Production of Antitumor Agents from Novel Marine Actinomycetes Strains Isolated from Alexandria, Egypt

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Abstract

Production of antitumor agent(s) from alternative natural marine microbial resources may decrease or avoid the bad side effects of the used anti-tumour chemotherapeutic agents and may increase the specificity of the antitumor agents to be safer for human application. So, this study aimed to search for new alternative antitumor agent producers using two recommended bioassays (Red Potato Disc Bioassay and Sulfonhodamine B Bioassay). The obtained results indicated the isolation of three new marine actinomycetes strains from the western harbor of Alexandria, they identified using the 16S RNA gene sequence as Streptomyces sp. strain AMS11, Nocardiopsis halotolerans strain AMS10b and Nocardiopsis halotolerans strain AMS10a. The bio-toxicity test was carried out using the biomarker Artemia salina, they showed a moderate toxicity level (LC<sub>50</sub>&lt;1000 ppm). The antitumor activities of their supernatants against the colon cancer cell line (HCT116) and the liver cancer cell line (HEPG2) showed promising results in comparison to the recommended chemotherapeutic drug 5-flourouracil used for the treatment of both liver and colon cancer cells. The optimization for antitumor activity was performed using the Plackett–Burman experimental design.

Keywords: Red Potato Disc Bioassay; Sulfonhodamine B Bioassay; Plackett–Burman design; Streptomyces sp.; Nocardiopsis halotolerans; Liver cell line; Colon cell line

Introduction

Cancer is a leading cause of mortality worldwide and therefore a major focus of research made on the chemoprevention of cancer. This approach is a mean of cancer control where the induction of this disease partially prevented or the rate of its development slowed or reversed partially by the administration of one or more naturally occurring agents. The majority of such drugs are either natural products (NP), their derivatives (ND), natural product mimics (NPM) or semisynthetic derivatives (SSD) [1].

Over 70% of the earth’s surface covered by oceans and it believed life originated in the oceans. Additionally, the oceans considered as a source for natural products mainly accumulated in marine living organisms. Several bioactive compounds of therapeutic interest have been isolated from marine invertebrates and some of them reported to be of microbial origin. Numerous of these compounds show pharmacological activities and are helpful for the invention and discovery of bioactive compounds acting against deadly diseases like cancer, acquired immuno-deficiency syndrome [2].

The marine microorganisms especially actinomycetes are widely distributed in oceans all over the earth and acting as a great source for the discovery of different natural products. Further studies were also developed to obtain antitumor agents and many other compounds like enzyme inhibitors. Also, marine habitat has produced a significant number of very potent marine-derived agents to inhibit the growth of human tumor cells in vitro, in vivo and in humans [3,4].

Throughout this study a screening for antitumor marine microbial producers was occurred using seawater samples collected from different stations of the Western Harbour of Alexandria, the bioactivity was detected and confirmed using different bioassays as the Red Potato Disc Bioassay [5] and Sulfonhodamine B (SRB) assay using two cancer cell lines; colon cell line (HCT116) and liver cell line (HEPG2) where a comparison with a standard compound 5-flourouracil was investigated [6,7]. The bio-toxicity of the potent supernatants was performed using the biomarker Artemia salina [8]. Finally, the optimization to reach the ideal conditions for maximum bioactivity was carried out using a Plackett-Burman experimental design [9].

Materials and Methods

Isolation of different actinomycetes

Isolation of different marine actinomycetes was carried out from 10 stations of the western harbor of Alexandria, Egypt. Four recommended actinomycetes culture media; Oatmeal (shofan) Nitrate Medium, Starch Casein Nitrate Medium, Starch Nitrate Medium and Potato Dextrose Medium were tested in order to select the most suitable culture medium for antitumor production [10–12]. The supernatant of each actinomycetes isolate was obtained using a micro-centrifuge for 15 min at 12000 rpm then undergo antitumor activity test.

Screening for the antitumor activity

Red Potato Disc bioassay

Throughout this assay the potato discs has been infected by...
Molecular identification of the most potent actinomycetes

The most potent isolates were chosen for molecular identification using 16S rRNA gene sequencing molecular technique which carried out at Sigma Scientific Services Co., Cairo, Egypt.

DNA extraction

DNA was extracted from overnight pure culture of each marine actinomycetes isolate using GeneJET Genomic DNA purification kit (Thermo). The procedure was identical to that recommended by the manual instructions. The preparations were analyzed on a 0.7% agarose gel and then determined spectrophotometrically.

PCR-amplification and sequencing of 16S rRNA gene

The amplification of the genomic DNA was carried out through a PCR process using Maxima Hot Start PCR Master Mix (Thermo) as following:-

1) Gently vortex and briefly centrifuge Maxima’ Hot Start PCR Master Mix (2X) after thawing. 2: Add the following components for each 50 µl reaction at room temperature: 25 µl of Maxima’ Hot Start PCR Master Mix (2X), 1 ul (20 uM) of 16SrRNA Forward primer, 1 ul (20 uM) of 16SrRNA Reverse primer, 5 ul of Template DNA and 18 µl of Water, nuclease-free making a total volume 50 µl. The used primers were specific for actinomycetes; F: AGAGTTTGATClCTGGCTCAG and R: GGTTACCTTGTTACGACTT. The GeneJET column was used for cleaning up the PCR product.

Confirmation test for the antitumor activity of the potent actinomycetes

Scanning electron microscopy

The antitumor activity of the most potent actinomycetes was confirmed though scanning electron microscope examination compared to the normal potato cells, the infected potato cells with the A. tumefaciens and the infected potato cells with Ti plasmid of the A. tumefaciens. This process was carried out at the Faculty of Science, Alexandria University.

The Sulforhodamine B Bioassay (SRB)

The bio-toxicity experiment was carried out using Artemia salina as a recommended biomarker, for detecting the LC50 value for the three potent isolates according to Meyer et al. [17] using 24 hour old neuplli of Artemia salina. Different concentrations 250, 500, 750 and 1000 ppm [v/v] were made from the studied supernatants using 0.25, 0.5, 0.75 and 1.0 ml, respectively. These supernatants were distributed separately using clean and dry glass vials (12 ml) then total volume were completed to 10 ml using sterile seawater. Ten live neuplli of Artemia salina transferred to each vial. The number of the viable biomarker was counted after 24 hour of application. The mortality percentages and the half-lethal dose (LC50) were determined using the probit analysis method [8,18].

Optimization and verification for the culture medium and the physiological conditions using Plackett-Burman statistical design

Throughout this study Plackett-Burman design method was used to detect the optimum medium components of oatmeal (OM) nitrate culture medium as a basal medium and the optimum physiological conditions for liquid cultures to reach maximum antitumor production and activity. Seven independent variables (OM, K,HPO4, KNO3, MgSO4, Temp., pH and inoculum size (I.S)) were examined in 8 combinations in comparison to the basal medium (0) according to the Plackett-Burman design matrix. The main effect of each tested variable was presented as the difference between the antitumor activity % averages at both the high level (+) and the low level (-) of the examined variable [9].
### Results

#### Screening for antitumor agent producers using different culture media

Seven actinomycetes strains coded as; 2A, 6C, 8act, 9A, 9b, 9C and 10b were isolated from the western harbor of Alexandria using four different culture media. The obtained results in (Table 1) indicated the Oatmeal nitrate culture medium was most potent for the growth of the antitumor active marine actinomycetes isolates measured as increasing in the optical density at λ\text{max} nm.

#### The molecular identification process

The most active isolates for anti-tumor agent production were identified using the 16S rRNA gene sequence. They were found to be novel marine actinomycetes strains and reported in the GENBANK with new association numbers, as follows:

- 9A: *Streptomyces sp.* strain AMS11; (association #: KF964032), 10b: *Nocardiopsis halotolerans* strain AMS10a; (association #: KJ577539). The phylogenetic tree of these new marine actinomycetes strains presented in Figure 1.

#### Scanning electron microscopy examination

The anti-tumor activity of the supernatants of these three active marine actinomycetes was confirmed through the scanning electron microscopy examination. The obtained micrographs showed the effect of the *A. tumefaciens* and its Ti-plasmid on the potato cells and the starch granules formation. They led to the absence or the decrease in the granules number compared to that of the normal potato cells, where the infected cells showed no ability to form normal starch granules (Figure 2).

#### The cytotoxicity test

In case of the SRB assay using the liver cancer cell line (HePG2) IC\text{50}, the obtained results of the use of *N. halotolerans* strain AMS10a and *Streptomyces sp.* strain AMS11 supernatants showed a very promising treatment through three replicates, the IC\text{50} values were 0.74 ± 0.47 and 1.24 ± 0.56, respectively, comparing to the standard compound 5-fluorouracil (IC\text{50} 2.2 ± 1.6). While the supernatant of the *N. halotolerans* strain AMS10b showed a moderate effect against

### Table 1: Effect of different culture media on the growth of the antitumor active marine actinomycetes isolates measured as increasing in the optical density at λ\text{max} nm.

| Isolate code | 'Culture media' | Optical Density (OD) | Incubation period (h) |
|--------------|-----------------|----------------------|-----------------------|
|              |                 | 0  | 6  | 12 | 18 | 24 | 30 | 36 |
| 2A           | M₀ | 0.060 | 0.066 | 0.072 | 0.080 | 0.16 | 0.18 | 0.12 |
|              | M₃ | 0.02  | 0.02  | 0.02  | 0.02  | 0.08  | 0.09  | 0.11  |
|              | M₄ | 0.038 | 0.064 | 0.09  | 0.13  | 0.12  | 0.16  | 0.16  |
|              | M₅ | 0.136 | 0.368 | 0.6   | 0.6   | 0.6   | 0.64  | 0.928 |
| 6C           | M₁ | 0.12  | 0.128 | 0.136 | 0.18  | 0.176 | 0.24  | 0.24  |
|              | M₂ | 0.06  | 0.062 | 0.066 | 0.06  | 0.056 | 0.04  | 0.02  |
|              | M₃ | 0.09  | 0.104 | 0.12  | 0.12  | 0.2   | 0.25  | 0.2   |
|              | M₄ | 0.14  | 0.51  | 0.88  | 0.56  | 0.6   | 0.95  | 0.84  |
| 8act.        | M₁ | 0.014 | 0.02  | 0.02  | 0.11  | 0.104 | 0.12  | 0.18  |
|              | M₂ | 0.07  | 0.072 | 0.074 | 0.1   | 0.09  | 0.08  | 0.04  |
|              | M₃ | 0.082 | 0.103 | 0.124 | 0.04  | 0.122 | 0.2   | 0.16  |
|              | M₄ | 0.172 | 0.226 | 0.28  | 0.42  | 0.4   | 0.62  | 0.5   |
| 9A           | M₀ | 0.06  | 0.08  | 0.062 | 0.11  | 0.104 | 0.24  | 0.23  |
|              | M₁ | 0.066 | 0.068 | 0.072 | 0.066 | 0.06  | 0.06  | 0.05  |
|              | M₂ | 0.072 | 0.09  | 0.108 | 0.14  | 0.2   | 0.32  | 0.22  |
|              | M₃ | 0.184 | 0.306 | 0.43  | 0.534 | 0.648 | 1     | 0.98  |
| 9b           | M₁ | 0.084 | 0.088 | 0.094 | 0.106 | 0.12  | 0.124 | 0.2   |
|              | M₂ | 0.11  | 0.124 | 0.14  | 0.1   | 0.08  | 0.06  | 0.064 |
|              | M₃ | 0.03  | 0.044 | 0.06  | 0.12  | 0.24  | 0.22  | 0.3   |
|              | M₄ | 0.178 | 0.498 | 0.82  | 0.5   | 0.74  | 0.8   | 1     |
| 9C           | M₀ | 0.02  | 0.022 | 0.024 | 0.03  | 0.05  | 0.07  | 0.24  |
|              | M₁ | 0.02  | 0.022 | 0.014 | 0.046 | 0.058 | 0.08  | 0.12  |
|              | M₂ | 0.086 | 0.092 | 0.1   | 0.1   | 0.18  | 0.22  | 0.36  |
|              | M₃ | 0.2   | 0.564 | 0.93  | 1.06  | 1     | 1.5   | 1.48  |
| 10b          | M₀ | 0.06  | 0.064 | 0.07  | 0.064 | 0.136 | 0.1   | 0.102 |
|              | M₁ | 0.172 | 0.176 | 0.18  | 0.18  | 0.22  | 0.26  | 0.28  |
|              | M₂ | 0.088 | 0.108 | 0.128 | 0.166 | 0.18  | 0.18  | 1.7   |
|              | M₃ | 0.186 | 0.272 | 0.36  | 0.404 | 0.506 | 0.6   | 0.96  |

*M₁: Starch nitrate medium, M₂: Casein medium, M₃: Potato dextrose medium, M₄: Oatmeal nitrate medium.*
Figure 1: (A) The phylogenetic tree of the potent actinomycetes, *N. Halotolera* strain AMS10a; (B) *Streptomyces* sp. strain AMS11; (C) *N. halotolera* strain AMS10b.

The bio-toxicity test

The bio-toxicity experiment was carried out using *Artemia salina* as biomarker for testing the bio-toxicity of the potent supernatant of *N. halotolera* strain AMS10a, *Streptomyces* sp. strain AMS11 and *N. halotolera* strain AMS10b. The mortality percentages and the LC<sub>50</sub> values were estimated. The results indicated these supernatants had a moderate bio-toxicity level (LC<sub>50</sub>&lt;1000 ppm) they were 603.9, 517.6 and 583.4 ppm, respectively (Figure 4).

Optimization and verification of the antitumor activity of the tested marine actinomycetes using Plackett-Burman experimental design

The optimization of the seven variables affecting the oatmeal culture
Figure 2: (A and B) Micrographs show the starch granules of the infected cells with the Ti plasmid of*A. tumefaciens* (tumorized cells); (C) The infected cells with *A. tumefaciens* itself.
Figure 3: (A) Micrographs show the starch granules of the normal cells; the treated cells using the supernatant of marine actinomycetes; (B) N. Halotolerans strain AMS10a, (C) Streptomyces sp. strain AMS11; (D) N. halotolerans strain AMS10b.
Figure 4: The probit analysis show the bio-toxicity and the log LC50 values of the supernatants of \textit{N. halotolerans} strain AMS10a (A) \textit{Streptomyces} sp. strain AMS11 (B) and \textit{N. halotolerans} strain AMS10b (C) using \textit{Artemia salina} as a biomarker.

Table 2: The anticancer activity of the most potent marine actinomycetes according to their IC50 values (μM) against three replicate (R1,2,3) liver cell lines (HePG2) compared to the standard chemotherapeutic compound (5-Flourouracil).

| Tested Product                  | Colon cancer cell line (HCT116) |
|--------------------------------|---------------------------------|
|                                | R1     | R2     | R3     | SE     | Average ± SE |
| 5-Flourouracil                 | 4.92 E-08 | 1.24 E-06 | 5.31 E-06 | 1.5936 E-06 | 2.2 ± 1.6 |
| \textit{N. halotolerans} strain AMS10a | 1.634 | 0.03076 | 0.5629 | 0.47145 | 0.74 ± 0.47 |
| \textit{Streptomyces} sp. strain AMS11 | 1.14 | 0.3258 | 2.272 | 0.56431 | 1.24 ± 0.56 |
| \textit{N. halotolerans} strain AMS10b | 50 | 65 | 56 | 1.52752 | 57 ± 1.52 |

Table 3: The anticancer activity of the most potent marine actinomycetes according to their IC50 values (μM) against three replicates (R1,2,3) of colon cancer cell line (HCT116) compared to the standard chemotherapeutic compound (5-Flourouracil).
medium was carried out for a maximum antitumor activity using the matrix of the Plackett-Burman experimental design (Table 4). It was showed the concentration of Shofan, K₃HPO₄ and KNO₃ in addition to the pH must be adjusted at their low levels 0.15 g/100 ml, 0.025 g/100 ml, 0.01 g/100 ml and 6, respectively. Whereas the concentration of MgSO₄, the temperature and the inoculum size showed to be more suitable on presentation at their high levels (0.04 g/100 ml, 37°C and 4 ml/100 ml, respectively (Figure 5).

The elucidation of the obtained results and the evaluation of the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates to predict the near optimum levels of the tested independent variables. The data examined compared to the basal medium and the anti-optimized medium. The results indicated high antitumor activity (97.5%) was obtained on using the optimized medium where the antitumor activity showed to be more effective for the tumorized cells themselves [20].

![Figure 5: Elucidation of different cultured factors affecting the production of the antitumor agent produced by marine actinomycetes.](Image)

The elucidation of the obtained results and the evaluation of the accuracy of the applied Plackett-Burman statistical design, a verification experiment was carried out in triplicates to predict the near optimum levels of the tested independent variables. The data examined compared to the basal medium and the anti-optimized medium. The results indicated high antitumor activity (97.5%) was obtained on using the optimized medium where the anti-optimized medium showed low antitumor activity (59.5%). The optimized medium increased the antitumor activity by 25.8% compared to the basal medium (71.7%). In addition, the main effect values as well as the t-test values indicated the significant factors were MgSO₄ concentration and pH value at 85% confidence limit, also the temperature can be considered as an effective variable that control the antitumor activity (Table 5).

**Discussion**

Now a days it is very important to open more lines to search for novel antitumor agent (s) from natural resources to be more safe and healthy for patients, due to the rapid development of resistance to multiple chemotherapeutic drugs and also to face these challenges: 1) The development of the multidrug resistance phenomenon in patients. 2) The long-term treatment with antitumor drugs lead to severe side effects (Hair dropping, skin inflammation, Nausea and vomiting, early menopause, Weight gain, Memory problems immunodeficiency and gastrointestinal problems). 3) The un-selectivity of the used antitumor drugs to the tumorized cells themselves [19]. Also Adjuvant chemotherapy may be cause cardiotoxicity and or neurotoxicity: Examples include cyclophosphamide, ifosfamide, mitomycin and fluorouracil [20].

Over 18000 compounds have been isolated from marine sources and approximately 150 compounds are cytotoxic against the different tumor cells. Thus, it is excited that new groups of actinomycetes from unexplored or under exploited habitats be perused as resources of novel bioactive secondary metabolites. The marine actinobacteria have been looked upon as potential sources of bioactive compounds, and the work done earlier has shown that these microbes are the richest source of secondary metabolites [21-23].

Different studies shows that the marine actinomycetes which have bioactivity can be isolated on different media, the most used media were starch nitrate agar media, starch casein nitrate agar media, oat meal agar media, potato dextrose agar media, glycerol glycine agar medium and chitin agar medium [11,24,25]. Similarly, in this study a physiological adaptation made by using four different media; starch nitrate agar medium, starch casein nitrate agar medium, potato dextrose agar medium and oat meal agar medium. The obtained results revealed that oatmeal culture medium was the best medium for the
isolation of bioactive marine actinomycetes according to the obtained growth and bioactivity for 36 hour incubation period at 37°C and pH 7. Unlike the study of Attimad et al. [10] showed the starch-casein agar supplemented with nystatin and nalidixic acid was found to be suitable for isolating actinomycetes from marine sediments, which were collected from the coastal areas of Gokharna and Muradeshwara of Karnata state.

The results of the potato disk bioassay and the SRB assay in addition to the biotest-tic test and the obtained IC₅₀ values (<1000 ppm) of the tested supernatants of marine actinomycetes indicated that the Nocardiosis halotolerants strain AMS10a and Streptomyces sp. strain AMS11 are promising antitumor producers against the liver cell line (HePG2), the IC₅₀ values were 0.74 ± 0.47 and 1.24 ± 0.56, respectively, comparing with the standard chemotherapeutic drug 5-fluorouracil (IC₅₀= 2.2 ± 1.6). Whereas Nocardiosis halotolerans strain AMS10b showed a moderate antitumor activity against the colon cancer cell line (HCT116) the IC₅₀ value was 2.53 ± 2.49 comparing with the 5-fluorouracil (IC₅₀=1.9 ± 1.2). Unlikely, Schiman et al. [26] examined the cytostatic and cytotoxic activities of two novel extracts of Streptomyces antibioticus Tii 6040 on human cell lines. Both extracts exhibited a growth inhibition against gastric adenocarcinoma (HMO,) and mamma carcinoma (MCF-7) cells rather the inhibition towards the hepatocellular carcinoma (HEP G2) compared to the 5-fluorouracil. While El-Shatoury et al. [27] proved microorganisms associated with the marine shellfish are suggested to be potential source of bioactive metabolites. They studied sixty-three actinomycetes strains isolated from the marine shellfish Donax trunculus anatius and their metabolic extracts. They showed wide antimicrobial activities towards 11 reference and clinical cultures; and 17.5% showed antitumor activities against several sources, respectively, for optimum production of growth and bioactive metabolites.

**Table 5:** Verification of Plackett- Burman experimental design for antitumor activity of the tested marine actinomycetes.

| Tested Variables | OM (g/100 ml) | K₂HPO₄ (g/100 ml) | KNO₃ (g/100 ml) | MgSO₄ (g/100 ml) | Temp. | pH | **L.S. (ml/100 ml)** | Average activity (%) |
|------------------|---------------|-------------------|-----------------|------------------|-------|----|---------------------|---------------------|
| *Optimized medium* | 0.15          | 0.025             | 0.01            | 0.04             | 37    | 6  | 4                   | 97.5                |
| *Basal medium*   | 0.3           | 0.05              | 0.02            | 0.02             | 30    | 7  | 2                   | 71.7                |
| *Anti-optimized medium* | 0.6       | 0.1               | 0.04            | 0.01             | 25    | 8  | 1                   | 59.5                |

| Tested Variables | (Potato discs+sterile seawater) | (Potato discs+A. tumefaciens) | (Potato discs+ A. tumefaciens+Ampicillin) |
|------------------|---------------------------------|-------------------------------|------------------------------------------|
| Cₛ               | 0                               | 65                            | 60                                       |

| * T: Cₛ is the supernatant of marine antitumor active actinomycetes |
| ** Optical density =1.0 |

**Conclusion**

From this study, the sheep oat meal culture medium can be used for producing antitumor products from novel marine strains of Streptomyces sp. strain AMS11, Nocardiosis halotolerans strain AMS10a, Nocardiosis halotolerans strain AMS10b. According to the obtained results from the Red Potato Assay and the SulphoRhodamine-B (SRB) cytotoxicity assay they can be used as alternative resources for producing antitumor agents with a moderate biotoxicity in comparison with the standard antitumor drug 5-fluorouracil (5-FU) they showed promising results especially in the treatment of liver cancer cell line. However, it is good to continue the research on such products to know exactly the nature and the real chemical characterization of such marine natural products.

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