Effects of salt concentration on the production of cytotoxic geodin from marine-derived fungus *Aspergillus* sp.

Introduction

Fungi play an important role in drugs discovery, producing secondary metabolites having interesting bioactivities [1, 2]. Most fungi belong to terrestrial habitats and have been studied for the bioactive secondary metabolites such as antibiotics; however, those related to the harsh marine environment are recently under extensive study for the production of bioactive natural products [3, 4]. Fungi from the depth of the sea could be studied by culturing in the same artificial environment [4] and those isolated from the coastal areas of the sea produce a variety of valuable bioactive secondary metabolites such as polyketides, peptides, alkaloids, and terpenoids [5]. Fungi from the coastal regions are related either to coral or coral reefs, coastal plants such as mangroves, and rocks [6]. Filamentous fungi synthesize commercially beneficial byproducts like wine from rice by *Aspergillus oryzae*, and bean curd by *Monascus* while the pure bioactive secondary metabolites are used as medicines such as penicillin, cephalosporin, and lovastatin produced by *Aspergillus terreus* [9–12]. Geodin 1 has interesting biological activities such as antiviral [13], antimicrobial [14], glucose stimulator for rat adipocytes [15], enhancement of fibrinolytic activity [16], cytotoxic activity [17], and is an essential part of the first nonpeptide, having inhibitory activity of the galanin receptor subtype GALR1 [18, 19]. In our previous study, it showed moderate cytotoxic activities against various cancer cell lines such as breast cancer (BT474), large cell lung...
cancer (NCI-H460), non-small cell lung cancer (H-1975), lung cancer (A549), chronic myelogenous leukemia (K562) and prostate cancer (DU145) cells [20].

**Materials and methods**

The fungal strain was cultivated in 1,000 mL Erlenmeyer flasks containing about 60 g sterilized rice medium and the respective concentrations of sodium chloride (NaCl) salt at 0.0%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 5.0%, 8.0%, 10.0%, 15.0%, 20.0%, and 30.0%. The flasks were fermented at room temperature for 1, 2, 3, 4, and 5 weeks, and extracted thrice times with ethyl acetate (EtOAc). Each crude extract was evaporated under vacuum to dryness and subjected to normal phase silica gel column chromatography (mesh 200–300), eluting with linear gradients of petroleum ether (PE) – EtOAc. Friction 4, eluted with 40% PE-EtOAc, was further subjected to the re-crystallization in methanol and dichloromethane. As a result of re-crystallization, geodin 1 was obtained in pure form as a white crystalline solid. The electro spray ionization mass spectrometry (ESI-MS) spectra were measured on Ultra Performance Liquid Chromatographic Mass Spectrometer (Waters UPLC® system using a C18 column [ACQUITY UPLC® BEH C18, 2.1 × 50 mm, 1.7 µm; 0.5 mL/min]). Nuclear magnetic resonance (NMR) spectra were recorded on an Agilent DD2 NMR spectrometer (500 MHz for _1H_ and 125 MHz for _13C_ NMR) and tetra-methyl saline (TMS) as an internal standard while deuterated chloroform (CDCl3) as a solvent. The structure of compound 1 was confirmed based on ESI-MS m/z at 398.9 [M+H]^+ and NMR spectral data (Table 1) [20, 21]. The chemical structure of geodin 1 was elucidated by Barton and Scott in 1958 [9, 22]. It was re-confirmed based on ESI-MS and NMR spectra (Figure 1) [20, 21]. The fungal strain was identified as _Aspergillus_ sp., based on its morphology and molecular identification. The 512 base pair ITS sequence had 98% similarities with _Aspergillus_ sp. NRRL58570 (HQ288052.1). The sequence data were submitted to GenBank with accession number KY235298 and the strain was stored in the Key Laboratory, School of Medicine and Pharmacy, Ocean University of China, Qingdao, P.R.China, with Genbank accession number KY235298.

**Results and discussion**

Geodin was discovered by Raistrick & Smith in 1936 from _A. terreus_ and its structure with relative configuration was elucidated by Barton and Scott in 1958 [9, 22]. It was reported about 16 mg/L by Bizukojc and Ledakovicz [8] and 74.3 mg/L by Bizukojc and Pecyna [23] from _A. terreus_ through lactose and glycerol as a medium. Abd Rahim et al. used a cheap industrial crude glycerol and reported about 58.9 mg/L in shake flask; they also studied the effects of viscosity, friction, and sonication and obtained about 56.9 mg/L geodin from _A. terreus_ [12, 24]. It is for the first time to study the effect of salt concentration on the yield of geodin 1 isolated from the soft coral-derived fungus _Aspergillus_ sp. and its yield was improved to multi-gram quantity through a cheap fermentation medium.

Broth media such as potato dextrose broth (PDB), glucose peptone yeast (GPY), Chashi, and Starch were used under shaking and unshaking conditions, containing a series of NaCl salt concentrations. However, no improvement in the yield of geodin was observed. Furthermore, rice medium having different concentrations of NaCl salt and supplements such as MgSO4, NaCOOCH3, FeSO4, CuCl2, and KCl were also studied. However, the optimal conditions for the high yield of geodin were found as rice medium with 2% NaCl salt, room temperature, static condition, and 3 weeks incubation period. NaCl salt enhances the biosynthesis of geodin, controlling the osmotic pressure. A high concentration of NaCl salt increases osmotic pressure and thus the fungal cells expand, and hence numerous cells rupture which results in low production of metabolite [12]. Moreover, Na⁺ ions are comparatively unreactive and are responsible for metabolite production [25]. The less amount of water in the medium may interfere

**Table 1: Comparison of experimental NMR data with the literature [21] for geodin 1.**

<table>
<thead>
<tr>
<th>Positions</th>
<th>Data from literature</th>
<th>Data from experiment</th>
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<tbody>
<tr>
<td>δC δH</td>
<td>δC δH</td>
<td></td>
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<tr>
<td>1</td>
<td>108.8</td>
<td>108.8</td>
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<tr>
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<tr>
<td>6</td>
<td>146.6</td>
<td>146.6</td>
</tr>
<tr>
<td>7</td>
<td>18.7</td>
<td>2.57 (3H, s)</td>
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<tr>
<td>8</td>
<td>193.3</td>
<td>193.2</td>
</tr>
<tr>
<td>1’</td>
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<td>84.5</td>
</tr>
<tr>
<td>2’</td>
<td>137.0</td>
<td>137.0</td>
</tr>
<tr>
<td>3’</td>
<td>137.5</td>
<td>7.15 (1H, d, 1.5)</td>
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<tr>
<td>4’</td>
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<td>185.0</td>
</tr>
<tr>
<td>5’</td>
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<td>5.83 (1H, d, 1.5)</td>
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<tr>
<td>6’</td>
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<td>167.9</td>
</tr>
<tr>
<td>7’</td>
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<td>8’</td>
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<td>163.4</td>
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<tr>
<td>9’</td>
<td>53.1</td>
<td>3.71 (3H, s)</td>
</tr>
</tbody>
</table>

δC, chemical shift of carbon; δH, chemical shift of proton; NMR, nuclear magnetic resonance. *Measured in deuterated chloroform (CDCl3).
with physiological functions such as increasing membrane permeability and producing enzymes in the fungal cell [26]. Similarly, as the fungal cells are delicate and therefore in a static condition, porous and less compact mediums such as rice give rise to a high yield of the respective metabolite.

The yield of compound 1 was 137.2 mg/L after 3 weeks incubation period at 25 °C at optimal fermentation medium in static condition (Figure 2), while its yield was 3.4 mg/L in broth media in a shake flask. The factor of incubation period was also studied and it was found that the yield of geodin 1 increases with time, but after 3 weeks the gradual decrease in yield occurred and disappeared after 2 months. Furthermore, in the absence of NaCl salt, no chlorinated compounds were produced as we had isolated previously [20]. Thus, NaCl salt played an important role in the production of chlorinated secondary metabolites produced by this Aspergillus sp. SYM-02-005. Similarly, the hypothetical biosynthetic pathway [27] for geodin 1 was also confirmed (Figure 3).

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Figure 2: The yield of geodin 1 at various concentrations of NaCl after 3 weeks of incubation at 25 °C.

Figure 3: Biosynthetic pathway of natural product geodin 1 [27].
Competing interests: The authors state no conflict of interest.
Informed consent: Informed consent was obtained from all individuals included in this study.
Ethical approval: The local Institutional Review Board deemed the study exempt from review.

References