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Taxonomy and Characterization of Bioactive Metabolites from a New Marine Microorganism Strain MLA-21

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Marine actinomyces MLA-21 was isolated from Arctic sediment samples. The strain was identified by the methods of morphology, physiological and chemotaxonomic characterization and 16S rRNA gene sequence analysis. The results showed that the strain was a new marine variable species of *S. coelicolour*. Two active compounds were isolated and purified from the fermentation of the strain and their antitumor activity against HepG-2, MCF-7 and A549 cell lines were defined by *vitro* cytotoxic activity assay. Their structures were elucidated as methyl *p*-hydroxybenzoate (**1**) and 3-(4-propylphenoxy)-propanoic acid (**2**) by ^{1}H and ^{13}C NMR and EI-MS spectroscopic methods. Compounds **1** and **2** were isolated from marine microorganism for the first time.

Key Words: Bioactive metabolite, Isolation, Cytotoxic assay, Strain MLA-21.

INTRODUCTION

Streptomyces are Gram-positive bacteria characterized by a complex morphologic differentiation cycle which is accompanied by production of numerous bioactive secondary metabolites with diverse structures and biological activities[1], nearly 75 % of commercially and medically useful antibiotics came from this genus[2]. This fact has made *Streptomyces* the most attractive bacteria in searching for active pharmaceutical compounds.

However, the rate finding new bioactive compounds from the common sources have been decreased[3]. More and more researchers turn to the uncommon actinomycetes living in extreme environments[4]. The exceptional living environment of arctic, such as low temperature, intensity UV radiation and seasonal disparity of the sunlight could possibly induce the evolution of distinct biosynthesis pathways in the microorganism to create some novel compounds with unique chemical structure[5].

In present work, we isolated and purified two compounds by the guidance of cytotoxic activity *in vitro* assay from the strain MLA-21, which was isolated from an arctic sediment sample and identified as a new marine variable species of *Streptomyces coelicolour*.

Both compounds are elucidated by NMR and EI-MS spectroscopic methods, exhibited conspicuous antitumor activity against HepG-2, MCF-7 and A549 cell lines and the relevant IC_{50} values were defined by MTT method.

EXPERIMENTAL

Isolation and cultivation of the strain MLA-21: The sediment sample was collected near the arctic yellow river station (78º55'N, 11º56'E) and was deposited in an incubator at -20 ºC. According to the propensity of the psychrophilic microorganism, the isolating work was taken at 20 ºC employing soil dilution technique on Gause's synthetic agar medium prepare with artificial seawater[6,7] (KNO_3 1.0 g/L, Soluble starch 20.0 g/L, K_2HPO_4 0.5 g/L, $MgSO_4$ 0.5 g/L, NaCl 0.5 g/L, $FeSO_4$ 0.01 g/L, Agar B 20.0 g/L, PH 7.2-7.4). The single colony picked from the primary culture was purified for 3-5 times and the primary strain was kept in slant cultures at -20 ºC for further study.

Phylogenetic analysis: The strain of MLA-21 was incubated in Gause's synthetic media on shaker (220 rpm) at 20 ºC for 7 days, then the genomic DNA of the strain was extracted according to the method described by Rainey *et al.*[8]. The 16S rDNA gene was amplified through PCR with the modify primers of fD1 and rD[19,10], which were forward (5'-CGG AGA GTT TGA TCC TGG CTC AG-3') and reverse (5'-AAA GGA GGT GAT CCA GCC GCA-3'). The amplification was performed with the program as: pre-degeneration at 94 °C for 2 min followed by 30 cycles of denaturation at 94 ºC for 45 s, annealing at 54 ºC for 90 s and elongation at 72 ºC for 2 min, then the final elongation at 72 ºC for 10 min. The PCR product was separated on 1 % agarose gel and purified with gel extraction kit.

A nucleotide sequence database search in GenBank was preformed to find out the related species of the strain MLA-21, then the sequences data were obtained and the nucleotide substitution rates (K_{nuc}) was calculated using BLAST program. The sequences were aligned *via* Clustal X 2.1 software[11]. The phylogenetic tree was was constructed using the neighbour-joining method[12] by MEGA 4.1 program[13], the statistical significance of the tree topology was evaluated using bootstrap analysis.

Cultural and physiological studies: The cultural characters of strain MLA-21 were observed on ISP[14] (international *Streptomyces* project) including seven different types of medium and two non-ISP medium, Wakesman medium No. 2 and Gause's synthetic medium[15]. The strain of MLA-21 was inoculated on these media and incubated for 7-15 days at 20 ºC and the plates were examined daily for growth condition.

The carbon sources utiliazation of the strain was determined by the method, which was modified from the original reported by Shirling and Gottlieb[14]. Some carbohydrates *e.g.*, mannose, D-fructose, D-lactose, D-glucose, maltose, sucrose, D-xylose, raffinose, L-arabinose, L-rhamnose) were added respectively at the concentration of 0.01 % as sole carbon sources to liquid minimal medium. 50 µL spore suspension (a loopful spore floated in 1 mL steriled isotonic sodium chloride) was inoculated to 50 mL medium respectively then incubated at 20 ºC for 7 days. The carbon sources utilization was measured by the weight of dry mycelium.

The strain's tolerance of NaCl, ability to produce H_2S and different enzymes and some other physiological characteristics of the isolate were examined according to the standard protocols[16,17].

Cell wall chemotype analysis: The cell wall composition analysis was carried out to definite the chemotype of strain MLA-21. The hydrolyzates of the whole cell was obtained follow the method reported by Lechevalier and Lechevalier[18]. Then the TLC method was taken to analysis the amino acid and sugar in the hydrolyzates by compare to the standard sugar amino acid solution[19].

Fermentation: A loopful spore of the strain MLA-21 was inoculated into a 2 L of seed medium (1 % glucose, 1.2 % tyrosine and 0.2 % yeast extract dissolving in artificial seawater, pH 7.2). After 96 h of incubation on rotary shaker (220 rpm) at 20 ºC, the seed culture (5 % v/v) was transferred to the Gause's synthetic medium.

The culture filtrates (20 L) was obtained by cultivation of the strain in fermentation tank Biostat C20-3 (B. Brawn Biotech International, USA) for 168 h under condition as follows: rotation speed: 100 rpm, gas fluent: 4.5 L/min and temperature: 20 ºC.

Cytotoxic assay: The cytotoxic activity was determined by MTT assay[20]. Three cancer cell lines: Hep-G2 (human hepatoma cell line), MCF-7 (human mammary cancer cell) and A549 (human lung cancer cell) were used as the test cells (The cell strains are kept in Department of Marine Pharmacy, China Pharmaceutical University). The cells were cultured at 5,000 cell pre well in 96-well plate with DMEM medium including 10 % FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL) incubated in a atmosphere of 5 % CO_2 at 37 ºC. The dried elusions of macro porous resin were dissolved in H_2O to the concentration of 100 µg/mL. When the monolayer formed, the samples were added into the medium (20 µL pre well) respectively. Cisplatin (10 µg/mL) and Gause's synthetic media were used as the positive and negative control and there were 6 paralleled control for each group. The OD_{570} was measured after 48 h.

Isolation and identification of bioactive metabolites: After 7 days of the fermenting process, the broth was collected and centrifuged. Then the culture filtrate (20 L) was concentrated in vacuum to a volume of 2 L and the residue was subjected to macro porous adsorption resin D101 ($m^2/g = 400$, Nanjing University) eluting with the gradient solvent system of H_2O: EtOH (H_2O, 9:1, 7:3, EtOH, v/v) to afford four fractions (Fr. 1-Fr. 4). Fractions (Fr. 2, Fr. 3 and Fr. 4) were concentrated and tested for their cytotoxic activity. According to the result, Fr. 4 showed obviously activity and selected for further study.

Fr. 4 was subjected to ODS reversed-phase liquid chromatography using a mixture solvent system of MeOH-H_2O to give four fractions: Fr. 4.1 (1:4, v/v), Fr. 4.2 (1:2), Fr. 4.3 (3:4) and Fr. 4.4 (MeOH). Then the Fr. 4.3 was further purified by preparative HPLC column (250 × 10 mm, 5 µm) with MeOH-H_2O (7:3) to afford compound **1** (Rt: 20 min, 5 mg). Compound **2** was purified from the Fr. 4.4 employing Sephadex LH-20 column chromatography. The structures of compound **1** and **2** were elucidated on the ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and mass spectroscopic data.

RESULTS AND DISCUSSION

Phylogenetic analysis: The almost complete 16S rRNA gene sequences of the strain was a continuous stretch of 1433 nucleotides and the data was submitted to GenBank database with the accession number of HQ848083. Non-redundant search result of BLAST came from GenBank, EMBL, DDBJ and PDB demonstrated that the strain belonged to the genus *Streptomyces* and the phylogenetic tree (Fig. 1.) constructed on the 16S rRNA sequences of related species indicated that the strain MLA-21 and the strain *S. coelicolour* A3 (**2**) has a close relationship.

Cultural characteristics of strain MLA-21: Through inoculated on ISP and non-ISP medium, the cultural characteristics strain MLA-21 was record in Table-1. The strain showed good growth on ISP-1, ISP-2, ISP-3, ISP-4, ISP-7 and Gause's synthetic agar medium, showed moderate growth on ISP-6 medium, while the strain can't grow on ISP-5 and Wakesman medium. The strain MLA-21 developed white to grey aerial mycelia, white to yellow vegetative mycelia and grey spores on all the tested media, especially on Gause's synthetic agar medium. However, there were no diffusible pigments produced on any of the media mentioned.

Biochemical characteristics of strain MLA-21: The results of the biochemical characteristics of strain MLA-21were record in Table-2. The whole cell hydrolyzates are I, L-DAP and glycin and the sugar composition of whole cell hydrolysates is glucose and the result indicated that the cell wall is type I, the sugar is type C and the chemotype is type IV[19]. Strain MLA-21 can produce protease, amylase, catalase, cellulase and melanoid pigments, also made the gelatin liquefaction., but cannot produce H_2S and peptonize milk peptone.

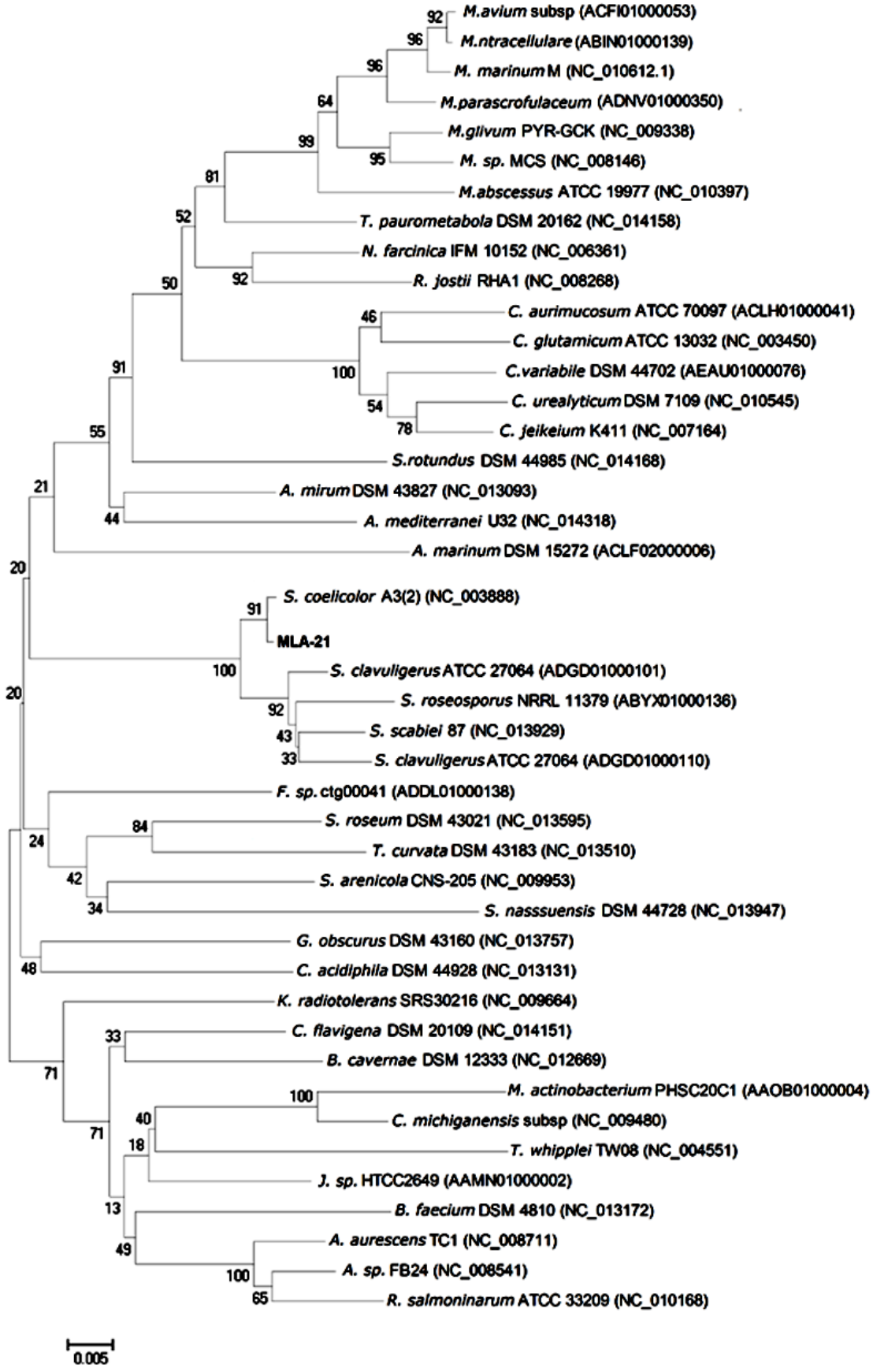

Fig. 1. Phylogenetic tree obtained by distance matrix analysis of 16S rRNA sequences and constructed using the neighbor-joining method, showing the phylogenetic position of strain MLA-21 among related species. Numbers on branch nodes are bootstrap values (1000 resamplings). Bar and 0.005 Knuc unit are shown at the branch points

TABLE-1
CULTURE CHARACTERISTICS OF STRAIN MLA-21

Medium	Growth condition	Vegetative mycelia	Aerial mycelia	Spores	Soluble pigment
ISP1	Good	Pale yellow	Ivory white	Pale grey	-
ISP2	Good	Yellow	White	Grey	-
ISP3	Good	Ivory yellow	Off-white	Pale grey	-
ISP4	Good	Off-white	White	Off-white	-
ISP5	-	-	-	-	-
ISP6	Moderate	Off-white	White	Pale grey	-
ISP7	Good	Pale yellow	Off-white	Grey	-
Wakesman medium No.2	-	-	-	-	-
Gause's synthetic agar medium	Good	Isabeling	Grey	Dark Grey	-

-, No data recorded

TABLE 2
PHYSIOLOGICAL PROPERTIES AND CHEMOTYPE OF STRAIN MLA-21

Test item	Result of MLA-21	*Streptomyces coelicolour* A3 (2)
Whole cell hydrolyzate	I, L-DAP and Glycin	I, L-DAP and Glycin
Whole cell sugar	C, Glucose	C, Glucose
Protease	+	+
Amylase	+	+
L-asparaginase	+	+
Catalase	+	+
Cellulase	+	+
Lipase	+	+
Nitrate reductase	+	+
Melanoid pigments	+	+
Liquefaction of gelatin	+	+
Peptonization of milk	+	+
Coagulation of milk	+	+
Production of H_2S	+	+
Mannose	+	+
D-Fructose	+	+
D-Lactose	+	+
D-Glucose	-	+
Maltose	+	+
Sucrose	+	+
D-Xylose	+	+
Raffinose	-	+
L-Arabinose	-	+
L-Rhamnose	+	+
Salt tolerance	≤14 %	+

+growth; - no growth

Strain MLA-21can utilizes L-rhamnose, D-lactose, D-fructose, D-xylose and sucrose, but cannot utilize D-glucose, raffinose and L-arabinose.

The phylogenetic analysis indicates that strain MLA-21 and strain *S. coelicolour* A3 (**2**) has a close relationship, but the biochemical characteristics of the two strains shows great differences. Strain MLA-21 can produce L-asparaginase, Lipase and nitrate reductase, can liquefy gelatin and exhibited a salt tolerance up to 14% while strain *S. coelicolour* A3 (**2**) cannot. The *S. coelicolour* A3 (**2**) can peptonize milk peptone, utilize D-glucose, raffinose and L-arabinose while strain MLA-21 cannot. On the bases of the biochemical characteristics and physiological characterizations and 16S rRNA sequence analysis, the strain MLA-21 was identified as a new marine variable species of *S. coelicolour*.

Cytotoxic assay: The cytotoxic activity of three elutions (Fr. 2, Fr. 3 and Fr. 4, 100 µg/mL) was measured by the inhibitory rate (IR), which was calculated as the follow equation:

$$\text{Inhibitor rate}\,(\%) = \left(1 - \frac{OD_{Sample} - OD_{Blank}}{OD_{Negtive} - OD_{Blank}}\right) \times 100\%$$

The result was shown in Fig. 2. The Fr. 4 showed good inhibitive active to these test cells. The inhibitory rate were 52.31 % to A549, 43.54 % to MCF-7 and 55.77 % to Hep-G2. IC_{50} values of the two compounds and positive control (Cisplatin) to Hep-G2, A549 and MCF-7 cell lines were determined as well, the results was shown in Table-3. It can be seen that two compounds showed favourable antitumor activity against the cells tested. Compound **1** is more effective compared to compound **2** with the IC_{50} of 2.27 µg/mL to Hep-G2, 6.32 µg/mL to MCF-7 and 14.86 µg/mL to A549 cell lines.

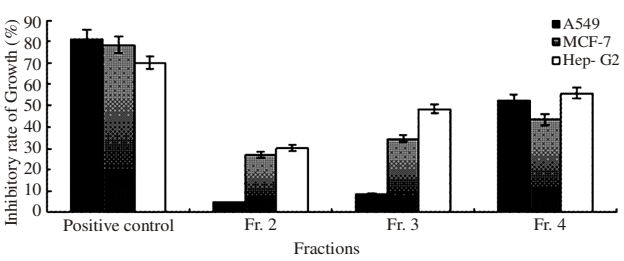

Fig. 2. Cytotoxic activity *in vitro* of three fractions (Fr. 2, Fr. 3, Fr. 4). The test concentration was 100 µg/mL with cisplatin (100 µg/mL) as the positive control (Data expressed as means ± standard error, n = 6)

TABLE-3
IC_{50} OF THE TWO ISOLATED COMPOUNDS

	IC_{50} (µg/mL)		
	Hep-G2	MCF-7	A549
Compound 1	2.27	6.32	14.86
Compound 2	5.94	16.87	26.85
Cisplatin[a]	0.36	2.71	2.27

[a]Positive control

Structure elucidation of the metabolites: The biological assay showed that the Fr. 4 of EtOH elution possessed a reasonable cytotoxic activity. Two compounds were separated from this fraction after several separation steps (Fig. 3).

Compound **1** (Fig. 4A.) was obtained as white amorphous powder. It was completely soluble in DMSO, acetone and slightly in hot water. EI-MS analysis of the compound gave a molecular ion at *m/z* 152.2 $[M]^+$. The ^1H NMR (DMSO, 500 MHz) spectrum of compound **1** showed 4 signals at 7.79 (2H, d, J = 8.6 Hz), 6.80 (2H, d, J = 8.6 Hz), 3.73 (3H, s) and 5.02 (1H, s), while ^{13}C NMR (DMSO, 125 MHz) depicted 6 signals at 166.08 (C-1'), 163.14 (C-1), 131.28 (C-5, C-3), 119.20 (C-4), 115.50 (C-2, C-6), 51.40 (C-2'). Based on the spectral data, bioactive compound **1** was identified as methyl *p*-hydroxybenzoate with the molecular formula $C_8H_8O_3$.

Compound **2** (Fig. 4B.) was found light yellow powder and it was completely soluble in $CHCl_3$ and MeOH, but

partially in water. In EI-MS analysis, the compound **2** exhibited a molecular ion at *m/z* 207.1 $[M]^+$. The ^1H NMR ($CDCl_3$, 500 MHz) spectrum of the compound depicted 8 signals at 7.07 (2H, d, J = 8.5 Hz), 6.76 (2H, d, J = 8.5 Hz), 4.96 (1H, s), 4.24 (2H, t, J = 7.1 Hz), 2.85 (2H, t, J = 7.1 Hz), 2.26 (2H, t, J = 7.45 Hz), 1.63 (2H, dt, J_1 = 7.45 Hz, J_2 = 7.4 Hz), 0.91(3H, t, J = 7.4 Hz) while ^{13}C NMR ($CDCl_3$, 125 MHz) inferred 9 signals at 173.73 (C-1'), 154.3 (C-1), 130.0 (C-3, C-5), 115.3 (C-2, C-6), 64.9 (C-3'), 36.2 (C-1"), 34.3 (C-2'), 22.6 (C-2") and 13.6 (C-3"). On the basis of the above spectral information, compound **2** was elucidated as 3-(4-propylphenoxy) propanoic acid with the molecular formula $C_{12}H_{16}O_3$.

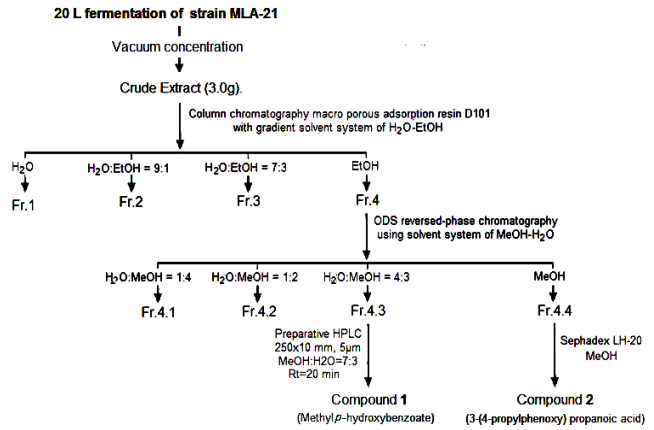

Fig. 3. Work-up scheme for the purification process of the bioactive metabolites from *Streptomyces sp*. MLA-21

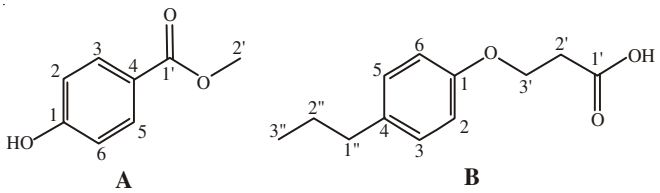

Fig. 4. Molecular structures of A: methyl-4-hydroxybenzoate (compound **1**), B: 3-(4-propylphenoxy) propanoic acid (compound **2**)

Conclusion

In this study, the strain MLA-21 isolated from arctic sediment has been identified as a new marine variable species of *S. coelicolour* based on the results of biochemical characteristics, physicological characterizations and 16S rRNA gene sequence analysis (accession No. HQ848083).

Two active compounds were isolated and purified from the fermentation of strain MLA-21, their structures were elucidated as methyl *p*-hydroxybenzoate (**1**) and 3-(4-propylphenoxy)-propanoic acid (**2**) by ^1H and ^{13}C NMR and EI-MS spectrometry. Compound **1** and **2** were firstly obtained from marine microorganism and displayed conspicuous antitumor activity against HepG-2, MCF-7 and A549.

Nevertheless, it has been reported by lots of works that several factors influence the secondary metabolites production[21,22]. In this case, we noticed that heating is inevitable in the separation process, so the stability of the broth under heating was tested as follows: Keep the broth in 70 ºC water bath for 3 h, then measure its inhibitory effect on Hep-G2. It was found that the inhibitory effect was still noticeable (IR % = 47.62 %). This result confirmed that the active component cannot be damaged during the heating process of the separation.

The two compounds obtained were both aromatic characterized by their common properties as low molecular weight and good membrane which make them exhibit a wide activity spectrum[23]. Some of these molecules have been found posses antitumor activity, inhibit micro bacterial growth and even promise therapeutic agents for AIDS[24]. Interestingly, a compound (4-(4-hydroxyphenoxy) butan-2-one) possessing a similar structure with compound **2** has been reported for its antibiosis activity[25]. Compound **1** and compound **2**, obtained from *Streptomyces sp*. MLA-21 showed an apparent cancer cell inhibitory activity without complicated chemical structures suggested the strain MLA-21 is still worth a further study for its biologic and pharmaceutical potentiality.

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