



Original Article

Asian Pacific Journal of Tropical Biomedicine



apjtb.org

doi: 10.4103/apjtb.apjtb_623_24

Anti-metastatic potential of OR3 pigment from *Streptomyces coelicolor* JUACT03 on MDA-MB-231 breast cancer *in vitro* and *in vivo*Somasekhara Derangula¹, Varalakshmi K Nadumane²✉¹Department of Biotechnology, School of Sciences, JAIN (Deemed-to-be-University), Bangalore, Karnataka, India²Center for Research in Pure and Applied Sciences, JAIN (Deemed-to-be-University), Bangalore 560078, Karnataka, India

ABSTRACT

Objective: To investigate the mechanism of anticancer activity of a pigment OR3 from *Streptomyces coelicolor* in *in vitro* and *in vivo* metastatic breast cancer models and to characterize the pigment.

Methods: The anticancer mechanism was analyzed in MDA-MB-231 cells using MTT, lactate dehydrogenase, caspase, DNA fragmentation, clonogenic, flow cytometry, Western blot, and scratch assays. The effects of OR3 on xenograft mouse models were evaluated by tumor volume measurement, hematological analysis, and histopathological observation. The characterization of OR3 was also performed using gas chromatography-mass spectrometry and nuclear magnetic resonance spectroscopy.

Results: OR3 exhibited potent cytotoxicity against MDA-MB-231 cells, with no observed effects on HEK-293 cells. Caspase-9 activation was detected in OR3-treated MDA-MB-231 cells. Flow cytometry showed a dose-dependent induction of apoptosis and cell cycle arrest at the sub-G₁ and S phases. Furthermore, OR3 completely inhibited MDA-MB-231 cell migration and demonstrated anti-proliferative effects by downregulating the protein expression of KPNA2, XPO1, RAB5B, and p38 MAPK. In *in vivo* studies, OR3 was non-toxic to mice, inhibited tumor xenograft growth, and maintained normal hematological parameters and tissue architecture. Nuclear magnetic resonance spectroscopy demonstrated the presence of a prodigiosin-like compound, while gas chromatography-mass spectrometry analysis identified additional compounds in OR3.

Conclusions: Our findings validate OR3 pigment as a promising compound for the treatment of metastatic breast cancer, warranting further studies.

KEYWORDS: Breast cancer; MDA-MB-231; Tumor growth inhibition; Apoptosis; OR3 pigment; *Streptomyces coelicolor*; Metastasis

1. Introduction

Breast cancer accounts for 24.5% of all cancer cases globally, with its incidence in India slightly lower at 13.5%[1]. Changes in cellular and molecular mechanisms contribute to the uncontrolled division of cancer cells, while cancer metastasis poses significant challenges to eradication[2]. The management of breast cancer involves various therapeutic approaches, including chemotherapy, radiation therapy, surgical interventions, and preventive measures[3,4]. However, many current anticancer treatments indiscriminately damage both cancerous and healthy cells[5]. In this context, natural products derived from diverse sources, such as medicinal herbs

Summary

Question: Is it possible to control metastatic breast cancer growth with a natural pigment isolated from *Streptomyces coelicolor*?

Findings: In the *in vitro* and *in vivo* studies of metastatic breast cancer (MDA-MB-231) cells, a yellow pigment (OR3) from *Streptomyces coelicolor* exhibits significant anti-metastatic properties and induces apoptosis *via* the downregulation of key signaling proteins.

Meaning: The applicability of potential natural pigments needs to be explored further for metastatic breast cancer therapy.

✉To whom correspondence may be addressed. E-mail: kn.varalakshmi@jainuniversity.ac.in

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-Non Commercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

©2025 Asian Pacific Journal of Tropical Biomedicine Produced by Wolters Kluwer-Medknow.

How to cite this article: Derangula S, Nadumane VK. Anti-metastatic potential of OR3 pigment from *Streptomyces coelicolor* JUACT03 on MDA-MB-231 breast cancer *in vitro* and *in vivo*. Asian Pac J Trop Biomed 2025; 15(2): 43-52.

Article history: Received 18 October 2024; Revision 20 November 2024; Accepted 17 December 2024; Available online 17 January 2025

and microorganisms, have proven effective. Over 60% of current anticancer and antitumor drugs originate from these natural reservoirs[6,7]. Among these, actinomycete bacteria are invaluable sources of biologically active natural products, with applications as antibiotics, as well as antiparasitic, anticancer, insecticidal, antiviral, antifungal, immunosuppressant, and hypersensitivity-modulating agents[8,9]. The genus *Streptomyces*, the largest genus of the Actinobacteria, is well-known for producing nearly 70% of commercial antibiotics. In cancer treatment, *Streptomyces* have already been proven as the main resources producing anticancer drugs like doxorubicin, mitomycin C, dactinomycin and bleomycin[10]. *Streptomyces coelicolor* (*S. coelicolor*) is amongst the best studied representatives of the genus *Streptomyces*. *S. coelicolor* produces the red pigment prodigiosin with profound anticancer activities[9]. The current study aimed to explore the effect of a yellow pigment (OR3), from a new isolate of *S. coelicolor* JUA03[11] on metastatic breast cancer.

2. Materials and methods

2.1. Pigment extraction and its partial purification

An Actinomycete strain with remarkable anticancer potential was distinguished through 16S rRNA sequencing and confirmed to be *S. coelicolor*[11]. Its genetic sequence was submitted to GenBank under the accession number MH817838. The pigment OR3 from this strain was extracted using the established method and partially purified using Thin Layer Chromatography (TLC) sheets (60 F 254, Merck) as documented[12].

2.2. Cell lines

The cell lines, HEK-293 and MDA-MB-231 were obtained from the National Centre for Cell Sciences, Pune, in India. They were maintained in high glucose Dulbecco's Modified Eagle Medium (HiMedia), enriched with 10% fetal bovine serum (HiMedia), and incubated at 37 °C and 5% CO₂ in a CO₂ incubator (ThermoFisher, USA).

2.3. In vitro anticancer activity analysis

2.3.1. Cytotoxicity screening

The anticancer activity was checked using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MDA-MB-231 and HEK-293 cells were cultured in 96-well microtiter plates for 24 h and added with various concentrations of OR3 pigment (0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 µg/mL) and doxorubicin (8 and 16 µg/mL), followed by incubation for 24, 48, and 72 h. After that, the

assay was performed as per the method described earlier[13].

2.3.2. Activity of lactate dehydrogenase (LDH)

The cytotoxicity of the pigment (OR3) was assessed at its IC₅₀ concentration (8 µg/mL) by analyzing the LDH enzyme activity. The LDH assay was performed following the Cytoscan™ LDH Assay Kit (G-Biosciences; cat. # 786-210) manual instructions, as per previous methods[12].

2.3.3. Caspase-9 activity assay

The effect of OR3 pigment on caspase 9 activity was assessed using the CasPASE Apoptosis Assay Kit (from Elabscience; cat. # E-CK-A313). HEK-293 and MDA-MB-231 cells were treated with the OR3 pigment and doxorubicin (8 µg/mL) for 24 h. Afterwards, as per the instructions provided in the manual, the caspase assay was performed[12].

2.3.4. Annexin V/AbFluor 488 apoptosis detection

Apoptosis detection was performed using the Annexin V-AbFluor™ 488 Apoptosis Detection Kit (KTA0002, Abbkine, Inc.) following the manufacturer's protocol. MDA-MB-231 cells (2×10^6 cells/mL), cultured for 24 h in tissue culture flasks, were treated with OR3 (4 and 8 µg/mL) for 24 h. Afterwards, they were rinsed with ice-cold phosphate buffered saline (PBS) and stained with 5 µL of AbFluor 488 Annexin V and incubated for 15 min in the dark, followed by the addition of 2 µL of propidium iodide (PI) and 400 µL of 1× binding buffer. Samples were analyzed immediately with a Cytomics FC500 flow cytometer, using FlowJo X software, and cells were classified into live, early apoptotic, and late apoptotic/necrotic populations.

2.3.5. Cell cycle analysis

The MDA-MB-231 were cultured for 24 h. Subsequently, OR3 (8 µg/mL) was added to the cells and incubated for 24 h. Following this, the treated and untreated cells were harvested and processed by PI staining for cell cycle analysis using a flow cytometer (BD FACSVerse). The DNA content was analyzed using the software, FCS version 6.2[14].

2.4. Western blot analysis

Changes in the proteins that regulate cancer cell proliferation and invasion of MDA-MB-231 cells after treatment with OR3 were analyzed by Western blot. Cells were initially seeded at a density of 2×10^6 cells/well in T-75 roux flasks and treated with the IC₅₀ concentration (8 µg/mL) of OR3. Total protein was isolated from the cells after 24 h, using ice-cold RIPA cell lysis buffer (ElabScience), and was subjected to Western Blot studies as per the standard methodology[15]. The antibodies used included p38 MAPK polyclonal antibody (Cat. No: E-AB-32461; 1:1 000 dilution),

RAB5B polyclonal antibody (Cat. No: E-AB-40198; 1:500 dilution), XPO1 polyclonal antibody (Cat. No: E-AB-17839; 1:1 000 dilution), KPNA2 polyclonal antibody (Cat. no: E-AB-1906; 1:1 000 dilution), and β -actin polyclonal antibody [Cat. No: E-AB-40517; 1:2 000 dilution (Elabscience)]. Densitometry of the bands was carried out using the Image J software.

2.5. Clonogenic analysis

Approximately 200 MDA-MB-231 cells were seeded per well in a six-well plate. After 24 h, they were treated with OR3 (4 and 8 μ g/mL) for another 24 h, using PBS as a negative control. Afterwards, the assay was carried out as per standard methodology[15]. Clonogenicity was measured as the number of colonies formed by treated cells relative to those formed by untreated cancer cells, expressed as a percentage.

2.6. Metastasis evaluation by scratch assay

MDA-MB-231 cells (2×10^5 cells/mL) were seeded in 6-well plates. After 24 h, they were washed with PBS[16]. Using a sterile 200 μ L tip, a scratch was made in the midline of the wells and OR3 was applied at concentrations of 0.5 and 1 μ g/mL, and at 0, 24, 48, 72, and 96 h, the dimensions of the scratch and the percentage inhibition of wound closure (cell migration) were assessed using Image J software.

2.7. Characterization of OR3

2.7.1. Gas chromatography–mass spectrometry (GC–MS) analysis

The yellow pigment (OR3), partially purified by TLC, was analyzed by GC-MS at the South India Textile Research Association, Coimbatore, Tamil Nadu, in India, as per the methodology provided[11]. Further, the compound identification was carried out using the database of the National Institute of Standards and Technology.

2.7.2. NMR spectroscopy for compound characterization

OR3 was further purified by HPLC and then analyzed at the NMR spectroscopy facility of Indian Institute of Science, Bengaluru, to identify the compounds as described earlier[17]. A total of 5 mg of the HPLC-purified OR3 fraction was used for NMR spectroscopy (^1H NMR and ^{13}C NMR).

2.7.3. Test for prodigiosin

Test for prodigiosin in the TLC-purified pigment fractions, ORI (pink) and OR3 (yellow) was carried out as follows: The TLC-purified pigments were divided into three portions. One part

was acidified with 0.1 mol/L HCl (pH 2.0), the second part was alkalinized with 0.1 mol/L NaOH (pH 10.0), and the third part was neutral (pH 7.0). The color development was visually observed. These pigments were analyzed for maximum UV-VIS absorbance at pH values of 2.0, 7.0, and 10.0 between 200 and 700 nm, in a UV-visual spectrophotometer (Shimadzu, Japan) using methanol as the blank[18].

2.8. In vivo studies

2.8.1. Animals

Female Balb/c mice aged 8-12 weeks (19.4-21.5 g) were used. Mice were housed under standard laboratory conditions of 22 °C and 46%-69% relative humidity, with free access to water and standard food, and maintained on a photoperiod of 12 h day and 12 h night. Acute class method as per OECD 423 Guidelines was followed to assess the toxicity of OR3 in mice. The nude mice for the efficacy study were obtained from Charles River, USA, and maintained under standard conditions [Temperature: (22 $\pm$ 3) °C; humidity: 50%-60%] in individually ventilated cages, accommodating 5 mice in each cage. *Ad libitum* access to water and food was provided to the mice. Every experiment was performed in a BSL-2 biosafety cabinet, and all pre-sterilized chemicals and reagents were used.

2.8.2. Ethical statement

In vivo study on mice was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals. For these *in vivo* studies, ethical clearance was obtained from the Institutional Animal Ethics Committee (IAEC) committee of Skanda Life Sciences under IAEC number IAEC-SLS-2022-073.

For toxicity assessment, the mice were grouped into four, each comprising five animals. OR3 was orally administered to G2, G3, and G4 groups on Day 1 of the study at concentrations of 50, 300, and 1000 mg/kg body weight, respectively. The G1 group served as the negative control, with animals orally fed the vehicle. Individual checks were conducted for all groups daily over 14 d. The next day, all animals were humanely euthanized, and a comprehensive gross necropsy was performed. The organs were dissected and preserved in 10% neutral buffered formalin for subsequent histopathological analysis.

2.8.3. Tumor induction in mice

MDA-MB-231 cells were purchased from the ATCC facility in the USA, subcultured, and maintained in RPMI media along with 10% fetal bovine serum. Penicillin (100 IU/mL) and streptomycin (100 μ g/mL) were used to prevent contamination.

MDA-MB-231 cells (5×10^7 cells/mL) as a suspension in serum-free media along with matrigel in a 1:1 combination, was injected (0.2 mL) subcutaneously into mice above the right flank region.

Mice were observed daily for tumor growth and once the tumor size reached the desired volume of 100–150 mm³ (2 weeks), their volumes were noted down and they were randomly sorted into 4 groups, comprising of 5 mice each.

2.8.4. Experimental groups

Experimental groups include group I, the control group of tumor mice, to which normal saline was given orally; group II, the positive control group which received 5 mg/kg cisplatin; groups III and IV received oral doses of OR3 at 10 mg/kg and 30 mg/kg, respectively, for 21 d.

2.8.5. Hematological parameters

Experimental animals were sacrificed using xylazine and ketamine (4.5 and 45.0 mg/kg b.wt, respectively). About 0.6 to 0.7 mL of blood was collected and was used for complete blood count (CBC) analysis by a hematology analyzer (Erba transgesia; H360).

2.8.6. Tumor volume and percentage of tumor growth inhibition (%TGI)

The tumor volume was calculated by measuring the width and length of the tumor using a vernier caliper. The percentage tumor growth inhibition was calculated with the formula: %TGI = 1 – (MTV of treated mice/MTV of control mice) × 100, in which MTV is the mean tumor volume.

2.8.7. Histopathological study

A histopathological study was performed on tissues including the liver, lungs, spleen, heart, and kidney as well as tumor using routine methods[19]. Tissue sections (5 µm) stained with hematoxylin and eosin were observed under a binocular microscope (Labomed Model Lx300) and photographed.

2.9. Statistical analysis

The data were expressed as mean ± SE and all tests were conducted in triplicates, and their significance was analyzed statistically using Graph Pad Prism software. One-way ANOVA was performed and a *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Cytotoxic effect of OR3

In MTT assay, the viability of MDA-MB-231 cells after 24 h of treatment with 16 µg/mL of OR3 and doxorubicin was 38% and 32%, respectively, which further reduced to 31% and 21%, respectively after 72 h (Figure 1A). The IC₅₀ value of OR3 was calculated as 8 µg/mL. OR3 demonstrated potent anticancer activity against MDA-MB-231 cells in a dose- and time-dependent manner, which was comparable to doxorubicin, while showing lower cytotoxicity on HEK-293 cells (Figure 1B).

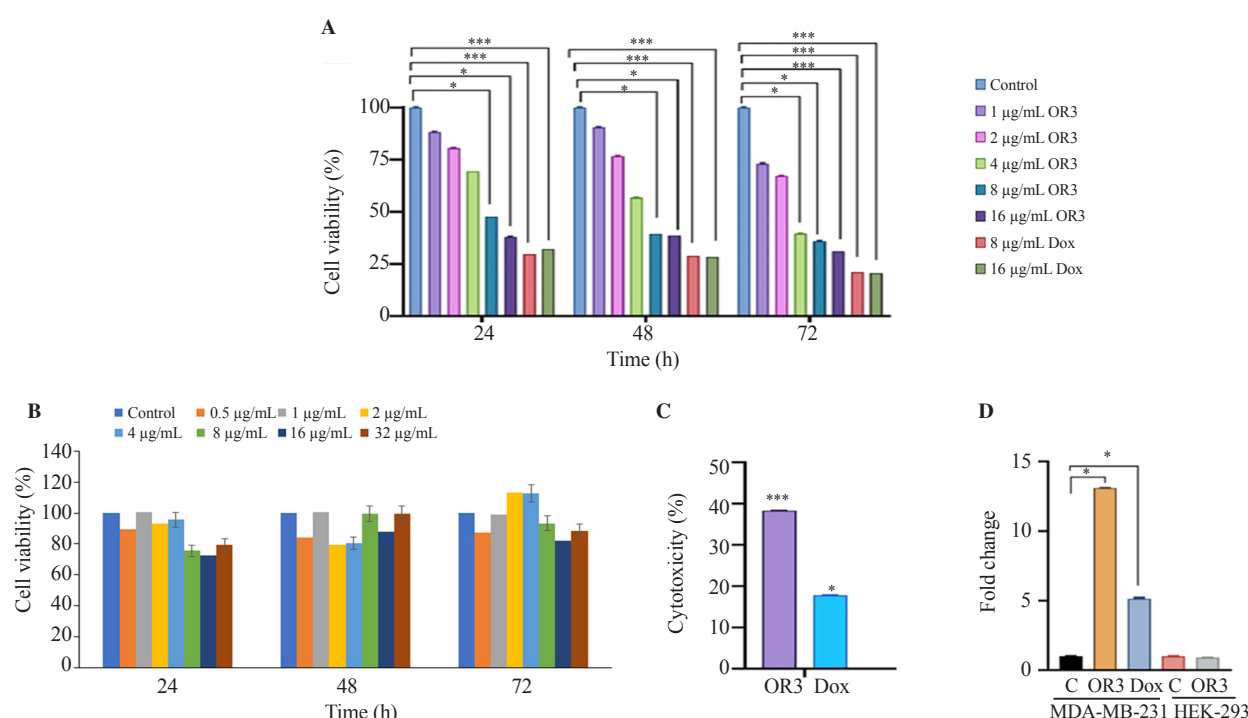


Figure 1. MTT, lactate dehydrogenase, and caspase assay results after treatment with OR3 pigment from *Streptomyces coelicolor* in MDA-MB-231 cells. The effect of OR3 pigment on (A) MDA-MB-231 cells and (B) HEK-293 cells at 24, 48, and 72 h using MTT assays. (C) Lactate dehydrogenase activity in MDA-MB-231 cells treated with OR3 pigment (8 µg/mL). (D) Caspase 9 activity. The data are expressed as mean ± SE and analyzed by one-way ANOVA analysis. **P* < 0.05 and ****P* < 0.001 compared with the control. Dox: doxorubicin; C: control.

According to LDH results, the cytotoxicity was 38% for MDA-MB-231 cells after treatment with 8 $\mu\text{g/mL}$ of OR3 (Figure 1C). In contrast, doxorubicin (8 $\mu\text{g/mL}$) showed 18% cytotoxicity to MDA-MB-231 cells. In comparison with the standard drug, OR3 exhibited greater cytotoxicity to the breast cancer cells.

3.2. Caspase 9 activities in OR3-treated cells

The caspase-9 activity in MDA-MB-231 after treatment with 8 $\mu\text{g/mL}$ of OR3 was upregulated by 12-folds. Treatment with the same concentration of doxorubicin resulted in a 5-fold upregulation of caspase 9 in MDA-MB-231 cells. In comparison to the standard drug

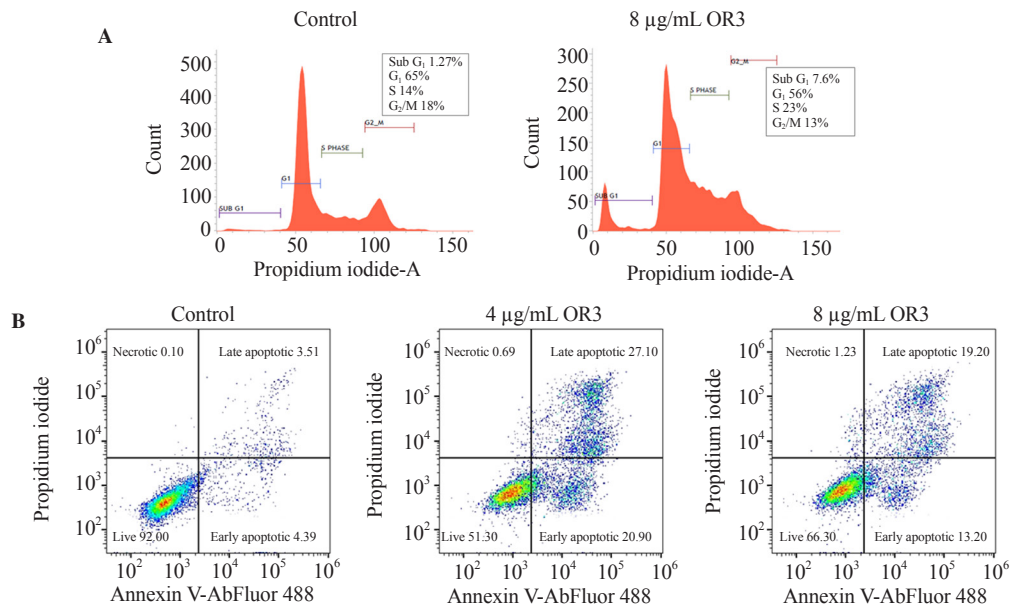


Figure 2. Effect of OR3 pigment from *Streptomyces coelicolor* on apoptosis of MDA-MB-231 cells. (A) Cell cycle analysis after OR3 treatment (8 $\mu\text{g/mL}$) in MDA-MB-231 cells. (B) Annexin V/AbFluor 488 propidium iodide apoptosis study of OR3 (4 and 8 $\mu\text{g/mL}$)-treated MDA-MB-231 cells.

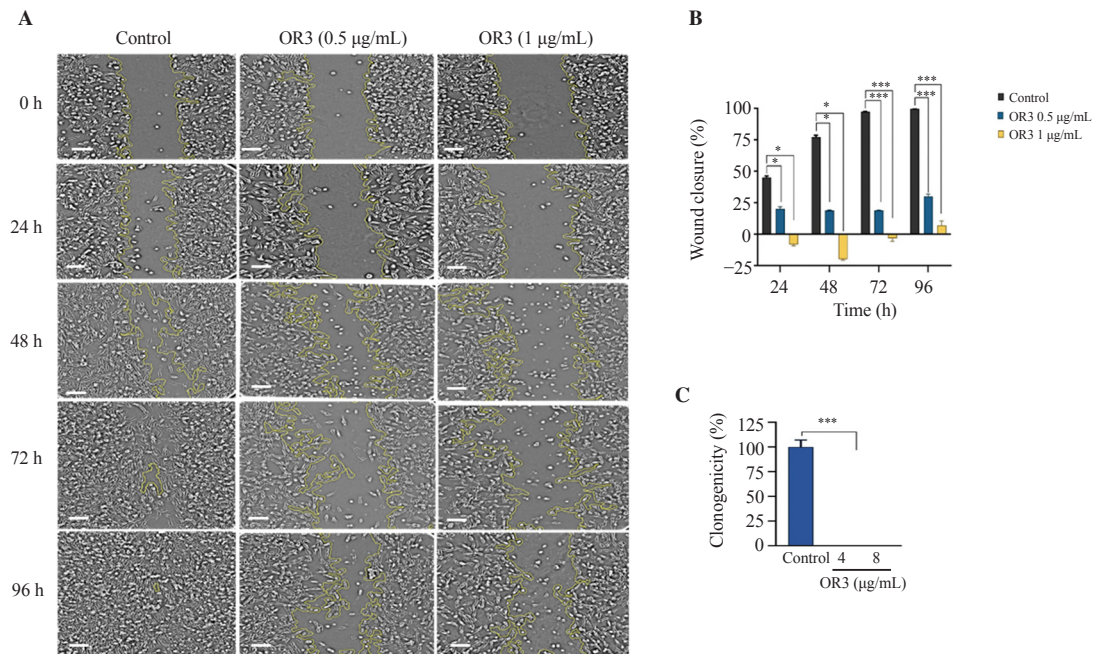


Figure 3. Effect of OR3 pigment on clonogenicity and migration of MDA-MB-231 cells by colony formation assay and scratch assay. (A) Images from the scratch assay showing inhibition of wound closure in OR3-treated MDA-MB-231 cells (magnification: $\times 40$; scale bar: 120 μm). (B) Bar diagram showing the wound closure as a relative (%) value in comparison to the control. (C) Bar diagram showing clonogenicity (%) of MDA-MB-231 cells. The data are shown as mean $\pm$ SE of triplicate experiments and analyzed by one-way ANOVA analysis. * $P < 0.05$ and *** $P < 0.001$ compared with the control group.

doxorubicin, OR3 exhibited a higher apoptosis-inducing property in the breast cancer cell line, while showing no significant effect on HEK-293 cells (Figure 1D).

3.3. OR3 induces cell cycle arrest

We observed that OR3 inhibited the proliferation of MDA-MB-231 cells by inducing sub-G₁ phase arrest, with 7.60% of cells in sub-G₁ compared to 1.27% in control cells. S phase arrest was also evident, with 23% of cells compared to 14% in control cells, typically leading to the death of cancer cells (Figure 2A).

3.4. OR3 induces breast cancer cell apoptosis

Quantification of apoptotic cells in MDA-MB-231 cells treated with OR3 was performed by annexin V-AbFluor 488/PI staining, utilizing a flow cytometer. The results are depicted in Figure 2B. Treatment with OR3 at concentrations of 4 and 8 µg/mL led to an increase in the percentage of apoptotic cells in MDA-MB-231 cells, with values of 48.0% and 32.4%, respectively, while the necrotic cells comprised 0.69% and 1.23%, respectively.

3.5. OR3 inhibits clonogenicity of MDA-MB-231 cells

At the end of 3 weeks, no colonies were observed in MDA-MB-231 cells treated with OR3, while the untreated control cells formed a large number of visible colonies. The colony inhibition rate by OR3 was 100% when compared to control cells (Figure 3C).

3.6. OR3 prevents the migration of breast cancer cells

As observed in Figure 3A & 3B, both 0.5 and 1 µg/mL of OR3 significantly inhibited the migration of MDA-MB-231 cells. The inhibition rate of OR3 ranged from 75% to 90%. The wound closure was only 18% by 0.5 µg/mL of OR3 at 72 h, while there was no wound closure by 1.0 µg/mL of OR3 at 72 h. Moreover, there was only 6% wound closure by 1.0 µg/mL of OR3 at 96 h, as opposed to the 100% closure in the control cells. The results indicate that OR3 has highly effective inhibitory effects on the metastasis of MDA-MB-231 cells.

3.7. OR3 downregulates the expressions of invasion and proliferation-related proteins in MDA-MB-231 cells

OR3 treatment notably downregulated the expression of proteins involved in the RAN pathway, such as KPNA2 and XPO1 (Figure 4). Besides, the expression of RAB5B, a protein essential for vesicle-mediated transport, was decreased by OR3 treatment. The p38 MAPK, which is majorly involved in cancer cell invasion and metastasis, was also downregulated in OR3-treated MDA-MB-231 cells.

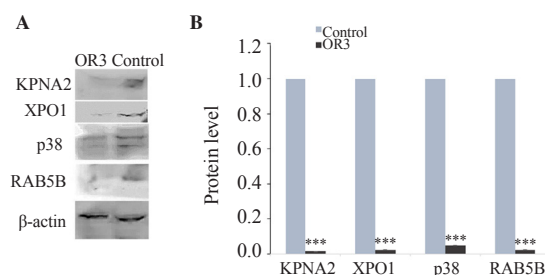


Figure 4. (A) Western blot analysis. β -actin was used as an internal control. (B) Bar diagram representing protein fold changes in Western blot. *** $P < 0.001$ compared with the control.

3.8. Characterization of OR3

3.8.1. GC-MS analysis

GC-MS analysis identified some compounds in OR3 (Figure 5). According to the chromatogram, five major peaks at different retention times (RT) were observed. The mass spectral analysis of the major peak at RT 3.16 min corresponds to the compound 2,4-difluorobenzene, peak at RT 3.51 min to butane, 1-chloro, peak at RT 10.76 min to quinoline 3 bromo, peak at RT 24.21 min to pentadecanoic acid methyl ester, as well as peaks at RT 25.21 min and RT 25.63 to hexadecanoic acid methyl ester.

3.8.2. NMR spectroscopy of purified OR3

Proton and carbon NMR analyses revealed that the major compound in the fraction is prodigiosin[17]. The ¹H NMR spectrum displayed characteristic peaks at 8.520 ppm, indicating aromatic protons, and at 4.5 ppm and 2.5 ppm, corresponding to aliphatic protons typical of methylene (CH₂) groups. Additionally, a peak at 3.351 ppm suggested the presence of functional group protons, such as methoxy (-OCH₃) or methyl (-CH₃) groups. Methyl protons were also observed at lower ppm values (0.889 ppm, 0.870 ppm, 0.850 ppm, 0.834 ppm), further supporting the identification of prodigiosin (Supplementary Figure 1).

The ¹³C NMR spectrum confirmed the presence of prodigiosin, with a peak at 72.517 ppm indicating a carbon atom in a unique chemical environment, likely associated with specific functional groups or structural motifs with electron-withdrawing or deshielding effects. Another peak at 63.050 ppm indicated a different chemical environment for another carbon atom. Peaks in the range of 40.122 ppm to 38.870 ppm suggested the presence of carbon atoms in aliphatic chains or substituents, such as methylene (CH₂) groups, with their multiplicity and intensity, providing insights into the branching or symmetry of the carbon chain. A peak at 25.565 ppm indicated the presence of a carbon atom in an aliphatic moiety, likely a methyl (-CH₃) group, with a relatively low chemical shift, indicating a shielding effect due to nearby electron-donating groups (Supplementary Figure 1).

3.8.3. Test for prodigiosin

When the OR3 (yellow) and OR1 (pink) fractions separated in TLC were checked for the presence of prodigiosin, OR1 exhibited distinct color changes at pH values of 2.0, 7.0, and 10.0, appearing pink, red, and yellow, respectively, characteristic of prodigiosin, while OR3 showed very light colorations corresponding to prodigiosin (Supplementary Figure 1). The maximum absorption spectra (λ -max) of the pink OR1 showed notable peaks: at pH 2.0, the peak is at 528 nm (characteristic of prodigiosin), while at pH 7.0 and 10.0, it shifted to 469 nm along with an additional peak at 358 nm, indicating a novel compound. The OR3 pigment consistently showed maximum absorbance at 358 nm across all pH values, suggesting the presence of a novel compound or prodigiosin analog.

3.9. In vivo studies

3.9.1. OR3 toxicity to mice

The acute toxicity study showed that there were no visible changes in the behavior of mice during the 14 days of study.

3.9.2. Anti-tumor effect of OR3

Figure 6 depicts the tumor inhibition and tumor volume after treatment for 21 d. At the end of the experiment, the tumor volume was 1592.47 mm³ in the control group, but 10 and 30 mg/kg of OR3 markedly decreased tumor volume to 704.87 mm³ and 499.01 mm³, respectively ($P < 0.05$). In addition, OR3 at 30 mg/kg showed a promising tumor inhibition potential with 69% inhibition (Figure 6B).

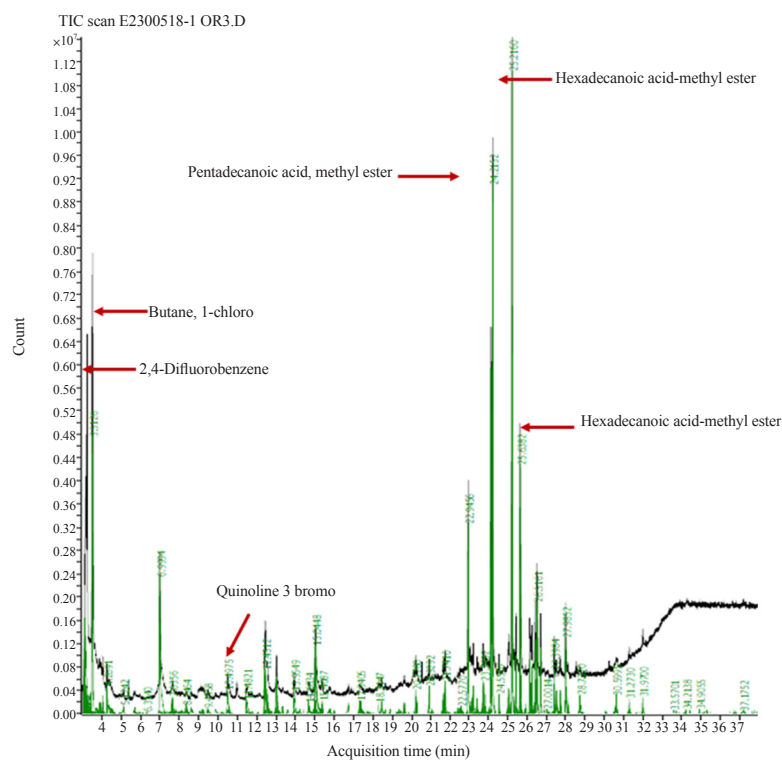


Figure 5. GC-MS chromatogram of TLC purified OR3 pigment showing five major peaks: 2,4-difluorobenzene (RT: 3.16 min), butane, 1-chloro (RT: 3.51 min), quinoline 3 bromo (RT: 10.76 min), pentadecanoic acid, methyl ester (RT: 24.21 min), and hexadecanoic acid-methyl ester (RT: 25.21 min and 25.63 min).

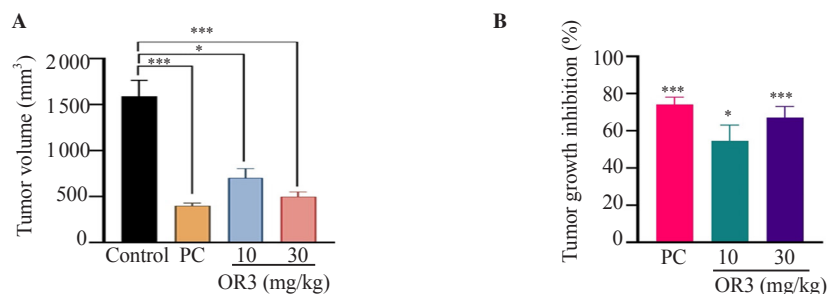


Figure 6. (A) Tumor volume (mm³) and (B) tumor growth inhibition (%) in tumor-bearing mice after OR3 treatment. The data are shown as mean $\pm$ SE, $n=3$, and analyzed by one-way ANOVA. * $P < 0.05$ and *** $P < 0.001$ compared with the control. PC: positive control (cisplatin 5 mg/kg).

3.9.3. Effect of OR3 on blood cells

Treatment of tumor-bearing mice with OR3 resulted in a significant increase in the hemoglobin level compared to the tumor control mice (Supplementary Table). The white blood cell count was found at normal levels in the OR3 treatment groups. The red blood cell count of the OR3 treated groups increased significantly compared to the control mice. All other parameters such as the platelet count, neutrophils, lymphocytes were normal in the OR3 treatment groups. These results indicate that OR3 is relatively safe for *in vivo* use.

3.9.4. Histopathological results

Histopathological evaluation of the heart, kidney, liver, spleen, lungs, and tumor tissues of mice treated with OR3 (10 and 30 mg/kg) revealed normal structures without noticeable alterations in their cellular and histological characteristics. The heart tissue sections revealed unaltered cellular structures of cardiac muscle cells and intact connective tissue architecture across all treatment groups, indicative of preserved cardiac function. The liver section of all 4 groups of tumor mice exhibited a normal structure with lobes and normal hepatocytes (Supplementary Figure 2). Kidney sections showed normal architecture with mild congestion (blue arrow) in vehicle control mice and OR3-treated mice. Moderate mononuclear cell infiltration (black arrow), congestion (blue arrow), and focal necrosis (orange arrow) were observed in a few parts of the kidney in cisplatin-treated mice. In the lungs of all 4 groups, normal bronchiole, blood vessel, alveolar duct, and alveoli were observed. Histopathology of tumor tissue section showed more densely arranged tumor cells in control mice, complete necrosis of tumor cells in cisplatin-treated mice, few tumor cells with mild necrosis of tumor in 10 mg/kg OR3 treated mice, few cells with moderate necrosis of tumor in 30 mg/kg OR3 treated mice.

4. Discussion

To address metastatic breast cancer with a potent anticancer natural compound, we assessed the antitumor potential of the pigment OR3, derived from *S. coelicolor* JUACT03, against MDA-MB-231 (metastatic breast cancer) cell line and in *in vivo* mouse models of metastatic breast cancer. Cytotoxic effects and the activation of apoptotic mechanisms are pivotal cellular events that enhance the efficacy of potential anticancer drugs, preventing cancer metastasis[20]. In this study, we employed MTT and LDH assays to analyze cytotoxicity, while caspase, cell cycle analysis, DNA fragmentation, and Annexin V/AbFluor 488 staining techniques were used to determine apoptosis.

According to the MTT results, OR3 exhibited potent cytotoxicity towards MDA-MB-231 cells. Interestingly, the IC₅₀ value of OR3 (8 µg/mL) was quite close to that of the anticancer drug doxorubicin (7.28 µg/mL) against MDA-MB-231 cells. The cytotoxic activity of OR3 aligns with reports of similar compounds from *Streptomyces* species[21]. Several antitumor compounds have been documented

from *Streptomyces* species, such as chromomycin SA from *Streptomyces* sp. KML-2, with remarkable antitumor activity on MCF-7 cells, having an IC₅₀ of 12.6 µg/mL, and streptokordin from *Streptomyces* sp. KORDI-3238 exhibited anti-tumor activity towards MDA-MB-231 cells with an IC₅₀ of 7.5 µg/mL[22,23].

OR3 caused direct cytotoxic effects on cancer cell lines, as indicated by LDH activity analysis. In OR3-treated MDA-MB-231 cells, a significant LDH release (38%) was observed compared to the standard drug doxorubicin. These results highlight that OR3 exhibits specific cytotoxicity towards cancer cells, unlike doxorubicin.

Under normal conditions, cell cycle events including G₁, S, G₂/M, and M phases are tightly regulated by several checkpoints to maintain a balance between cell division and cell death. During carcinogenesis, these checkpoints are disrupted, leading to uncontrolled cell divisions[24]. Flow cytometric analysis revealed that OR3 induced cell cycle arrest, as indicated by a higher percentage of cells in the sub-G₁ and S phases, supporting the conclusion that OR3 activates the apoptotic machinery in MDA-MB-231 cells.

Annexin-V/PI staining followed by flow cytometric analysis is a valuable method for distinguishing and quantifying apoptotic cells from necrotic cells[25]. In the present study, Annexin-V/PI staining confirmed the presence of early and late apoptotic cells resulting from OR3 treatment in MDA-MB-231 cells. At an IC₂₅ concentration, OR3 induced a higher percentage of early and late apoptotic cells, while at an IC₅₀ concentration, it led to an increased proportion of necrotic cells. These findings validate that OR3 induces cancer cell death *via* multiple mechanisms.

Caspase activation prompts apoptosis, with cytochrome c release from the mitochondria activating caspase-9, the initiator caspase. Activated caspase-9 subsequently triggers effector caspase-3, which is responsible for DNA fragmentation and morphological changes[26]. In our study, OR3 predominantly induced apoptosis *via* the mitochondrial pathway, as evidenced by a significant increase in caspase-9 activity in MDA-MB-231 cells. Importantly, OR3 did not trigger caspase-9 activation in normal HEK-293 cells, confirming the cancer-specific action of OR3.

DNA fragmentation is a hallmark of apoptosis, offering valuable insights into the mechanisms of anticancer compounds[27]. Our analysis of DNA from OR3-treated MDA-MB-231 cells revealed that OR3 induced apoptosis to a lesser extent and necrosis to a greater extent. Interestingly, doxorubicin treatment produced similar results to OR3.

We also conducted a colony formation assay with OR3-treated MDA-MB-231 cells, and the results demonstrated that OR3 completely inhibited colony formation in treated cells. These findings highlight the strong anti-proliferative effects of OR3 on metastatic breast cancer.

Cancer spreads *via* cell migration and tissue invasion, allowing tumor cells to enter the bloodstream and facilitate metastasis. Metastatic cancer is a major cause of relapse, making it particularly challenging to cure[28]. Previous investigations have reported that specific angucycline glycosides and moromycin B, isolated from

Streptomyces species, induce apoptosis and inhibit the invasive potential of metastatic breast cancer cells[29,30]. To evaluate the anti-migratory potential of OR3 on MDA-MB-231 cells, we performed a scratch assay. The results confirmed that OR3 significantly inhibits both the invasion potential and migration capacity of breast cancer cells. This was further supported by Western blot analysis, which revealed significant downregulation of key proteins involved in tumor cell proliferation, invasion, and metastasis, including p38 MAPK, KPNA2, XPO1, and RAB5B, compared to untreated cancer cells.

In addition to *in vitro* studies, *in vivo* studies in mice with tumors were carried out. Based on the results of the acute toxicity study, optimal doses of OR3 (10 and 30 mg/kg) were selected for testing against tumors in mice. Hematological studies revealed that OR3 administration improved certain hematological parameters like the hemoglobin and red blood cell counts, in tumor-bearing mice compared to the vehicle control group. Previous studies have linked cancer progression in mice to significant hematological changes, including reduced hemoglobin and red blood cell counts, as well as increased white blood cell counts. Traditional cancer chemotherapy often leads to complications such as myeloid destruction and anemia[31]. Our results indicate that OR3 treatment reversed tumor-induced hematological alterations by elevating hemoglobin levels and total red blood cell counts while maintaining total white blood cell counts. These findings suggest that OR3 does not cause the common side effects associated with chemotherapy. Moreover, OR3 demonstrated comparable efficacy to cisplatin in reducing solid tumor volume. Tumor inhibition by 30 mg/kg of OR3 reached 69%, closely matching the 74.9% inhibition observed with 5 mg/kg of cisplatin.

Chemotherapy often induces tissue damage or organ necrosis, which can be evaluated through histopathological examination of tissue sections. Histopathological assessment of the liver, spleen, lungs, kidney, and heart sections from tumor-bearing mice treated with OR3 revealed normal structures and architectures. In contrast, analysis of tumor tissues confirmed that OR3 induced cell death by inducing apoptosis and necrosis.

These findings indicate that the OR3 pigment is not only effective in tumor inhibition but also safe and non-toxic to normal, healthy tissues and organs, which highlights its potential as an anti-cancer agent, particularly for treating metastatic breast cancer.

Though our study reports significant findings, some limitations need to be mentioned. First, our investigation focused solely on a metastatic breast cancer cell line and a xenograft metastatic breast cancer mouse model; other cancer types were not explored. Secondly, the characterization and identification of the bioactive compound in the OR3 pigment were only partially accomplished. While our *in vivo* studies provide evidence for the antitumor efficacy of OR3, the *in vivo* mechanism of tumor inhibition was not evaluated. Despite these shortcomings, our study represents a significant step forward, highlighting the potential utility of this natural pigment for further research aimed at developing an anti-metastatic drug. Future

studies will address these gaps to fully characterize OR3 and explore its therapeutic potential.

In conclusion, OR3 from *S. coelicolor* demonstrated promising cytotoxic and anti-metastatic efficacy on MDA-MB-231 cells and in xenograft mouse models of metastatic breast cancer. The presence of a novel compound, similar to prodigiosin, was confirmed in OR3. These findings highlight the potential of OR3 for further characterization and drug development targeting metastatic breast cancer.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

The authors are grateful to JAIN (Deemed-to-be University), Bengaluru for providing the infrastructural facilities to carry out the research. The author, Mr. Somasekhara D would like to acknowledge the financial support by way of fellowship provided by CSIR, Govt. of India.

Funding

The authors received no extramural funding for the study.

Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

VKN and SD contributed to the conceptualization. SD designed the experiment, investigations, methodology and formal analysis. SD contributed to writing the initial draft. Both VKN and SD contributed to the final version of the manuscript, and approved it. VKN supervised the project.

References

- [1] Mehrotra R, Yadav K. Breast cancer in India: Present scenario and the challenges ahead. *World J Clin Oncol* 2022; **13**(3): 209-218.
- [2] Liang Y, Zhang D, Tse GM, Lin H, Krut FAE. Editorial: The molecular mechanisms of metastasis and therapeutic resistance in breast cancer. *Front Oncol* 2023; **13**. doi: 10.3389/fonc.2023.1194858.

- [3] Kavya D, Nadumane VK. A combination of semi-purified *L*-methioninase with tamoxifen citrate to ameliorate breast cancer in athymic nude mice. *Mol Biol Rep* 2023; **50**: 2925-2932.
- [4] Hoshyar R, Mohaghegh Z, Torabi N, Abolghasemi A. Antitumor activity of aqueous extract of *Ziziphus jujube* fruit in breast cancer: An *in vitro* and *in vivo* study. *Asian Pac J Reprod* 2015; **4**(2): 116-122.
- [5] Kroschinsky F, Stolz F, von Bonin S, Beutel G, Kochanek M, Kiehl M, et al. New drugs, new toxicities: Severe side effects of modern targeted and immunotherapy of cancer and their management. *Crit Care* 2017; **21**(1): 89.
- [6] Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 40 years. *J Nat Prod* 2020; **83**(3): 770-803.
- [7] Atanasov AG, Zotchev SB, Dirsch VM, Orhan IE, Banach M, Rollinger JM, et al. Natural products in drug discovery: Advances and opportunities. *Nat Rev Drug Discov* 2021; **20**(3): 200-216.
- [8] Tan LT, Ser HL, Yin WF, Chan KG, Lee LH, Goh BH. Investigation of antioxidative and anticancer potentials of *Streptomyces* sp. MUM256 isolated from Malaysia mangrove soil. *Front Microbiol* 2015; **6**. doi: 10.3389/fmicb.2015.01316.
- [9] Finger M, Sentek F, Hartmann L, Palacio-Barrera AM, Schlembach I, Rosenbaum MA, et al. Insights into *Streptomyces coelicolor* A3(2) growth and pigment formation with high-throughput online monitoring. *Eng Life Sci* 2022; **23**(1). doi: 10.1002/elsc.202100151.
- [10] Law JW, Law LN, Letchumanan V, Tan LT, Wong SH, Chan KG, et al. Anticancer drug discovery from microbial sources: The unique mangrove *Streptomyces*. *Molecules* 2020; **25**(22). doi: 10.3390/molecules25225365.
- [11] D S, Dammali M, Nadumane VK. Proteomic analysis of human breast cancer MCF-7 cells to identify cellular targets of the anticancer pigment OR3 from *Streptomyces coelicolor* JUA03. *Appl Biochem Biotechnol* 2023; **195**(1): 236-252.
- [12] Prashanthi K, Suryan S, Varalakshmi KN. *In vitro* anticancer property of yellow pigment from *Streptomyces griseoaurantiacus* JUA01. *Braz Arch Biol Technol* 2015; **58**(6): 869-876.
- [13] Venkatachalam P, Nadumane VK. Overexpression of p53 and Bax mediating apoptosis in cancer cell lines induced by a bioactive compound from *Bacillus endophyticus* JUPR15. *Process Biochem* 2018; **73**: 170-179.
- [14] Pozarowski P, Darzynkiewicz Z. Analysis of cell cycle by flow cytometry. In: Schönthal AH (ed.) *Checkpoint controls and cancer. Methods in molecular biology*. USA, New Jersey: Humana Press; 2004, p. 281, 301-311.
- [15] Buranrat B, Junking M. Piperine suppresses growth and migration of human breast cancer cells through attenuation of Rac1 expression. *Asian Pac J Trop Biomed* 2022; **12**(1): 39-46.
- [16] Bolla SR, Mohammed Al-Subaie A, Yousuf Al-Jindan R, Papayya Balakrishna J, Kanchi Ravi P, Veeraghavan VP, et al. *In vitro* wound healing potency of methanolic leaf extract of *Aristolochia saccata* is possibly mediated by its stimulatory effect on collagen-I expression. *Heliyon* 2019; **5**(5). doi: 10.1016/j.heliyon.2019.e01648.
- [17] Arivuselvam R, Dera AA, Parween Ali S, Alraey Y, Saif A, Hani U, et al. Isolation, identification, and antibacterial properties of prodigiosin, a bioactive product produced by a new *Serratia marcescens* JSSCPM1 strain: Exploring the biosynthetic gene clusters of *Serratia* species for biological applications. *Antibiotics* 2023; **12**(9): 1466. doi: 10.3390/antibiotics12091466.
- [18] Faraag AH, El-Batal AI, El-Hendawy HH. Characterization of prodigiosin produced by *Serratia marcescens* strain isolated from irrigation water in Egypt. *Nat Sci* 2017; **15**(5): 55-68.
- [19] Salim N, Santhiagu A, Joji K. Purification, characterization, and anticancer evaluation of l-methioninase from *Trichoderma harzianum*. *3 Biotech* 2020; **10**: 501. doi: 10.1007/s13205-020-02494-w.
- [20] Lopez J, Tait SW. Mitochondrial apoptosis: Killing cancer using the enemy within. *Br J Cancer* 2015; **112**: 957-962.
- [21] Ravikumar S, Fredimoses M, Gnanadesigan M. Anticancer property of sediment actinomycetes against MCF-7 and MDA-MB-231 cell lines. *Asian Pac J Trop Biomed* 2012; **2**(2): 92-96.
- [22] Aftab U, Zechel DL, Sajid I. Antitumor compounds from *Streptomyces* sp. KML-2, isolated from Khewra salt mines, Pakistan. *Biol Res* 2015; **48**: 58. doi: 10.1186/s40659-015-0046-3.
- [23] Jeong SY, Shin H, Kim T, Lee HS, Park SK, Kim HM. Streptokordin, a new cytotoxic compound of the methylpyridine class from a marine-derived *Streptomyces* sp. KORDI-3238. *J Antibiot* 2006; **59**: 234-240.
- [24] Hughes CC, MacMillan JB, Gaudencio SP, Jensen PR, Fenical W. The ammosamides: Structures of cell cycle modulators from a marine-derived *Streptomyces* species. *Angew Chem Int Ed Engl* 2009; **48**(4): 725-727.
- [25] Poon IK, Hulett M D, Parish CR. Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death Differ* 2010; **17**(3): 381-397.
- [26] Elmallah MI, Micheau O. Marine drugs regulating apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *Mar Drugs* 2015; **13**: 6884-6909.
- [27] Dutta S, Mitra SK, Bir A, T RP, Ghosh A. Enhancing anti-cancer activity: Green synthesis and cytotoxicity evaluation of turmeric-gold nanocapsules on A549 lung cancer cells. *Cureus* 2023; **15**(8). doi: 10.7759/cureus.43087.
- [28] Narod SA, Sopik V. Is invasion a necessary step for metastases in breast cancer? *Breast Cancer Res Treat* 2018; **169**: 9-23.
- [29] Qu XY, Ren JW, Peng AH, Lin SQ, Lu DD, Du QQ, et al. Cytotoxic, anti-migration, and anti-invasion activities on breast cancer cells of angucycline glycosides isolated from a marine-derived *Streptomyces* sp. *Mar Drugs* 2019; **17**. doi: 10.3390/md17050277.
- [30] Vicente J, Stewart AK, van Wagoner RM, Elliott E, Bourdelais AJ, Wright JL. Monacyclinones, new angucyclinone metabolites isolated from *Streptomyces* sp. M7_15 associated with the Puerto Rican sponge *Scopalina ruetzleri*. *Mar Drugs* 2015; **13**(8): 4682-4700.
- [31] Alapati V, Noolvi MN, Manjula SN, Pallavi KJ, Patel HM, Tippetwamy BS, et al. *In vivo* anti-tumour activity of novel quinazoline derivatives. *Eur Rev Med Pharmacol Sci* 2012; **16**(13): 1753-1764.

Publisher's note

The Publisher of the *Journal* remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Anti-metastatic potential of OR3 pigment from *Streptomyces coelicolor* JUACT03 on MDA-MB-231 breast cancer *in vitro* and *in vivo*

Running title : Anti-metastatic potential from *Streptomyces coelicolor*

Somasekhara Derangula¹, Varalakshmi K Nadumane^{2*}

Department(s) and institution(s)

¹Department of Biotechnology, School of Sciences, JAIN (Deemed-to-be-University), Bangalore, Karnataka, India

²Center for Research in Pure and Applied Sciences, JAIN (Deemed-to-be-University), Bangalore 560078, Karnataka, India

Corresponding Author: Varalakshmi K Nadumane

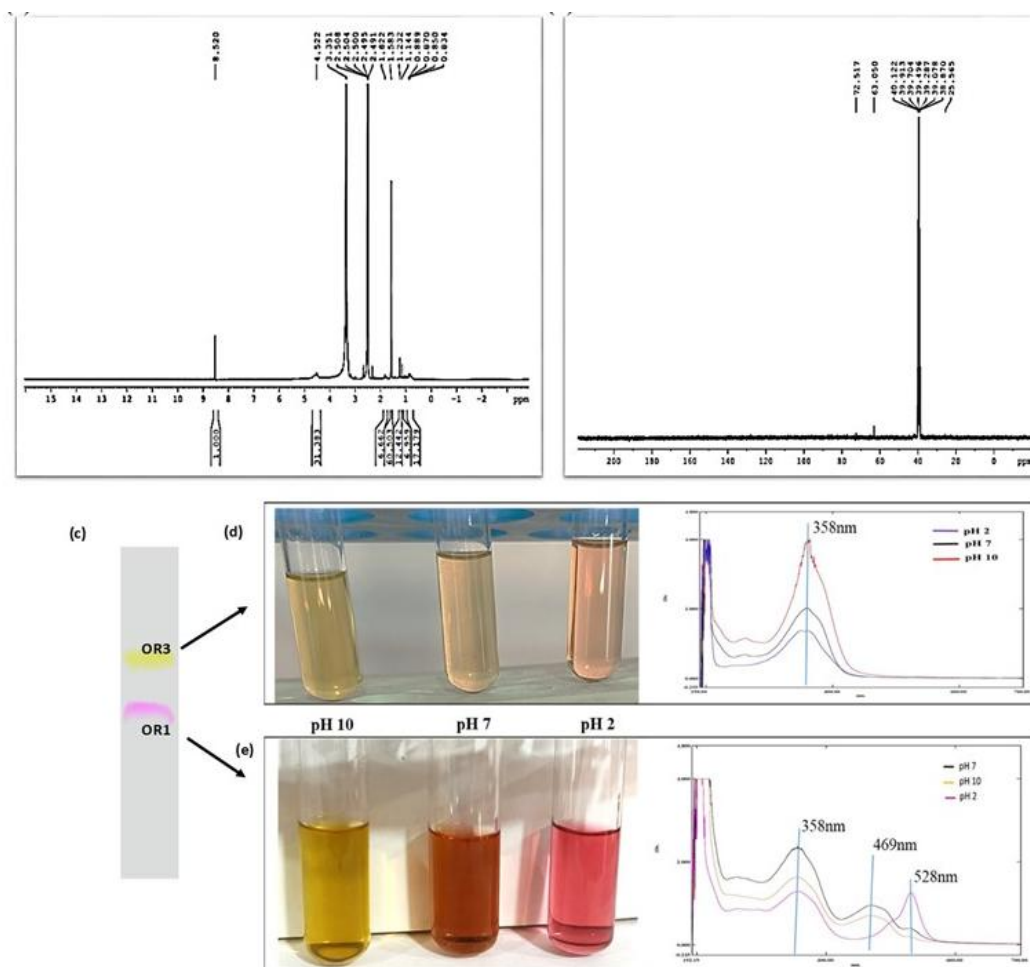
E-mail address: kn.varalakshmi@jainuniversity.ac.in

Supplementary Table. Effects of OR3 on the blood parameters of MDA-MB-231 tumor-bearing mice.

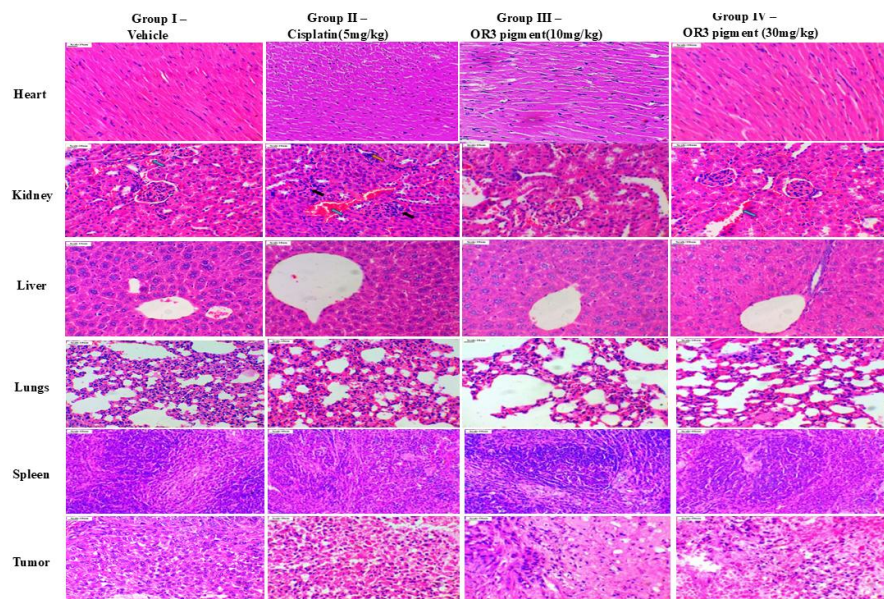
Parameters	Tumor control	Cisplatin	OR3 pigment (10 mg/kg)	OR3 pigment (30 mg/kg)
Hb (g%)	9.63 ± 0.44	9.50 ± 0.74	11.05 ± 0.5*	10.89 ± 1.17*
WBC (cells/cumm)	2 270.8 ± 97.33	1 992.47 ± 53.32*	2 183.47 ± 358.85	2 291.68 ± 93.90
Neutrophils (%)	4.51 ± 1.10	3.87 ± 2.51*	5.63 ± 1.93	4.58 ± 0.61
Lymphocytes (%)	57.31 ± 2.05	53.86 ± 3.26	56.15 ± 2.91	55.27 ± 0.67
Eosinophils (%)	1.83 ± 0.28	5.28 ± 0.84	3.52 ± 0.86	3.34 ± 0.67
Monocytes (%)	5.49 ± 0.81	7.39 ± 0.2	5.1 ± 0.53	7.22 ± 0.44
Platelet count (Lakhs/cumm)	7.58 ± 0.64	6.68 ± 1.07	6.24 ± 0.46	8.08 ± 0.44
RBC count (mill/cumm)	5.71 ± 0.36	5.61 ± 0.34	6.30 ± 0.20*	6.22 ± 0.66*
PCV (%)	27.74 ± 1.49	27.81 ± 1.92	31.86 ± 1.45	32.21 ± 3.68
MCV (fl)	35.34 ± 0.56	34.85 ± 0.45	30.8 ± 4.47	36.43 ± 0.53
MCH (pg)	11.83 ± 0.26	11.44 ± 0.34	11.79 ± 0.34	11.97 ± 0.00

MCHC (%)	23.66 ± 0.28	23.58 ± 0.35	23.94 ± 0.00	23.76 ± 0.53
-----------------	--------------	--------------	--------------	--------------

The values are given as mean ± SE (n = 5) and analyzed by one-way ANOVA analysis. *P < 0.05 compared with the control group. MCV: mean corpuscular volume, Hb: hemoglobin, MCHC: mean corpuscular hemoglobin concentration, MCH: mean corpuscular hemoglobin, PVC: packed cell volume, WBC: white blood cells, RBC: red blood cells.



Supplementary Figure 1. (A) ^1H NMR spectrum; (B) ^{13}C NMR spectrum of the HPLC purified OR3 pigment. Chemical shifts with corresponding functional groups, represented as being similar to those found in prodigiosin or its analogs in the OR3 fraction; (C) TLC fractionation of pigment extracted from *Streptomyces coelicolor*; (D) Different coloration of the OR3 pigment at pH 2, pH 7, and pH 10, along with the maximum absorption spectra (λ_{max}) of OR3 pigment at these pH levels. (E) Different coloration of the OR1 pigment at pH 2, pH 7, and pH 10, along with the maximum absorption spectra of OR1 pigment at these pH levels.



Supplementary Figure 2. Photomicrographs of the different tissue sections stained with hematoxylin and eosin in mice (magnification: $\times 40$; scale bar: $18\ \mu\text{m}$). Heart, kidney, liver, lungs, spleen, and tumor, belonging to I) group 1-vehicle control II) group 2-cisplatin(5mg/kg) III) group 3-OR3 fraction(10mg/kg) and IV) group 4-OR3 fraction (30mg/kg) treated. In the histopathology of the kidney, moderate mononuclear cell infiltration (black arrow), congestion (blue arrow) and focal necrosis (orange arrow) are found in few parts in group 2, while very less congestion (blue arrow) is observed in group 1 and 4. In the histopathology of tumor, more densely arranged tumor cells in group 1, complete necrosis of tumor cells in group 2, few tumor cells with mild necrosis of tumor in group 3, and few cells with moderate necrosis of tumor in group 4 are observed (scale bar: $18\ \mu\text{m}$).