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Structure of prodigiosin from *Serratia marcescens* NJZT-1 and its cytotoxicity on TSC2-null cells

Yinjuan ZHAO[1#], Qi CHENG[2#], Zizhu SHEN[1], Ben FAN[1], Yan XU[1], Yuting CAO[1], Fanxi PENG[1], Jingkai ZHAO[1], Bin XUE[2,3]*

Abstract

Prodigiosin, a secondary metabolite extracted from *Serratia marcescens* (*S. marcescens*), could induce apoptosis in various cancer cells, with however low toxicity on normal cells. The red pigment was extracted from a strain *S. marcescens* NJZT-1 isolated from soil, which had antibacterial activity. Spectral analyses (LC-ESI-MS, UV-VIS spectrophotometry, infrared spectra and HPLC) and TLC indicated the presence of prodigiosin in the extracellular bacterial culture extracts. The red pigment effectively killed the TSC2-null cells, whose mutation resulted in the progressive and systemic disease LAM. Our findings provide interesting evidence and important basis for the development of new therapeutic compounds with high potential effects against TSC2-null cells.

Keywords: natural products; prodigiosin; *Serratia marcescens* (*S. marcescens*) NJZT-1; anticancer activity; LAMTSC2-null cells.

Practical Application: *Serratia marcescens* natural products might be applied as a potential anticancer drug for the treatment of LAM patients in clinic.

1 Introduction

Prodigiosin is a secondary metabolite alkaloid with unique tripyrrole chemical structure, which is a red pigment isolated from *Serratia*, *Pseudomonas* and *Streptomyces* (Casullo de Araújo et al., 2010; Sam & Ghoreishi, 2018; Younis et al., 2016). Prodigiosin has a molecular formula of $C_{20}H_{25}N_3O$ and a molecular weight of 323.44 Da (Hu et al., 2016), with three rings, forming a pyrrolylpyrromethane skeleton with a C-4 methoxy group. Moreover, prodigiosin is moderately soluble in alcohol and ether, and soluble in chloroform, methanol, acetonitrile and DMSO (Hu et al., 2016; Kimata et al., 2017). It has been shown that prodigiosin has a broad range of biological activities, including the antibacterial, antifungal, and antimalarial effects (Hage-Hülsmann et al., 2018; Harris et al., 2004; Lazović et al., 2017; You et al., 2016), as well as the antibiotic, immunosuppressant (Pandey et al., 2007; Yu et al., 2015), antidiabetic, and anti-cancer activities (Genes et al., 2011; Harris et al., 2004; You et al., 2016). Moreover, prodigiosin has also been used as potential dye for food, textile, cosmetics, pharmaceutics and polyolefins (Danevčič et al., 2016; Davient et al., 2018; Dong et al., 2014; Genes et al., 2011; Goncharova et al., 2012).

Nowadays, it is popular to extract the biological products from the plants and microorganism, which have been used in the protection of plants or human beings, due to the natural friendliness (Dai et al., 2017; Dong et al., 2014; Zhao et al., 2017). Prodigiosin is a critical bioactive compound with common applications in the food, pharmaceutical, cosmetic and textile industries. New strains are still being isolated from the surroundings, because different natural products extracted from different strains might have novel bioactivities (Auparakkitanon, 2014; Darshan & Manonmani, 2016; Dhar Purkayastha et al., 2018; Sturz et al., 1997; Suryawanshi et al., 2015).

S. marcescens is a rod-shaped, Gram negative bacterium belonging to the *Enterobacteriaceae* family, which is characterized by the ability of producing the red pigment prodigiosin (Ramaprasad et al., 2015). The chromogenic species are usually isolated from the water, soil, plants and/or insects within the environment. Moreover, *S. marcescens* is a facultative microorganism, and therefore the pigment could be produced under both aerobic and anaerobic conditions (Zabot et al., 2017). Although the role of prodigiosin has not yet been defined in the physiological and biological processes of the strains, it indeed could exert the antifungal, antibacterial and antiprotozoal activities, which might have potential to be used in clinic (Yip et al., 2019). Moreover, both prodigiosin and its synthetic derivatives have potent and specific immunosuppressive activities, with new targets clearly distinct from the other drugs (Woodhams et al., 2018). Furthermore, prodigiosin is effective in inhibiting the tumor cells and inducing the cellular apoptosis, involving a broad range of cancer cell lines (Su et al., 2016; You et al., 2016; Yu et al., 2015). Prodigiosin can inhibit the Wnt/β-catenin signaling pathway and exert the anticancer activity in the breast cancer cell (Wang et al., 2016) and reactivate the p53 family-dependent transcriptional activity in the p53-deficient human colon cancer cells (Prabhu et al., 2016).

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[1]*Collaborative Innovation Center of Sustainable Forestry in Southern China, Institute of Forest Protection, College of Forestry, Nanjing Forestry University, Nanjing, Jiangsu, China*
[2]*Core Laboratory, Sir Run Run Hospital, Nanjing Medical University, Nanjing, Jiangsu, China*
[3]*State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, Jiangsu, China*
[#]*These authors contributed equally to this study.*
*Corresponding author: xuebin@njmu.edu.cn

Lymph angioleio myomatosis (LAM) is a rare, progressive and systemic disease that would typically result in the cystic lung destruction, almost exclusively affecting the females, which would be often misdiagnosed as asthma. LAM occurs either in the tuberous sclerosis complex (TSC-LAM) disease, or in the sporadic form (sporadic LAM). In both these two forms, genetic evidence indicates that LAM would be caused by the inactivation or *loss-of-function* mutations in the TSC1 or TSC2 genes (Liu et al., 2019; Valianou et al., 2019). More and more attention has been paid to finding the resolution and treatment drugs for the disease. Otherwise, the inhibition or apoptosis in the TSC2-null cancer cells triggered by prodigiosin, or the action mechanism underlying the apoptosis triggering activities of the bacterial prodigiosin in human LAM, have not been reported.

In this study, a special *Serratia* strain was established. The natural product of this strain was subjected to the identification and characterization. Moreover, apoptosis of the TSC2-null cells was also detected to seek for a potential anticancer drug to LAM.

2 Materials and methods

2.1 Microorganism and culture conditions

A pigmented strain of *S. marcescens* NJZT-1 was isolated from the soil sample from the yard of the Nanjing forestry university, which had the ability to inhibit bacteria. The strain was maintained and cultured on the nutrient agar medium. To obtain an appropriate amount of bacterial biomass and pigment, the strain was transferred into a 1000-mL Erlenmeyer flask, containing 300 mL Luria Bertani (LB) broth medium, which was incubated at 28 °C, pH 7.2 for 72 h at 200 rpm. The produced pigment was extracted from the biomass containing *S. marcescens*, by the centrifugation and methanol digestion. The plates were incubated at 28 °C for 72 h until the appearance of red pigmentation.

2.2 Pigment extraction, purification, and identification

Pigmented cultures were scraped from the surface of the Petri dishes. The biomass was extracted and collected from the pigmented cultures by the centrifugation at 9,000 ×g for 10 min. Pigment was extracted with the acidified methanol. The crude extract was evaporated by heating in water bath at 60 °C, and the dried pigment was re-dissolved in methanol.

The extracted red pigment was purified by the thin layer chromatography (TLC) in the GF250 silica gel plates. The solvent system used was dichloromethane: Ethyl acetate (1:5). Separated spot of pigment was recovered by eluting from the silica gel with methanol, which was dried by evaporation. The pigment concentration was determined as gram/% on the dry weight (w/v). The dried product was further dissolved in DMSO, and subjected to the UV-visible spectrophotometry in the range of 840-190 nm (Shimadzu UV 1800). Then, the pigment was characterized using the Fourier transform infrared (FTIR) spectrophotometer (BRUKER TENSOR 37, Germany). After drying, the pigment was mixed with the KBr powder and compressed into the pellet for the FTIR spectroscopy with the frequency range of 4,000-400 cm^{-1}.

The characterization of the purified product was performed with the LC-MS system (Waters Quattro Premier Micromass). The prodigiosin dissolved in methanol was injected into the LC-MS, and MS was performed using the positive ion electrospray ionization, with the following settings: capillary voltage, 3.4 V; cone voltage, 30 V; and source temperature, 100 °C.

2.3 Cell lines and cell culture

The TSC2-null cancer cells were kindly donated by Dr. John Blenis (Weill Cornell Medical College, New York, NY, USA). The cells were cultured in the Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), containing 10% fetal bovine serum (FBS; Biological Industries, Bat Haemek, Israel), supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), in a 37 °C, 5%CO_2 incubator.

2.4 Cell counting kit 8 (CCK8) assay

Cell proliferation and cytotoxicity was measured with the CCK8 assay. Cells in the logarithmic growth phase were collected and inoculated onto the 96-well plate, at the density of 1×10^3/well, which were cultured in a 37 °C, 5%CO_2 incubator. After 24 h, 100 μL complete medium containing prodigiosin (at different dosages) was added into each well to incubate the cells for 48 h and 72 h, respectively. Then, totally 10 μL CCK-8 solution was added into each well, followed by incubation in dark at 37 °C for 2 h. The optical density at 450 nm was read with a microplate reader (Bio-Rad Laboratories).

2.5 Flow cytometry

Cellular apoptosis and necrosis were detected by flow cytometry with the apoptosis kit (Harlingen; BD Biosciences, Franklin Lakes, NJ, USA). After the treatment of prodigiosin at different dosages for 48 h, the cell culture supernatant was discarded, and the cells were centrifuged at 2000 rpm for 5 min. After washing and centrifugation, the cells were re-suspended in 500 μL 1× binding buffer. Then the cells suspension was treated with 5 μL Annexin V and propidium iodide (PI) in turn, followed by incubation in dark at room temperature for 15 min. The fluorescence was then determined using a FACSCalibur flow cytometer, and the percentage of apoptotic cells were counted and calculated.

3 Results

3.1 Strain isolation and product extraction, charaterization and identification

The *S. marcescens* NJZT-1 strain was isolated, with the temperature-stability according to the previous study, and the red pigment could be synthesized at 37 °C. Moreover, the strain could inhibit bacteria, including the *fusarium* and *bacillus cereus mycoides*. Our results showed that the colony of *S. marcescens* NJZT-1 was different with other *S. marcescens* strains, due to the red exudate around the colony (Figure 1A). It has been known that most metabolites of the *S. marcescens*

are fat-soluble and insoluble in water, including prodigiosin (Hu et al., 2016; You et al., 2016). Our results showed that the red exudate surrounding the colony in the tablet was soluble in water, which might be prodigiosin. This strain might produce and synthesize the red pigment prodigiosin.

The cell mass was cultured in the Luria Bertani (LB) broth medium and collected through centrifugation. The obtained crude prodigiosin extract was first purified by the thin layer chromatography (TLC). The TLC was performed for the isolated pigment on standard silica gel Gf254 plates, with the dichloromethane:ethyl acetate (1:5) as the solvent. Our results showed that the Rf value of the sample and standard was 0.6 (Figure 2). Moreover, the pigment from the TLC had a strong absorption band with the absorption maximum at 535 nm (Figure 2).

In addition, the FTIR was also performed for the product characterization. As shown in Figure 3, our results showed that the red pigment from *Serratia* had strong and broad absorptions at 3,325 cm^{-1} (N-H stretch), 2,941 cm^{-1} (C-H and C=O stretches). A previous study has shown that prodigiosin exhibits similar absorptions in CHCl3 at 1,660 cm^{-1} and 1,602 cm^{-1} (Lazović et al., 2017). Moreover, the N-H group and phenyl rings were evident at the fingerprint region, which was characterized by the weak intensity at 1 cm^{-1} (aromatic C=0). Furthermore, the absorptions at 1,022 cm^{-1} and 1,114 cm^{-1} showed the C-N bend (amines) and C-O (carboxylic) stretch, while the 846 cm^{-1} and 737 cm^{-1} indicated the C-H phenyl ring bend, and the weak absorption at 1,461 cm^{-1} indicated the bending of C-H.

The red pigment extracted from strain NJZT-1 were next determined by the high performance liquid chromatography

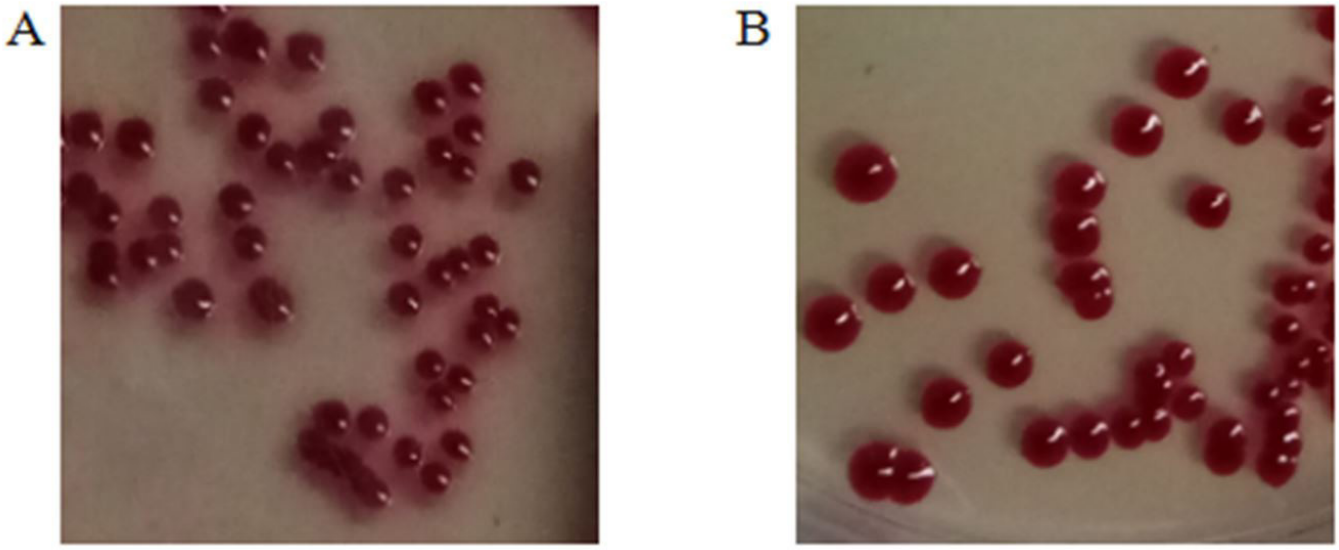

Figure 1. *Serratia marcescens* (*S. marcescens*) growing on the LB tablet (A: *S. marcescens* NJZT-1in our study B: *S. marcescens* in common).

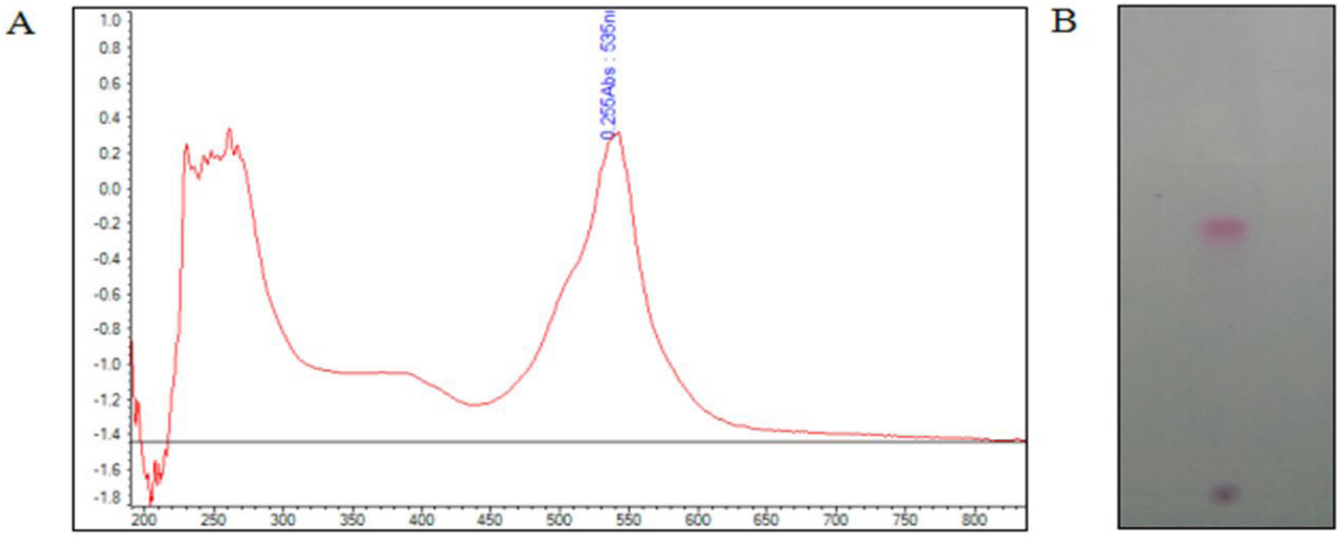

Figure 2. Characterization of the red pigment extract from *S.marcescens* NJZT-1. (A) Ultraviolet absorption spectrum graph; (B) The thin cell chromatography (B).

coupled with mass spectrometry (HPLC-MS). Our results from the HPLC analysis showed that, the major product was eluted at 14.323 min (Figure 4A), similar to the pervious investigation of prodigiosin (Li et al., 2016). Moreover, as shown in Figure 4B, there was a molecular ion peak M + H of prodigiosin at m/z 324, which corresponded to the molecular weight of prodigiosin ($C_{20}H_{25}N_3O$, 323 kD).

3.2 Anticancer effects of the extracted prodigiosin

Our precious work has shown the antimicrobial activities of the red pigment extracted from the *S. marcescens* NJZT-1. Moreover, prodigiosin has been well accepted for its anticancer property and apoptosis-triggering effects of malignant cancer cells. Herein, the anticancer effects of the extracted prodigiosin were investigated with the TSC2-null cells (a typical *in vitro* model for LAM). As shown in Figure 5, our results showed that the extracted prodigiosin effectively inhibited the growth of the TSC2-null cells, in a dose- and time-dependent manner.

To detect whether the extracted prodigiosin affected the proliferation of TSC2-null cells, the CCK8 assay was performed. Our results showed that the prodigiosin significantly inhibited the vitality and proliferation of TSC2-null cells. Moreover, there

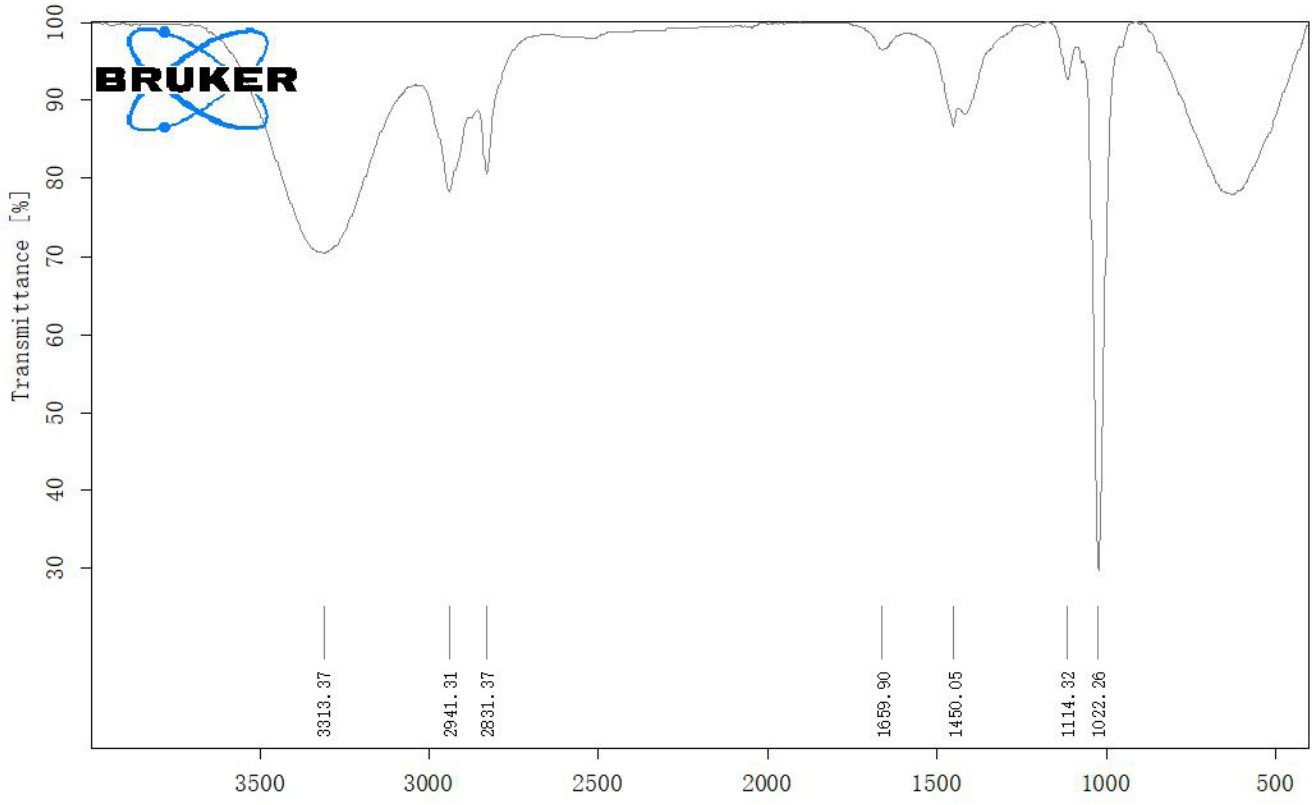

Figure 3. Infrared spectrum for the red pigment.

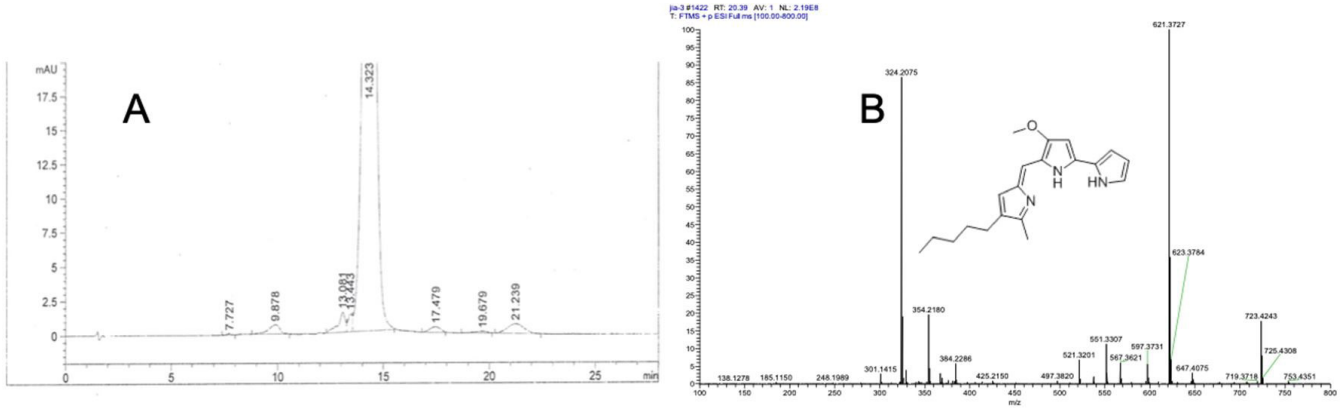

Figure 4. Characterization of the red pigment. (A) HPLC; (B) Mass spectrum.

was positive correlation between the inhibiting rate and the concentration, except for P1/8 (Figure 6) .

3.3 Apoptosis-triggering effects of the extracted prodigiosin

To further explore how the prodigiosin killed the cells effectively, the TSC2-null cells treated with various concentration of the extracted prodigiosin were subjected to the apoptosis detection with flow cytometry. As shown in Figure 7, our results showed that, the prodigiosin significantly triggered the apoptotic processes of the TSC2-null cells, in a dose-dependent manner. These results suggest that the bacterial prodigiosin could induce apoptosis in the cancer cells, even necrosis.

4 Discussion

The red pigment was isolated from the *S.marcescens* NJZT-1 strain, which was isolated from soil, with the temperature-stability. *S. marcescens* is the main strain producing prodigiosin, as a secondary metabolite and natural product. The temperature is the key factor for the prodigiosin production. It has been shown that, *S.marcescens* can produce this kind of pigment at

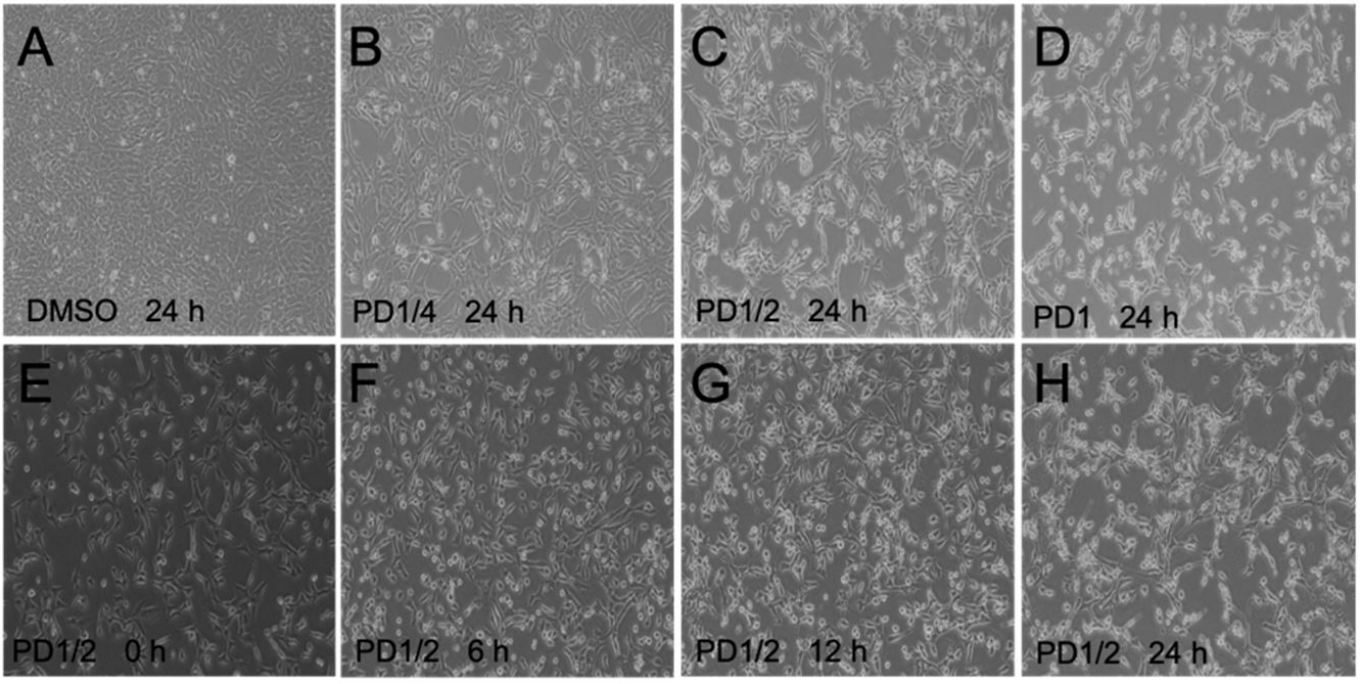

Figure 5. Prodigiosin killed TSC2-null (Tuberous sclerosis complex) cells in a dose-dependent and time-dependent manner. (A) The TSC2-null cells kept in DMSO (Dimethyl sulfoxide) for 24 h were used as control; (B-D) Prodigiosin was applied to treat the TSC2-null cells, at indicated concentrations, for 24 h; (E-H) The cells treated with 1/2 prodigiosin, for different durations, were also compared. TSC (Tuberous sclerosis complex), DMSO (Dimethyl sulfoxide).

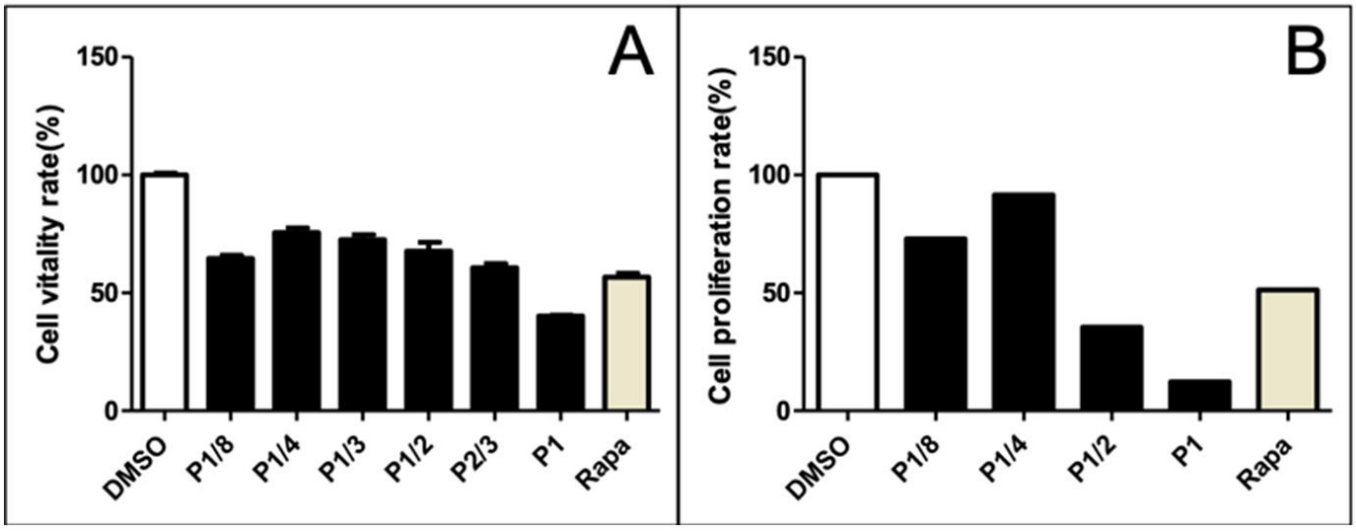

Figure 6. Prodigiosin inhibited the vitality (A) and proliferation (B) of TSC2-null cells. DMSO (Dimethyl sulfoxide).

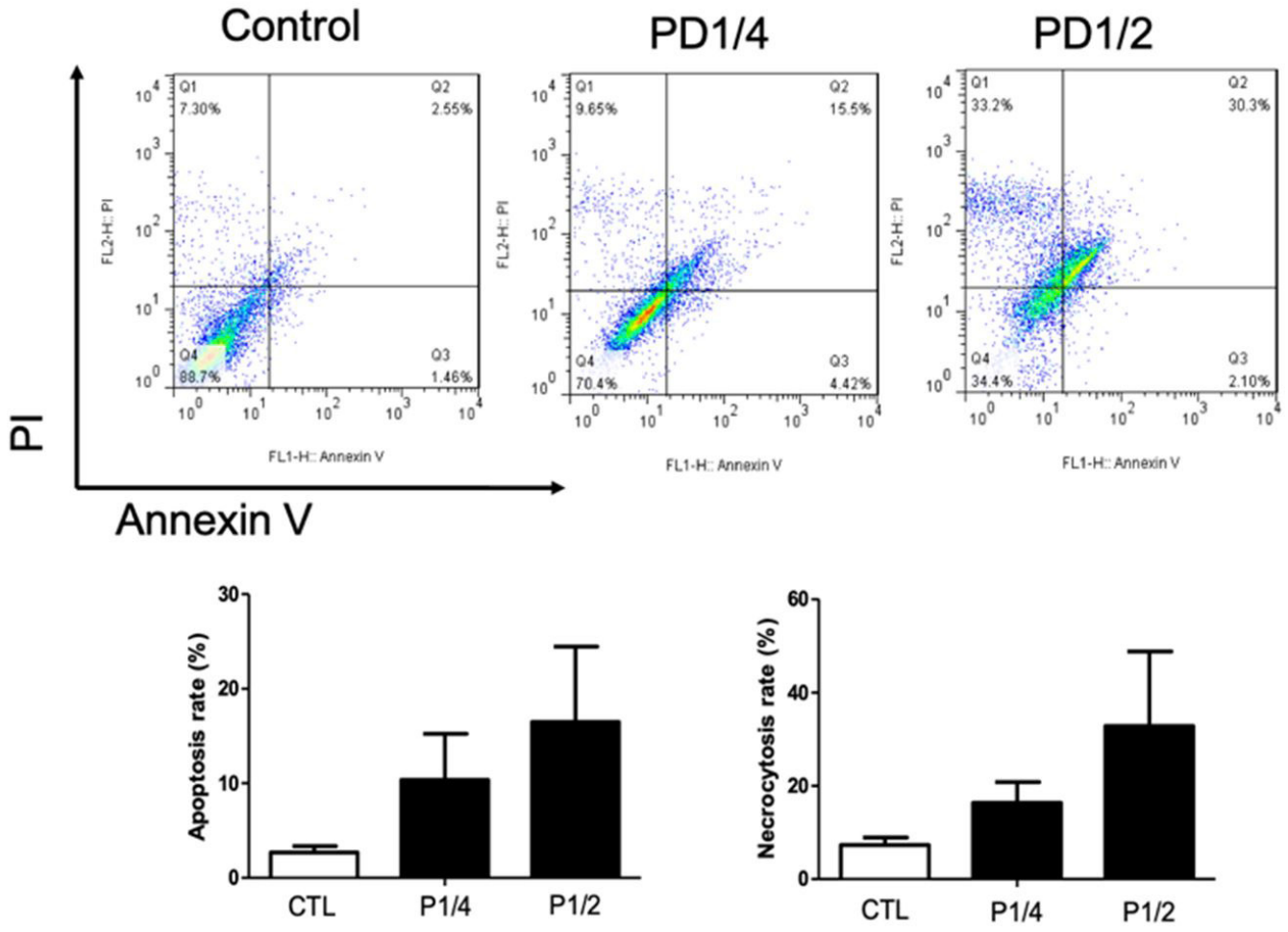

Figure 7. Apoptosis triggering activities of prodigiosin from *S. marcescens* NJZT-1 *in vitro*. The TSC2-null cells were subjected to the dual staining of Annex V and PI, and the fluorescence was detected with the flow cytometry. The Annex V+/PI-cells indicated the cells undergoing early apotosis. CTL: control.

about 25 °C, which however could not produce the pigments at elevated temperatures, especially till 37 °C (Tanikawa et al., 2006). The NJZT-1 strain in this study was not so sensitive to the temperature. It has a good ability to produce pigments in a wide ranged of temperature, i.e., from 20 °C to 35 °C, even at 37 °C.

Our results showed that, a red halo appeared on the plate, which did not exist in other *S. marcescens* strains. As is known to all, the red pigment produced by *S. marcescens* is the prodigiosin, which is considered as a fat-soluble substance (Williamson et al., 2006). But the pigment produced by the *S. marcescens* NJZT-1 could penetrate into the plate, indicating that it had good water solubility. Therefore, it is likely that there are some structural differences. However, the structure of this red pigment was not significantly different from the prodigiosin according to the spectral analyses (LC-ESI-MS, UV-VIS spectrophotometry, infrared spectra and HPLC). It would be possible that the red pigment produced by the bacterium would contain prodigiosin and/or its analogues (the soluble analogues might account for a small proportion), resulting the final product that was identified as prodigiosin.

The effects of the extracted product on the vitality and proliferation of the TSC2-null cells were also investigated. In these cells, the gene mutations result in the progressive and systemic disease of LAM. LAM is a rarely seen progressive mesenchymal neoplasm mainly affecting childbearing-age women (Baldi et al., 2014; McCormack, 2008; Kelly & Moss, 2001; Peron & Northrup, 2018). The LAM cases are sometime difficult to diagnose because the disease symptoms are similar to other lung diseases (such as asthma, emphysema, and bronchitis). Up to now, there is still no effective therapies that could cure LAM, while only the lung transplantation can save the life of LAM patients. During 2000-2006, some studies have found that the somatic mutation of TSC2 gene in the LAM patients would cause abnormal proliferation of LAM cells (Carsillo et al., 2000; Muzykewicz et al., 2009). In this study, our results showed that the extracted prodigiosin effectively killed the TSC2-null cells by inducing the cellular apoptosis and necrosis. These findings might provide an important and interesting basis for the development of the new therapeutic compound with high potential against the TSC2-null cells.

In this study, our results showed that the prodigiosin was a potential medical compound for inhibiting the growth of TSC2-null cells. However, further in-depth studies are still need to address the specific anticancer mechanism of *S. marcescens*

NJZT-1 prodigiosin. Because the mTOR pathway has been shown to be abnormally activated in the TSC2-null cells, the p-S6K and p-S6 could be chosen as the targets to investigate whether the prodigiosin inhibits the proliferation of TSC2-null cells through the mTOR pathway (Li et al., 2016). Besides, the elevated expression of cleaved-PARP1 could be induced by prodigiosin, indicating that prodigiosin can induce the TSC2-null cell apoptosis, which also deserves further analysis in the future.

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