cells was assessed. Furthermore, the effect of the extract on the expression profile of 84 genes commonly involved in various cellular processes important for cell motility was investigated.

The results showed that, *C. roseus* extract strongly inhibited the migration and invasion of MDA-MB-231 cells at non-cytotoxic concentrations. This inhibition was associated with decreased levels and activities of MMP-2 and MMP-9 in a dose-dependent manner. At molecular levels, the extract downregulated 52 motility-related genes, which included MMP-2, MMP-9 and MMP-14, amongst other proteases. Matrix metalloproteinases (MMPs) play an important role in tumour invasion and metastasis due to their capabilities to process the ECM components (Jabłońska-Trypuć et al. 2016; Tauro and Lynch 2018). The overexpression of MMP members, such as MMP-2, MMP-9 and MMP-14, is associated with the invasion and metastasis of mammary tumours (Di et al. 2018; Li et al. 2017). Moreover, the inhibition of these MMPs is linked to the inhibition of invasive capability of MDA-MB-231 cells (Ling et al. 2017; Mali et al. 2012). Previous studies have reported the role of plant and plant-derived substances in the inhibition of migration and invasion of MDA-MB-231 cells through the suppression of MMP-2, MMP-9 and MMP-14 activities and expression (Al Dhaheri et al. 2013; Li et al. 2014; Nho et al. 2015; Mali et al. 2012; Tieng et al. 2019). Likewise, our findings speculated that *C. roseus* extract may inhibit invasion, at least partly, by affecting the ability of the cells to degrade the ECM through regulating the secretion, activities and transcriptions of MMPs.

In addition to the downregulation of ECM proteases, the extract significantly downregulated other genes, which are mainly under RHO family GTPases, focal adhesions and invadopodia formation categories. RHO family GTPase proteins play a fundamental role in cancer cell metastasis by controlling several aspects of cell migration (Haga and Ridley 2016). The classical GTPase subfamilies RHO, RAC and CDC42 signaling molecules are well known for their function in the regulation of actin cytoskeleton, which is necessary for many cellular processes in cancer cell motility (Fife et al. 2014). Targeting the RHO, RAC and CDC42 signaling pathways provides several opportunities to discover effective therapeutics to inhibit cancer dissemination. The inhibition of

Table 2 Biological processes for the downregulated genes (≥2-fold) in MDA-MB-231 cells. Gene ontology analysis was performed using DAVID

Biological process	Count	Genes
Signal transduction	14	LIMK1, MET, RASA1, ARHGEF7, EGFR, EGF, FGF2, IGF1R, PAK4, PXN, PLCG1, PLAUR, RAC2, STAT3
Positive regulation of cell migration	12	BCAR1, ACTN4, CSF1, DIAPH1, EGFR, HGF, IGF1R, ILK, MMP14, MYLK, PAK1 and PRKCA
cell adhesion	11	BCAR1, RND3, FAP, ITGA4, ITGB2, ITGB3, MYH10, PXN, PRKCA, RAC1, VCL
Ephrin receptor signaling pathway	9	ACTR2, RASA1, ARHGEF7, WASL, CDC42, MMP2, MMP9, PAK1, RAC1
Movement of cell or subcellular component	9	ACTR2, WASL, MSN, PAK4, PLAUR, RAC1, STAT3, TLN1, VCL
Cell-matrix adhesion	7	ILK, ITGA4, ITGB2, ITGB3, PXN, RAC1, VCL
Actin cytoskeleton organisation	7	LIMK1, RND3, CDC42, DIAPH1, PFN1, RAC1, RAC2
Extracellular matrix disassembly	5	CAPN1, CAPN2, MMP14, MMP2, MMP9
integrin-mediated signaling pathway	5	BCAR1, ILK, ITGA4, ITGB2, ITGB3

Table 3 Pathway analysis for the downregulated genes (≥2-fold) in MDA-MB-231 cells. Pathway analysis was performed using KEGG

Pathways	Count	Genes
Regulation of actin cytoskeleton	25	BCAR1, LIMK1, ARHGEF7, WASL, ACTN3, ACTN4, CDC42, DIAPH1, ENAH, EGFR, EGF, EZR, FGF2, ITGA4, ITGB2, ITGB3, MSN, MYLK, PAK1, PAK4, PXN, PFN1, RAC1, RAC2, VCL.
Focal adhesion	23	BCAR1, MET, ACTN3, ACTN4, CAPN2, CDC42, DIAPH1, EGFR, EGF, HGF, IGF1R, ILK, ITGA4, ITGB3, MYLK, PAK1, PAK4, PXN, PRKCA, RAC1, RAC2, TLN1, VCL.
RAP1 signaling pathway	17	BCAR1, MET, CDC42, CSF1, EGFR, EGF, FGF2, HGF, IGF1R, ITGB2, ITGB3, PLCG1, PFN1, PRKCA, RAC1, RAC2, TLN1.
RAS signaling pathway	16	MET, RASA1, CDC42, CSF1, EGFR, EGF, FGF2, HGF, IGF1R, PAK1, PAK4, PLCG1, PLD1, PRKCA, RAC1, RAC2.
Pathways in cancer	14	MET, CDC42, EGFR, EGF, FGF2, HGF, IGF1R, MMP2, MMP9, PLCG1, PRKCA, RAC1, RAC2, STAT3
Adherens junction	11	MET, WASL, ACTN3, ACTN4, CDC42, EGFR, IGF1R, PTPN1, RAC1, RAC2, VCL.
PI3K-Akt signaling pathway	11	MET, CSF1, EGFR, EGF, FGF2, HGF, IGF1R, ITGA4, ITGB3, PRKCA, RAC1.
MAPK signaling pathway	9	RASA1, CDC42, EGFR, EGF, FGF2, PAK1, PRKCA, RAC1, RAC2.

cancer cell motility by regulating the expression of RHO, RAC and CDC42 molecules has been reported previously. Acevedo-Díaz et al. (2019) reported that *Ganoderma lucidum* extract suppresses the migration and invasion of MDA-MB-231 cells by reducing the expression of RAC signaling molecules and CDC42 and inhibiting lamellipodium formation. Likewise, Kasorn et al. (2018) reported that terrein, a metabolite isolated from *Aspergillus terreus*, inhibits the migration and invasion of breast cancer cell lines by suppressing the expression of RHO GTPases and MMPs. In the same context, the flavone apigenin inhibits the motility of breast cancer cells by downregulating the expression of the RHO

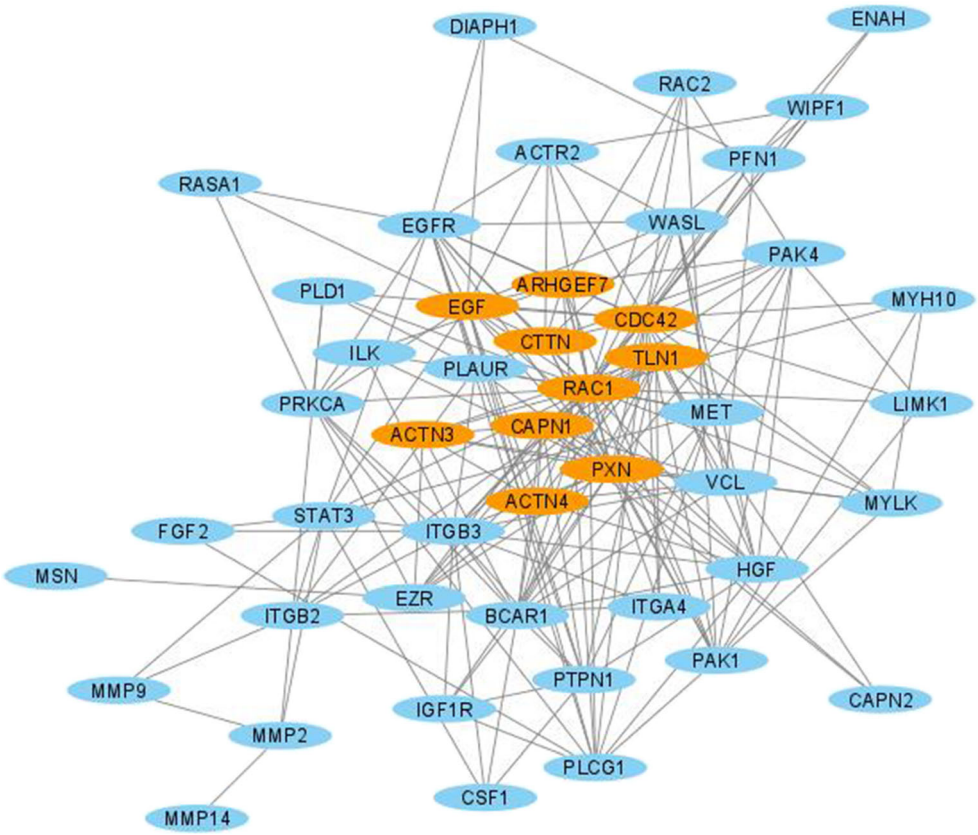

Fig. 7 Protein-protein interaction network of *C. roseus* downregulated genes. The network was constructed using STRING and visualised using Cytoscape software. The map shows significant interactions between the downregulated genes (the average node = 7.88, average local clustering coefficient = 0.515, enrichment p-value < 1.0e-16). The top 10 hub proteins were coloured in orange

GTPases RAS, RAC1, CDC42 and RHOA (Shih 2017). In our study, the extract downregulated the expression of 18 genes important to RHO, RAC and CDC42 signaling pathways. These results suggest that, *C. roseus* may partially exhibit its anti-migratory effect by suppressing RHO GTPase genes expression.

Studies have shown that microtubules targeting drugs such as vinca alkaloids exert anti-metastatic properties such as anti-migratory and anti-invasive effects (Bijman et al. 2006; Bates and Eastman 2017). Recently Wang et al. (2019) have shown that, microtubule-binding agents such as vinblastine, inhibited the migration of human osteosarcoma cancer cells (U2OS) through altering microtubule dynamics. This study showed that, low concentrations of *C. roseus* extract inhibited the migration and invasion of MDA-MB-231 cells through inhibition of various cellular processes including cytoskeletal reorganization. The inhibition of cytoskeletal reorganization could be due to the presence of vinblastine and vincristine in the extract. Study is ongoing to separate and identify the bioactive compounds responsible for anti-migratory and anti-invasive effect of *C. roseus* extract on MDA-MB-231 cells.

Notably, the most significantly downregulated molecule amongst the RHO GTPase genes was P21-activated kinase 1 (PAK1). *C. roseus* significantly suppressed the expression of PAK1 by 5.43-fold. PAK1, a member of the serine/threonine protein kinase family, is one of the major downstream effectors of the RAC and CDC42 signaling pathways (Manser et al. 1994). In addition to its essential role in transmitting signals controlling cytoskeleton reorganisation, it is implicated in the phosphorylation of many cellular proteins with functions in cell motility and invasion (Kumar et al. 2006; Radu et al. 2014). The role of PAK1 in cancer cell invasion through regulating the expression and activity of MMPs, particularly MMP-2 and MMP-9 has been documented (Chen et al. 2019; Rider et al. 2013). The importance of PAK1 as a therapeutic target in breast cancer has been highlighted by various research groups (Korobeynikov et al. 2019; Hirokawa et al. 2005; Ong et al. 2015). The inhibition of PAK1 by IPA-3 inhibitor significantly inhibits the migration of the MDA-MB-231 cell line in a time- and dose-dependent manner (Fajardo et al. 2015). In the present study, the downregulation of PAK1 by *C. roseus* may be a mechanism behind the suppression of the migration and invasion capabilities of MDA-MB-231 cells.

Focal adhesions are integrin-containing subcellular structure that facilitate cell-ECM crosstalk and represent scaffold for many biochemical signaling essential for tumour cell motility. It has been revealed that, formation of focal adhesions is essential for migration and invasion of cancer cells (Shen et al. 2018). Th e regulation of focal adhesions proteins is important to inhibit the migration of highly invasive TNBC cells (Schlienger et al. 2015; Shen et al. 2018). It has been reported that, the secondary metabolite afzelin inhibited the migration of MDA-MB-231 cells through suppression of focal adhesions formation (Rachmi et al. 2020). In the current study, 10 genes implicated in focal adhesions formation were significantly downregulated following *C. roseus* exposure. This result indicates the ability of *C. roseus* components to inhibit breast cancer migration and invasion by regulating focal adhesions formation.

The formation of cellular protrusions, such as filopodia, lamellipodia and invadopodia, are essential for cell motility. Invadopodia are finger-like, actin-rich projections formed by highly invasive cancer cells to degrade and invade the ECM (Murphy and Courtneidge 2011). The inhibition of invadopodium development can effectively decrease the invasiveness of breast cancer cells and prevent metastasis (Eckert and Yang 2011; Tieng et al. 2019). Invadopodium formation is a complex process involving initiation, stabilisation and maturation, which are regulated by various molecules. In our study, 17 genes, including CDC42, EGF, SH3PXD2A, WASL, MMP-2, MMP-9 and MMP-14, were downregulated by *C. roseus*. The above-mentioned downregulated genes are some of the key regulators of invadopodium process (Hoshino et al. 2013; Jacob and Prekeris 2015). Our findings were in line with previous studies reporting the role of medicinal plants in the inhibition of TNBC invasion by preventing invadopodium formation (Fu et al. 2016; Harun et al. 2018). This result suggested that the regulation of invadopodia-related genes was a mechanism utilised by *C. roseus* to halt the invasion of MDA-MB-231 cells.

In order to predict the most enriched biological functions and pathways of the downregulated genes following *C. roseus* exposure, GO and KEGG pathway analyses were performed using DAVID. DAVID provides bioinformatics and functional annotation tools to interpret the biological mechanisms associated with the regulated genes and has been commonly used in biological research (Shah et al. 2016). GO analysis results indicated that exposure to *C. roseus* extract resulted in the enrichment of important biological processes related to cell motility, such as the positive regulation of cell migration, cell adhesion, actin cytoskeleton organisation and ECM disassembly. Similarly, KEGG pathway annotation analysis revealed that the differentially expressed genes were mainly enriched in the regulation of actin cytoskeleton, focal adhesion, RAP1 and RAS signaling pathways. GO and KEGG pathway analyses confirmed the molecular targets of *C. roseus* and its potential role as inhibitor of breast cancer motility through the regulation of several cellular events. Using the STRING and Cytoscape, ten genes (RAC1, CTTN, CDC42, CAPN1, ACTN3, ARHGEF7, TLN1, EGF, PXN and ACTN4) were identified as hub genes highly correlated with the other genes in the network. Various recent studies support the importance of these identified hub genes in the migration and invasion of breast cancer cells (Tian et al. 2018; Montalto et al. 2019; Liang et al. 2018; Yin et al. 2017; Wang et al. 2017; Acevedo-Díaz et al. 2019).

In summary, the data showed that *C. roseus* inhibited the migration and invasion of the highly invasive TNBC cell line MDA-MB-231 at non-cytotoxic doses. The inhibitory effect may involve altering the expression profile of various genes implicated in ECM degradation, cytoskeleton reorganisation, focal adhesions and invadopodia formation. Our study provides a preliminary view of the potential molecular targets of *C. roseus* phytochemicals in MDA-MB-231 cells, and these results may aid in the design and development of effective combination therapies for the treatment of invasive breast cancer.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.2478/s11756-020-00641-5.

Acknowledgements We wish to acknowledge Universiti Sains Malaysia (USM) and The World Academy of Sciences (TWAS) for USM-TWAS Postgraduate Fellowship (FR number 3240275091) and financial support to Nagla.

Funding This study was funded by RUI grant (1001/PFARMASI/8011003), Universiti Sains Malaysia and FRGS grant (203.PFarmasi.6711764) Ministry of Higher Education.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

References

Acevedo-Díaz A, Ortiz-Soto G, Suárez-Arroyo IJ, Zayas-Santiago A, Martínez Montemayor MM (2019) *Ganoderma lucidum* extract reduces the Motility of breast cancer cells mediated by the RAC–Lamellipodin axis. Nutrients 11:1116. https://doi.org/10.3390/nu11051116

Ajuru MG, Ajuru G, Nmom FW, Worlu CW, Igoma PG (2019) Acute toxicity study and determination of median lethal dose of *Catharanthus roseus* in Wistar Albino rats. J Appl Sci 19:217–222. https://doi.org/10.3923/jas.2019.217.222

Al Dhaheri Y, Attoub S, Arafat K, AbuQamar S, Viallet J, Saleh A, Al Agha H, Eid A, Iratni R (2013) Anti-metastatic and anti-tumor growth effects of *Origanum majorana* on highly metastatic human breast cancer cells: inhibition of NFκB signaling and reduction of nitric oxide production. PLoS One 8:e68808. https://doi.org/10.1371/journal.pone.0068808

Bates D, Eastman A (2017) Microtubule destabilising agents: far more than just antimitotic anticancer drugs. Br J Clin Pharmacol 83:255–268. https://doi.org/10.1111/bcp.13126

Bijman MN, van Nieuw Amerongen GP, Laurens N, van Hinsbergh VW, Boven E (2006) Microtubule-targeting agents inhibit angiogenesis at subtoxic concentrations, a process associated with inhibition of Rac1 and Cdc42 activity and changes in the endothelial cytoskeleton. Mol Cancer Ther 5:2348–2357. https://doi.org/10.1158/1535-7163.MCT-06-0242

Chagas CM, Alisaraie L (2019) Metabolites of vinca alkaloid vinblastine: tubulin binding and activation of nausea-associated receptors. ACS Omega 4:9784–9799. https://doi.org/10.1021/acsomega.9b00652

Chen L, Bi S, Hou J, Zhao Z, Wang C, Xie S (2019) Targeting p21-activated kinase 1 inhibits growth and metastasis via Raf1/MEK1/ERK signaling in esophageal squamous cell carcinoma cells. Cell Commun Signal 17:31. https://doi.org/10.1186/s12964-019-0343-5

Choudhari AS, Mandave PC, Deshpande M, Ranjekar P, Prakash O (2020) Phytochemicals in cancer treatment: from preclinical studies to clinical practice. Front Pharmacol 10:1614. https://doi.org/10.3389/fphar.2019.01614

Denkert C, Liedtke C, Tutt A, von Minckwitz G (2017) Molecular alterations in triple-negative breast cancer—the road to new treatment strategies. Lancet 389:2430–2442. https://doi.org/10.1016/S0140-6736(16)32454-0

Di D, Chen L, Guo Y, Wang L, Wang H, Ju J (2018) Association of BCSC-1 and MMP-14 with human breast cancer. Oncol Lett 15:5020–5026. https://doi.org/10.3892/ol.2018.7972

Eckert MA, Yang J (2011) Targeting invadopodia to block breast cancer metastasis. Oncotarget 2:562–568. https://doi.org/10.18632/oncotarget.301

Eltayeb NM, Ng SY, Ismail Z, Salhimi SM (2016) Anti-invasive effect of *Catharanthus roseus* extract on highly metastatic human breast cancer MDA-MB-231 cells. J Teknol 78:35–40. https://doi.org/10.11113/jt.v78.9870

Fajardo AM, Browne T, Graff H, Kleier K, Neltner K, McCall C, Meyer B, Douglass L, Carter J (2015) Targeting PAK1 activity in breast cancer: Inhibition of cell growth, survival, motility, and signaling. Cancer Res 75:1024. https://doi.org/10.1158/15387445.AM20151024

Fields GB (2019) The rebirth of matrix metalloproteinase inhibitors: moving beyond the dogma. Cells 8:984. https://doi.org/10.3390/cells8090984

Fife CM, McCarroll JA, Kavallaris M (2014) Movers and shakers: cell cytoskeleton in cancer metastasis. Br J Pharmacol 171:5507–5523. https://doi.org/10.1111/bph.12704

Fernández-Pérez F, Almagro L, Pedreño MA, Gomez Ros LV (2013) Synergistic and cytotoxic action of indole alkaloids produced from elicited cell cultures of *Catharanthus roseus*. Pharm Biol 51:304–310. https://doi.org/10.3109/13880209.2012.722646

Fu H, Wu R, Li Y, Zhang L, Tang X, Tu J, Zhou W, Wang J, Shou Q (2016) Safflower yellow prevents pulmonary metastasis of breast cancer by inhibiting tumor cell invadopodia. Am J Chin Med 44:1491–1506. https://doi.org/10.1142/S0192415X1650083X

Haga RB, Ridley AJ (2016) Rho GTPases: Regulation and roles in cancer cell biology. Small GTPases 7:207–221. https://doi.org/10.1080/21541248.2016.1232583

Harun S, Israf D, Tham C, Lam KW, Cheema MS, Md Hashim NF (2018) The molecular targets and anti-invasive effects of 2, 6-bis-(4-hydroxyl-3methoxybenzylidine) cyclohexanone or BHMC in MDA-MB-231 human breast cancer cells. Molecules 23:865. https://doi.org/10.3390/molecules23040865

Hirokawa Y, Arnold M, Nakajima H, Zalcberg J, Maruta H (2005) Signal therapy of breast cancers by the HDAC inhibitor FK228 that blocks the activation of PAK1 and abrogates the tamoxifen-resistance. Cancer Biol Ther 4:956–960. https://doi.org/10.4161/cbt.4.9.1911

Hoshino D, Branch KM, Weaver AM (2013) Signaling inputs to invadopodia and podosomes. J Cell Sci 126:2979–2989. https://doi.org/10.1242/jcs.079475

Jabłońska-Trypuć A, Matejczyk M, Rosochacki S (2016) Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. J Enzyme Inhib Med Chem 31:177–183. https://doi.org/10.3109/14756366.2016.1161620

Jacob A, Prekeris R (2015) The regulation of MMP targeting to invadopodia during cancer metastasis. Fron Cell Dev Biol 3:4. https://doi.org/10.3389/fcell.2015.00004

Kapinova A, Kubatka P, Golubnitschaja O, Kello M, Zubor P, Solar P, Pec M (2018) Dietary phytochemicals in breast cancer research: anticancer effects and potential utility for effective chemoprevention. Environ Health Prev Med 23:36. https://doi.org/10.1186/s12199-018-0724-1

Kasorn A, Loison F, Kangsamaksin T, Jongrungruangchok S, Ponglikitmongkol M (2018) Terrein inhibits migration of human breast cancer cells via inhibition of the Rho and Rac signaling pathways. Oncol Rep 39:1378–1386. https://doi.org/10.3892/or.2018.6189

Korobeynikov V, Borakove M, Feng Y, Wuest WM, Koval AB, Nikonova AS, Serebriiskii I, Chernoff J, Borges VF, Golemis EA, Shagisultanova E (2019) Combined inhibition of Aurora A and p21-activated kinase 1 as a new treatment strategy in breast cancer. Breast Cancer Res Tr 177:369–382. https://doi.org/10.1007/s10549-019-05329-2

Kumar R, Gururaj AE, Barnes CJ (2006) p21-activated kinases in cancer. Nat Rev Cancer 6:459. https://doi.org/10.1038/nrc1892

Li C, Zhao Y, Yang D, Yu Y, Guo H, Zhao Z, Zhang B, Yin X (2014) Inhibitory effects of kaempferol on the invasion of human breast carcinoma cells by downregulating the expression and activity of matrix metalloproteinase-9. Biochem Cell Biol 93:16–27. https://doi.org/10.1139/bcb-2014-0067

Li H, Qiu Z, Li F, Wang C (2017) The relationship between MMP-2 and MMP-9 expression levels with breast cancer incidence and prognosis. Oncol Lett 14:5865–5870. https://doi.org/10.3892/ol.2017.6924

Liang Y, Chen H, Ji L, Du J, Xie X, Li X, Lou Y (2018) Talin2 regulates breast cancer cell migration and invasion by apoptosis. Oncol Lett 16:285–293. https://doi.org/10.3892/ol.2018.8641

Ling B, Watt K, Banerjee S, Newsted D, Truesdell P, Adams J, Sidhu SS, Craig AW (2017) A novel immunotherapy targeting MMP-14 limits hypoxia, immune suppression and metastasis in triple-negative breast cancer models. Oncotarget 8:58372–58385. https://doi.org/10.18632/oncotarget.17702

Mali AV, Wagh UV, Hegde MV, Chandorkar SS, Surve SV, Patole MV (2012) In vitro anti-metastatic activity of enterolactone, a mammalian lignan derived from flax lignan, and down-regulation of matrix metalloproteinases in MCF-7 and MDA MB 231 cell lines. Indian J Cancer 49:181–187. https://doi.org/10.4103/0019-509X.98948

Manser E, Leung T, Salihuddin H, Zhao ZS, Lim L (1994) A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature 367:40–46. https://doi.org/10.1038/367040a0

McSherry EA, Donatello S, Hopkins AM, McDonnell S (2007) Molecular basis of invasion in breast cancer. Cell Mol Life Sci 64:3201–3218. https://doi.org/10.1007/s00018-007-7388-0

Montalto FI, Giordano F, Chiodo C, Marsico S, Mauro L, Sisci D, Aquila S, Lanzino M, Panno ML, Andò S, De Amicis F (2019) Progesterone receptor B signaling reduces breast cancer cell aggressiveness: role of cyclin-D1/Cdk4 mediating paxillin phosphorylation. Cancers 11:1201. https://doi.org/10.3390/cancers11081201

Murphy DA, Courtneidge SA (2011) The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. Nat Rev Mol Cell Biol 12:413–426. https://doi.org/10.1038/nrm3141

National Institutes of Health (NIH) (2020) Image J, Image J download for windows. http://rsb.info.nih.gov/ij/download.html. Accessed 5 Jan 2020

Nho KJ, Chun JM, Kim DS, Kim HK (2015) *Ampelopsis japonica* ethanol extract suppresses migration and invasion in human MDAMB231 breast cancer cells. Mol Med Rep 11:3722–3728. https://doi.org/10.3892/mmr.2015.3179

Ong CC, Gierke S, Pitt C, Sagolla M, Cheng CK, Zhou W, Jubb AM, Strickland L, Schmidt M, Duron SG, Campbell DA (2015) Small molecule inhibition of group I p21-activated kinases in breast cancer induces apoptosis and potentiates the activity of microtubule stabilizing agents. Breast Cancer Res 17:59. https://doi.org/10.1186/s13058-015-0564-5

Pezzani R, Salehi B, Vitalini S, Iriti M, Zuñiga FA, Sharifi-Rad J, Martorell M, Martins N (2019) Synergistic effects of plant derivatives and conventional chemotherapeutic agents: an update on the cancer perspective. Medicina 55:110. https://doi.org/10.3390/medicina55040110

Rachmi E, Purnomo BB, Endharti AT, Fitri LE (2020) Afzelin inhibits migration of MDA-MB-231 cells by suppressing FAK expression and Rac1 activation. J Appl Pharm Sci 10:077–082. https://doi.org/10.7324/JAPS.2020.101010

Radu M, Semenova G, Kosoff R, Chernoff J (2014) PAK signalling during the development and progression of cancer. Nat Rev Cancer 14:13–25. https://doi.org/10.1038/nrc3645

Redig AJ, McAllister SS (2013) Breast cancer as a systemic disease: a view of metastasis. J Intern Med 27:113–126. https://doi.org/10.1111/joim.12084

Rider L, Oladimeji P, Diakonova M (2013) PAK1 regulates breast cancer cell invasion through secretion of matrix metalloproteinases in response to prolactin and three-dimensional collagen IV. Mol Endocrinol 27:1048–1064. https://doi.org/10.1210/me.2012-1322

Schlienger S, Ramirez RA, Claing A (2015) ARF1 regulates adhesion of MDA-MB-231 invasive breast cancer cells through formation of focal adhesions. Cell Signal 27:403–415. https://doi.org/10.1016/j.cellsig.2014.11.032

Shah P, Djisam R, Damulira H, Aganze A, Danquah M (2016) Embelin inhibits proliferation, induces apoptosis and alters gene expression profiles in breast cancer cells. Pharmacol Rep 68:638–644. https://doi.org/10.1016/j.pharep.2016.01.004

Sharma A, Amin D, Sankaranarayanan A, Arora R, Mathur AK (2019) Present status of *Catharanthus roseus* monoterpenoid indole alkaloids engineering in homo-and hetero-logous systems. Biotechnol Lett 42:11–23. https://doi.org/10.1007/s10529-019-02757-4

Shen J, Cao B, Wang Y, Ma C, Zeng Z, Liu L, Li X, Tao D, Gong J, Xie D (2018) Hippo component YAP promotes focal adhesion and tumour aggressiveness via transcriptionally activating THBS1/FAK signalling in breast cancer. J Exp Clin Cancer Res 37:175. https://doi.org/10.1186/s13046-018-0850-z

Shih YW (2017) Apigenin regulates matrix metalloproteinase-2/9 and Rho Gtpase family through FAk signal to reduce breast cancer MCF-7 cells metastasis. Int J Biotech Bioeng 3:258–267. https://doi.org/10.25141/2475-3432-2017-7.0249

Shin SA, Moon S, Kim WY, Paek SM, Park HH, Lee CS (2018) Structure-based classification and anti-cancer effects of plant metabolites. Int J Mol Sci 19:2651. https://doi.org/10.3390/ijms19092651

Siddiqui MJ, Ismail Z, Saidan NH (2011) Simultaneous determination of secondary metabolites from *Vinca rosea* plant extractives by reverse phase high performance liquid chromatography. Pharmacogn Mag 7:92–96. https://doi.org/10.4103/0973-1296.80662

Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, Simonovic M, Roth A, Santos A, Tsafou KP, Kuhn M (2015) STRING v10: protein–protein interaction networks, integrated over the tree of life. Nucleic Acids Res 43:D447–D452. https://doi.org/10.1093/nar/gku1003

Tauro M, Lynch C (2018) Cutting to the chase: how matrix metalloproteinase-2 activity controls breast-cancer-to-bone metastasis. Cancers 10:185. https://doi.org/10.3390/cancers10060185

Tian Y, Xu L, He Y, Xu X, Li K, Ma Y, Gao Y, Wei D, Wei L (2018) Knockdown of RAC1 and VASP gene expression inhibits breast cancer cell migration. Oncol Lett 16:2151–2160. https://doi.org/10.3892/ol.2018.8930

Tieng FYF, Latifah SY, Md Hashim NF, Khaza'ai H, Ahmat N, Gopalsamy B, Wibowo A (2019) Ampelopsin E reduces the invasiveness of the triple negative breast cancer cell line, MDA-MB-

231. Molecules 24:2619. https://doi.org/10.3390/molecules24142619

Vutukuri VR, Das MC, Reddy M, Prabodh S, Sunethri P (2017) Evaluation of acute oral toxicity of ethanol leaves extract of *Catharanthus roseus* in Wistar albino rats. J Clin Diag Res 11: FF01–FF04. https://doi.org/10.7860/JCDR/2017/24937.9325

Wang N, Wang Q, Tang H, Zhang F, Zheng Y, Wang S, Zhang J, Wang Z, Xie X (2017) Direct inhibition of ACTN4 by ellagic acid limits breast cancer metastasis via regulation of β-catenin stabilization in cancer stem cells. J Exp Clin Cancer Res 36:1–19. https://doi.org/10.1186/s13046-017-0635-9

Wang T, Jiang R, Bai J, Zhang K (2020) Integrative bioinformatic analyses of genome-wide association studies for understanding the genetic bases of human height. Biologia 1–8. https://doi.org/10.2478/s11756-020-00550-7

Wang X, Decker CC, Zechner L, Krstin S, Wink M (2019) In vitro wound healing of tumor cells: inhibition of cell migration by selected cytotoxic alkaloids. BMC Pharmacol Toxicol 20:4. https://doi.org/10.1186/s40360-018-0284-4

Yamaguchi H, Condeelis J (2007) Regulation of the actin cytoskeleton in cancer cell migration and invasion. Biochim Biophys Acta Mol Cell Res 1773:642–652. https://doi.org/10.1016/j.bbamcr.2006.07.001

Yao Y, Chu Y, Xu B, Hu Q, Song Q (2019) Risk factors for distant metastasis of patients with primary triple-negative breast cancer. Biosci Rep 39:BSR20190288. https://doi.org/10.1042/BSR20190288

Yin M, Ma W, An L (2017) Cortactin in cancer cell migration and invasion. Oncotarget 8:88232. https://doi.org/10.18632/oncotarget.21088

Zhou X, Seto SW, Chang D, Kiat H, Razmovski-Naumovski V, Chan K, Bensoussan A (2016) Synergistic effects of Chinese herbal medicine: a comprehensive review of methodology and current research. Front Pharmacol 7:201. https://doi.org/10.3389/fphar.2016.00201