

Screening of Myxobacteria Strains Producing Bioactive Substances against Breast Cancer

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This research was financially supported by Hebei Province Basic Research Project titled "Extraction, purification and structure analysis of antitumor bioactive substances from myxobacteria" (Grant No. 09966430D).

Abstract

[Objective] To screen myxobacteria strains producing secondary metabolites with anti-cancer activity. [Method] Fermentation culture the stains of myxobacteria, extract secondary metabolites with methanol, and detect the antitumor activity of secondary metabolites from myxobacteria and its toxicity to normal cells with the modified MTT (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide) method. [Results] Inhibition rate of myxobacteria strain 93431 fermentation original solutions, 2 fold dilution and 10 fold dilution on MCF-7 cells was 53.32%, 49.35%, and 46.50%, respectively, and their inhibition rate on MRC cells was 2.95%, 5.90%, and 1.97%, respectively. [Conclusion] Secondary metabolites produced from strains 93431 could inhibit MCF-7 cells specifically while had little effects on MRC cells and the bioactive substances from its secondary metabolites could be natural target anti-cancer agents.

Keywords: Myxobacteria, Breast cancer, MTT method, Secondary metabolites

Myxobacteria were a kind of complicated gram-negative bacterium and could perform cooperative feeding, movement and development into fruit bodies. It has been more than 200 years of investigation ever since myxobacteria were discovered. It could result in rich secondary metabolites, and was one of the important sources to develop antimicrobial agents (Reichenbach, 2001, PP. 149-156). In the secondary metabolites of myxobacteria, most of them were compounds with novel structure, and possessed high pharmacological and biological activity (Gurmeet, 2006, PP. 235-242).

Cancer is one of the major diseases that seriously damaged to human health, and the approach to heal cancer has been the research attention all over the world. Currently, clinical chemotherapeutic drugs have huge toxicity, and could bring poor life quality to patients, such as nausea, vomiting; myelosuppression, serious impairment of cardinal physiological organs, such as heart, liver, kidney, nervous system and so on. Breast cancer is one of common malignant tumors in women. According to statistics, the global number of new diagnosis of breast cancer was more than 12 million cases with 5 million cases of mortality (Curtis, 1999, PP. 138-144). In recent years, incidence of breast cancer showed an increasing trend in China, and the age of onset tended to be younger and younger.

Due to the implementation of surgery, chemotherapy, radiotherapy and other treatments, the mortality of breast cancer began to decrease. However, a considerable proportion of patients would recur and transfer finally, and the patients had to need a permanent medication. Consequently, drugs against breast cancer with high efficacy

and low toxicity tended to be particularly critical. Myxobacteria were rich in metabolic pathways, could generate a large number of metabolites with novel structure and unique function, and thus could be the important source of anticancer bioactive substances (Gerth, 2003, PP. 233-253).

1. Materials and methods

1.1 Materials

1.1.1 Strains

20 strains used in the present study were isolated and preserved in our laboratory.

1.1.2 Cell lines

MCF-7 (human breast cancer) cell lines and MRC (human embryonic lung fibroblast) cell lines were offered as a gift from Collection Center of China Type Culture, Wuhan University.

1.1.3 Reagents and apparatus

CAS medium: 1% enzymatic casein, 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.2

VY/2 medium: 1% yeast extract, 0.1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5mg/L VB12, pH 7.2

D312 macroporous resin: Shandong Lukang Pharmaceutical Group Co., Ltd.

RPMI-1640 culture medium: Gibco, USA

FBS: Hangzhou Evergreen Biological Engineering Materials Co., Ltd.

CO2 incubator: Thermo HERA cell 150, Germany

ELISA analyzer: BMG FLUOstar OPTIMA, Germany

Automatic Centrifugal Concentrator: miVac QUC-23050-100, UK

Membrane rotary evaporator: Heidolph, LABOROTA 4000, German

Analytical HPLC: Hitachi L-2000, DAD detector, Japan

Column: Thermo ODS-2, 5 μm , 250mm $\times$ 4.6mm

1.2 Methods

1.2.1 Fermentation culture

Strains preserved in glycerol were inoculated into the slope of VY/2, and cultured in biochemical incubator at 28°C till fruit bodies. The resultant fruit bodies were selected to inoculate into sterile CAS medium, and then shaking cultured at 28°C at 200r/min. After seed fluid was completed and microscopic examination, 10% seed fluid was inoculated into VY/2 fermentation medium containing D132 macroporous resin by pipette, and shaking cultured for 7 days at 28°C at 200r/min.

1.2.2 Extraction of fermentation products

After centrifuging fermentation products and discarding fermentation solutions, the strains and resin were collected, added 10 times methanol of their volume and placed in shaking table. After overnight extraction and centrifugation, strains and resin were removed. Methanol extracts of fermentation products were gathered, and concentrated by automatic centrifugal concentrator. Therefore, crude extracts of fermentation products were obtained.

1.2.3 Fermentation activity and toxicity detection

Crude extracts were dissolved by PBS buffer containing containing 5% DMSO and 5% ethanol. After filtrated by 0.22 μm sterile membrane, the resultant solution was diluted into 1/2 and 1/10 of its stock solution by RPMI-1640 medium containing 10% fetal calf serum respectively.

Based on the MTT method established by Mosmann (1983, PP. 55-65), its modification was applied in the present paper. MCF-7 cells were harvested with 0.25% trypsin, and cell viability was measured by 0.4% trypan blue; when their viability were above 90%, they were counted, and diluted into a density of approximately $1 \times 10^4/\text{mL}$ by RPMI-1640 medium containing 10% fetal bovine serum. Cells were seeded into 96-well plates, 180 μL each well, and incubated for 24h at 37°C in a 5% CO_2 incubator. Before adding the sample into 96-well plates, RPMI-1640 medium was removed, plates were rinsed by PBS (enough to cover the cell surface) in order to discard suspended cells. Samples were undertaken 3 gradients and 4 replicates, and 20ml prepared samples were added into each well, 20 μL 1mg/mL cisplatin as positive control and the same volume of RPMI-1640 medium as blank control, and incubated for another 24h continuously at 37°C in a 5% CO_2 incubator. After

completion of the treatment, cells in 96-well plates were centrifuged for 10 min at 1000 r/min in order to discard samples and medium and washed with PBS, and 20 μ L of 5 mg/mL MTT was added to the cells and incubated for another 4 h at 37°C. After 4 h, 96-well plates were centrifuged at 1000 r/min in order to remove the uninvolved MTT and the precipitated formazan was subsequently dissolved in 200 μ L dimethyl sulfoxide (DMSO) and gently shaken for 10 min. The absorption was measured at 492nm, with ELISA analyzer (Germany). The inhibiting rate was calculated using the equation below (Wang, 2009, PP. 755-760):

$$\text{Inhibiting rate} = (1 - \text{OD positive control} / \text{OD blank control}) \times 100\%$$

Toxicity determination used MRC cell model, and the medium changed into DMEM containing 10% fetal calf serum. Method was the same with inhibition rate. Inhibition rate of samples on MRC cells were measured and calculated.

1.2.4 Analysis of bioactive substances in fermentation products

Fermentation solutions with higher inhibition rate on MCF and lower on MRC were selected, and analyzed by Thermo ODS-2 analytical HPLC to ascertain components of secondary metabolites in fermentation solutions. A gradient elution was carried out using the following solvent systems: mobile phase: solvent A, 20%-80% methanols; solvent B, water.

2. Results

2.1 Bioactivity and toxicity

OD₄₉₂ values in the table were shown as the means and standard deviations of 4 replicates, and data was analyzed by SPSS 16.0 software. One-way ANOVA was used to test the significance of difference in measured variables between control and treatment in combination with Dunnett method. Results were listed in Table 1 in detail. As well known to us all, antitumor agents with potential development prospects should not only had strong inhibition effects on tumor cells, but also less toxicity to normal cells.

As seen from Table 1, the majority of myxobacteria fermentation metabolites could inhibit the growth of MCF-7 tumor cells in the present study. Among them, 16 strains had significant inhibition effects on MCF-7 tumor cells with positive rate over 80%, and inhibition rate of 9 strains attained more than 50%, accounting for 45% of the tested strains; Inhibition rate of myxobacteria strain 93431 fermentation original solution, 2 fold dilution and 10 fold dilution on MCF-7 cells was 53.32%, 49.35%, and 46.50%, respectively, and their inhibition rate on MRC cells was 2.95%, 5.90%, and 1.97%, respectively. Therefore, fermentation extracts of strain 93431 were selected to undertaken HPLC analysis to ascertain its secondary metabolites components with gradient elution.

2.2 Morphological variation of cells by inverted microscope

Normal MCF-7 cells attached to the wall to proliferate and showed an irregular multilateral angle with a large number of cells; in 96-well plates, after treated by strain 93431 fermentation solutions for 24h, cells in each group all had shrinkage, and separated from the surrounding cells. Nuclear contracted, cells that attached walls decreased, either suspension or shedding.

MRC cells attached to the wall to proliferate and showed a long-spindle with a large number of cells; in 96 well plates, after treated by strain 93431 fermentation solutions for 24h, a large majority of cells in each group showed a normal morphology, spreading and transparent.

2.3 Bioactive components separation by HPLC

Fermentation crude extracts of strain 93431 were undertaken HPLC analysis with gradient elution. Mobile phase and its proportion were listed in Table 2. Results showed that mixed components were easy to separate, and separation effects were listed in Figure 2. As seen from Figure 3, peaks at 38.65 min, 40.70 min, 43.63 min, 45.43 min, 48.06 min, 51.18 min, 64.54 min and 66.81 min represented secondary metabolites of strain 93431 fermentation products. Peaks at 8.75 min, 15.41min, 17.03 min and 23.75 min need further investigation to figure out.

3. Discussions

In the present study, using MCF-7 and MRC cell lines as an experimental model, we investigated the antitumor activity of secondary metabolites from myxobacteria and its toxicity to normal cells. Results indicated that myxobacteria strains 93431 had strong inhibitory effects on MCF-7 cells while had little toxicity to MRC cells. Accordingly, bioactive substances in strains 93431 fermentation products had selective inhibitory effects on tumor cells, and had potential development prospects. After HPLC analysis, results suggested that peaks at peaks at 38.65 min, 40.70 min, 43.63 min, 45.43 min, 48.06 min, 51.18 min, 64.54 min and 66.81 min represented

secondary metabolites of strain 93431 fermentation products, but they still need further separation and purification. In addition, functional targets of inhibiting tumor cell growth and its action mechanism still should need further investigation.

Streptomyces, fungi and bacillus were recognized as the big three drug sources. Currently, bioactive substances such as heterocyclic, quinine, polyether, macrocyclic, vinyl, peptides and other types, nearly 400 kinds were discovered in myxobacteria. With the increasing discovery of more and more secondary metabolites with pharmacological and biological activity, myxobacteria exceeded bacillus, and rose to the third place. Myxobacteria tended to be an important microbial resource, and would shed light on the screening and application of antitumor agents. Myxobacteria resources were abundant in China, but hard to isolate, purify and incubate. All those characteristics tended to be critical factors that impeded the research and development of myxobacteria. With the profound investigation of its metabolites, it would be certain to find some novel target antitumor agents with unique functions.

References

- Curtis, M. (1999). Global Breast Cancer Mortality Statistics. *CA Cancer J Clin*, 49:138-144.
- Gerth, K., Pradella, S., & Perlova, O., et al. (2003). Myxobacteria: proficient producers of novel natural products with various biological activities past and future biotechnological aspects with the focus on the genus *Sorangium*. *Biochemical Journal*, 106:233-253.
- Gurmeet, K., & Melinda, H. (2006). Biological evaluation of tubulysin A: a potential anticancer and antiangiogenic natural product. *Biochemical Journal*, 396:235-242.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods*, 55-65.
- Reichenbach, H. (2001). Myxobacteria producers of novel bioactive substances. *Journal of Industrial Microbiology & Biotechnology*, 27:149-156.
- Wang, D.H., & Tao, W.Y. (2009). Antitumor activity in vitro and volatile components of metabolites from myxobacteria *Stigmatella WNXJ-B*. *African Journal of Microbiology Research*, 3(11):755-760.

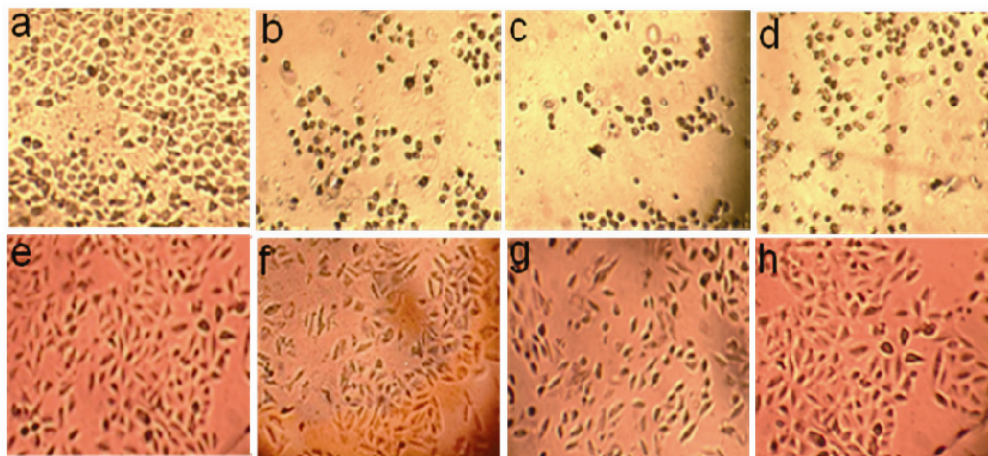
Table 1. OD₄₉₂ values and inhibition rate of bioactive substances

Strain	MCF-7 cells OD ₄₉₂ Values and inhibition rate (%)			MRC cells OD ₄₉₂ Values and inhibition rate (%)		
	Original solution	1/2 Original solution	1/10 Original solution	Original solution	1/2 Original solution	1/10 Original solution
92006	0.684±0.023* 15.10	0.496±0.026** 38.40	0.507±0.031** 37.05	0.249±0.008** 38.81	0.385±0.016 5.47	0.381±0.010* 6.33
92152	0.438±0.010** 45.67	0.689±0.041** 14.48	0.736±0.060 8.68	0.215±0.008** 47.17	0.247±0.009** 39.43	0.376±0.014* 7.60
92153	0.757±0.043 6.01	0.681±0.072** 15.47	0.731±0.056* 9.24	0.219±0.006** 46.22	0.368±0.009* 9.69	0.407±0.017 0.00
92154	0.491±0.011** 39.08	0.728±0.062* 9.65	0.772±0.038 4.16	0.232±0.007** 42.96	0.372±0.014* 8.65	0.407±0.011 0.00
92155	0.805±0.047 0.07	0.616±0.009** 23.59	0.793±0.040 1.53	0.236±0.006** 41.98	0.400±0.021 1.72	0.401±0.027 1.47
92163	0.792±0.009 1.69	0.780±0.007 3.17	0.758±0.009 5.91	0.305±0.010** 25.1	0.283±0.003** 30.37	0.296±0.006** 27.36
92213	0.725±0.041* 9.97	0.585±0.032** 27.40	0.549±0.012** 31.84	0.265±0.017 34.94	0.270±0.013 33.58	0.265±0.013 34.91
93428	0.527±0.025** 34.60	0.517±0.021** 35.84	0.529±0.016** 34.29	0.304±0.010** 25.30	0.324±0.022** 20.35	0.341±0.017** 16.15
93429	0.366±0.011** 54.63	0.355±0.016** 55.91	0.381±0.040** 52.68	0.259±0.013** 36.38	0.284±0.009** 30.25	0.400±0.018 1.79
93430	0.747±0.047 7.22	0.795±0.032** 1.47	0.714±0.020** 11.42	0.372±0.022* 8.70	0.405±0.016 0.02	0.406±0.009 0.25
93431	0.376±0.044** 53.32	0.408±0.014** 49.35	0.431±0.026** 46.50	0.395±0.006 2.95	0.383±0.011 5.90	0.399±0.013 1.97
93434	0.627±0.031** 22.21	0.338±0.034** 58.06	0.462±0.019** 42.67	0.246±0.022** 39.61	0.312±0.015** 23.46	0.378±0.024* 7.22
94236	0.336±0.042** 58.35	0.383±0.024** 52.45	0.395±0.014** 51.00	0.331±0.012** 18.69	0.361±0.031** 11.26	0.375±0.025* 7.85
94237	0.428±0.022** 46.91	0.425±0.021** 47.19	0.286±0.004** 64.55	0.222±0.007** 45.55	0.224±0.007** 44.90	0.326±0.023** 19.97
94238	0.359±0.035** 55.43	0.667±0.149** 17.18	0.507±0.106** 37.01	0.299±0.019** 26.60	0.332±0.026** 18.34	0.368±0.034* 9.48
94229	0.461±0.031** 42.76	0.335±0.023** 58.37	0.543±0.040** 32.55	0.229±0.009** 43.78	0.228±0.011** 43.99	0.314±0.020** 22.87
94231	0.376±0.046** 53.34	0.269±0.020** 66.55	0.350±0.028** 56.53	0.251±0.011** 38.30	0.309±0.023** 24.14	0.336±0.012** 17.52
94232	0.329±0.040** 59.11	0.302±0.024** 62.53	0.245±0.006** 69.57	0.278±0.014** 31.77	0.266±0.015** 34.55	0.315±0.006** 22.71
94241	0.684±0.044** 15.04	0.543±0.022** 32.59	0.806±0.052 0.00	0.239±0.006** 41.35	0.263±0.020** 35.39	0.255±0.009** 37.47
94242	0.521±0.028** 35.34	0.481±0.044** 40.29	0.615±0.017** 23.68	0.242±0.007** 40.64	0.244±0.004** 39.95	0.283±0.018** 30.38
CK	0.806±0.042			0.407±0.005		

Note: Data are means ±SD, n=4 (*, P<0.05 po0.05; **, (P<0.01).

Table 2. HPLC gradient elution parameters

Time (min)	Methanol (%)	Water (%)
0	20	80
20	20	80
60	60	40
80	80	20
90	100	0



Note: a, MCF-7 cell; b, MCF-7 cells treated by fermentation original solution for 24h; c, MCF-7 cells treated by 1/2 fermentation original solution for 24h; d, MCF-7 cells treated by 1/10 fermentation original solution for 24h; e, MRC cell; f, MRC cells treated by fermentation original solution for 24h; g, MRC cells treated by 1/2 fermentation original solution for 24h; h, MRC cells treated by 1/10 fermentation original solution for 24h.

Figure 1. Cell morphological variation

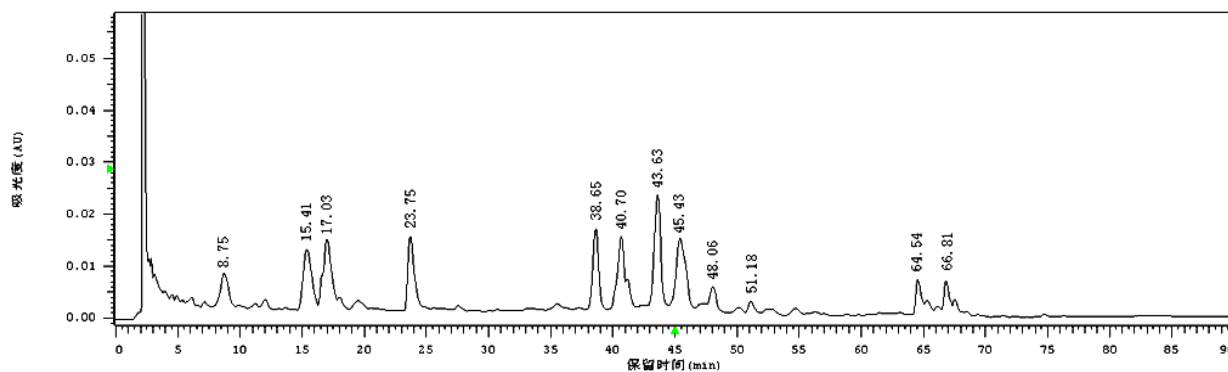


Figure 2. HPLC gradient elution chromatogram of strain 93431 fermentation products at 300 nm

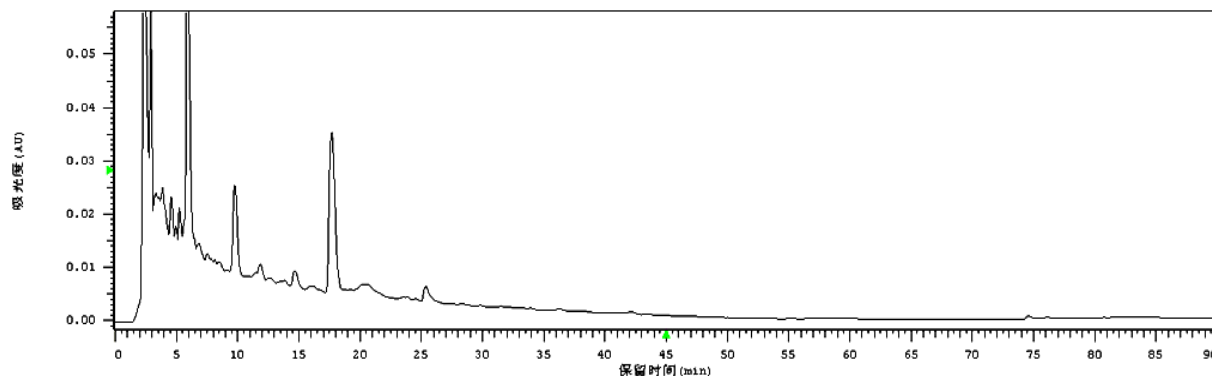


Figure 3. HPLC gradient elution chromatogram of blank medium at 300 nm