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EXOMETABOLITES OF STREPTOMYCETES ISOLATED FROM THE ODESA BAY EXHIBIT A TOXIC EFFECT AGAINST HUMAN CANCER CELL LINES

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The aim of the work was to determine the cytotoxic activity of marine actinobacteria exometabolites on the human larynx adenocarcinoma Hep-2 and rhabdomyosarcoma RD cell lines. Exometabolites of 6 strains of Streptomyces sp. were extracted with ethyl acetate and their composition was determined with UltiMate™ 3000 BioRS UPLC System coupled to mass spectrometer. Cytotoxic activity of exometabolites against cancer cells was determined after 24 hours of incubation by microscopic examination of cell morphological changes and the degree of cell monolayer degeneration. Exometabolites from Streptomyces sp. Myt7b strain showed the highest cytotoxic activity and at concentration of 500.0 μg/ml caused up to 90.0% death of RD and Hep-2 cells. The presence of 53 identified compounds with potential cytotoxic activity, including enterocin, 6-prenyltryptophol, medelamine A and turbinaric acid was detected in Streptomyces sp. Myt7b exometabolome. It is concluded that this strain is a promising producer of compounds with anticancer activity.

K e y w o r d s: marine streptomycetes, exometabolites, RD and Hep-2 cancer cells, cytotoxic activity.

Oncological diseases are the second most common spread cause of death after cardiovascular diseases in Europe and the USA. Despite significant efforts aimed at combating cancer, they remain one of the key problems for social medicine. The use of chemotherapy, one of the main methods of treating oncological diseases, is complicated by the severe side effects of chemotherapeutic drugs and the ability of many tumors to develop resistance to antitumor compounds [1, 2].

Soil microorganisms, primarily actinomycetes, are considered to be the most promising source of low molecular weight biologically active compounds, including antitumor ones. However, the gradual depletion of terrestrial biotopes as a source of new potential anticancer products, against the background of rather strict selection, which all potentially clinically significant compounds undergo, created the conditions for a comprehensive horizon expansion of the search for new compounds [3].

Marine microbiota are much older than land microbiota and are much less studied in all aspects. The study of the biological activity of low-molecular-weight substances of marine origin, in particular for antitumor and antimicrobial activity, can be called one of the main trends in applied biological research during the last two decades. The term “marine pharmacology” has even been proposed for this direction [4, 5].

The aim of the study was to determine the cytotoxic activity of exometabolites of Black Sea actinobacteria isolated from the biological fouling of seashells, concrete, and mussels, on the model of tumor cultures of RD and Hep-2 cells.

Materials and Methods

In the study, we used exometabolites of 6 Black Sea strains of actinobacteria isolated from the marine environment: *Streptomyces* sp. Myt5, *Streptomyces* sp. Myt7b, *Streptomyces* sp. Myt7w,

Streptomyces sp. Lim9.2, *Streptomyces* sp. Lim10, *Streptomyces* sp. Conc18.

To obtain exometabolites, cultures of actinobacteria were grown on Tryptic Soy Broth media (TSB, pancreatic hydrolyzed casein – 17.0; soy peptone – 3.0; sodium chloride – 5.0; potassium hydrogen phosphate – 2.5; glucose – 2.5 g/l) and Soy Glucose Broth (SG, glucose – 20.0; yeast extract – 5.0; soy peptone – 10.0; calcium carbonate – 2.0 g/l). At the first stage of cultivation, actinobacteria culture grown on a dense nutrient medium in Petri dishes was inoculated with one complete bacteriological loop to 15 ml of liquid sterile TSB nutrient medium in 100 ml flasks with glass balls and cultivated for 72 h at 28°C on a rotary shaker (Incubator Shaker Series INNOVA 40R, New Brunswick Scientific, USA) at 180 rpm.

At the second stage, 100 ml of liquid nutrient medium SG in 500 ml flasks with glass balls was inoculated with a 2 ml culture of actinobacteria grown in TSB medium. Cultivation was carried out at 28°C on a rotary shaker (Incubator Shaker Series INNOVA 40R, New Brunswick Scientific, USA) at 180 rpm for 7 days until the cultures reached the stationary phase of growth.

The culture liquid was separated from the cell mass of actinobacteria by preparative centrifugation (Labor Centrifuge Sigma 3-30K, Germany) at 10,000 g for 10 min at 18°C.

Exometabolites of actinobacteria were extracted from the culture liquid using ethyl acetate (J.T.Baker, Poland) in a ratio of 1:1. Extraction was carried out in a separatory funnel (SIMAX, Czech Republic) with a volume of 250 ml at room temperature on a horizontal shaker (GFL-3018, Germany) with light mixing at 110 rpm for 2 h.

After a clear layering, the extract was separated from the culture liquid using a separatory funnel, and the extractant was evaporated at 40°C in a stream of gaseous nitrogen of special purity 99.999% (INGAZ, Ukraine) under a pressure of no more than 2 psi using the Techne Sample Concentrator FSC 400D with DB 100/3 Dri-Block Heater (Cole-Parmer Ltd, United Kingdom). The mass of the concentrated and dried extract was determined on analytical scales (Pioneer Analytical Scales, Ohaus Corp. Pine Brook, NJ USA).

Stock solutions of extracted metabolites were prepared in dimethyl sulfoxide (DMSO) (Gaylord Chemical Corp., USA) at a concentration of 100 mg/ml. The use rates of solution of exometabolites were prepared in a nutrient medium for cell cultures DMEM (BioWest, France) at a concentration of 1 mg/ml. Prepared use rates of solutions of extracted metabolites were sterilized using membrane filters with a pore diameter of 0.22 μm (MILLEX GS Filter Unit with MF-Millipore MCE Membrane, MILLIPORE IRELAND Ltd).

The cytotoxic activity of exometabolites was studied on the model of a monolayer of transplanted cultures of malignant cells of human connective tissue – human rhabdomyosarcoma (RD) and tumor cells of the glandular epithelium of human laryngeal adenocarcinoma (Hep-2).

Cultures of Hep-2 and RD cells were plated in 48- and 96-well plates (4×10^4 cells per well) with 100 μl of DMEM medium (BioWest, France) supplemented with fetal bovine serum (FBS Premium) (BioWest, France) in the amount of 10% of the total volume of cell suspension in the well. Cell cultures were incubated at 36°C in a CO_2 incubator CCL-050T-8 (EscoMicroPteLtd, SINGAPORE) for 24 h.

After 24 h of cultivation, exometabolites of actinobacteria were added to each well in concentrations of 2.5; 25.0; 50.0; 100.0; 250.0 and 500.0 μg/ml. Intact cell cultures in DMEM medium and cell cultures in DMEM medium with the addition of DMSO in concentrations corresponding to the experimental ones – 0.5; 0.25; 0.1; 0.05; 0.025; 0.0025%, served as controls.

After 24 h of incubation, the culture medium was removed and the monolayer of cells was washed with 100 μl of Hanks solution (BioWest, France). Cells were fixed by adding 100 μl of 70% ethanol and incubated for 15 min at room temperature, then 100 μl of methylene blue dye (Genesis LLC, Ukraine) was added and incubated again for 15 min at room temperature. To remove excess dye, the tablet was washed three times with tap water and then incubated for 2 h at 37°C. The dye was eluted from the attached cells of the monolayer by adding 100 μl of 0.1 M HCl to each well and then incubated for 5 min at room temperature [6].

The cytotoxic effect of the exometabolites of marine bacteria was determined visually after 18-24 h of incubation by microscopic examination of the cell monolayer by morphological changes of individual cells and the degree of degeneration of the cell monolayer (Zeiss AxioScope A1 microscope, with a Zeiss Axiocam 503 color camera; Zen 2.0 microscope software; zoom 10x10). The degree of destruction of the monolayer was estimated by the

number of viable cells in the monolayer by the optical density indicator, which was measured using a spectrophotometer for microplates (μQuant™ Bio-Tek, USA) at 630 nm.

The degree of toxicity was assessed according to the following criteria:

- non-toxic – deviation of the optical density indicator from the control with DMSO <20%;

- the average level of toxicity – the deviation of the optical density indicator from the control with DMSO – from 21 to 50%;

- highly toxic – deviation of the optical density indicator from the control with DMSO >50%.

The composition of secondary metabolites was determined on a ThermoFischer Dionex UltiMate™ 3000 BioRS UPLC System coupled to a maXis II Bruker Daltonics mass spectrometer (4G hr-ToF). A Waters BEH C18 column (100 mm × 2.1 mm, 1.7 μm) was used at a temperature of 45°C and a flow rate of 0.5 ml/min. Solvents - A: 0.1% formic acid in water; - B: 100:20 acetonitrile + formic acid 0.1%. Mass detection took place in the range of 150-2000 m/z and 200-600 nm. The compounds were identified using the electronic version of "Dictionary of Natural Products" [7].

Mathematical processing of the obtained results was carried out using the Microsoft Office Excel-2016 program. Statistical indicators such as arithmetic mean (*M*) and standard measure of inaccuracy (*m*) were determined. The reliability of the differences between the average values was determined by the Student's *t*-test at a significance level of at least 95% ($P \leq 0.05$).

Results

Cytotoxic effect of exometabolites of Black Sea actinobacteria *Streptomyces* sp. Myt5, *Streptomyces* sp. Myt7b, *Streptomyces* sp. Myt7w, *Streptomyces* sp. Lim9.2, *Streptomyces* sp. Lim10, *Streptomyces* sp. Conc18 was determined on the model of transplanted cultures of human malignant cells - human rhabdomyosarcoma (RD) and human laryngeal adenocarcinoma (Hep-2) in the stationary phase of growth by indicators of morphological changes of cells, their death, and destruction of the monolayer. The results of the study are presented in Tables 1 and 2.

It was established that the secondary metabolites of the studied strains of *Streptomyces* sp. Myt5, *Streptomyces* sp. Myt7b, *Streptomyces* sp. Myt7w, at concentrations of 25.0-500.0 μg/ml, showed an acute cytotoxic effect on human RD rhabdomyosarcoma cell culture *in vitro.*

Already after 18 h of exposure, morphological changes of human RD rhabdomyosarcoma cells were detected – rounding of cells, vacuolization of cytoplasm, wrinkling and pyknosis of nucleus, destruction of the monolayer.

From the data presented in Table 1, it can be seen that exometabolites of strains of actinobacteria *Streptomyces* sp. Myt7b, *Streptomyces* sp. Myt5, *Streptomyces* sp. Myt7w were highly toxic to RD human rhabdomyosarcoma cell culture - at concentrations of 25.0-500.0 μg/ml, after 24 h of exposure, they caused the death of 49-88% of cells and almost complete destruction of the monolayer.

The level of cytotoxic effect of metabolites on RD cell culture depended on their concentration. So, for the secondary metabolites of *Streptomyces* sp. Myt7b, *Streptomyces* sp. Myt5, *Streptomyces* sp. Myt7w high level of cytotoxicity was inherent at concentrations from 25.0 to 500.0 μg/ml, average – for the lowest of the studied concentrations – 2.5 μg/ml.

It was established that in all studied concentrations (from 2.5 to 500.0 μg/ml) exometabolites of Black Sea strains of actinobacteria *Streptomyces* sp. Lim9.2 and *Streptomyces* sp. Lim10, and *Streptomyces* sp. Conc18 had a moderate level of cytotoxicity to a transplantable culture of human rhabdomyosarcoma cells RD. At the same time, the deviation from the control of the OD indicator of the monolayer after 24 h of exposure did not exceed 49%.

The results of the study of the effect of exometabolites of actinobacteria on the culture of Hep-2 cells according to the OD monolayer index are presented in Table 2.

From the presented data, it can be seen that exometabolites of the *Streptomyces* sp. strain showed an acute cytotoxic effect in the culture of Hep-2 human larynx adenocarcinoma cells in all tested concentrations after 24 h. Myt7b – the number of non-viable monolayer cells reached 70–90%.

Even at a minimum concentration of 2.5 μg/ml, the metabolites of this strain had pronounced antitumor activity – they caused the death of 53% of monolayer cells, unlike the *Streptomyces* sp. Myt7w, whose metabolites showed an acute cytotoxic effect on Hep-2 cell culture only at high concentrations from 100.0 to 500.0 μg/ml.

The subsequent metabolome analysis of *Streptomyces* sp. Myt7b, which showed the highest cyto-

Table 1. The effect of exometabolites of actinobacteria on cell viability in the RD culture monolayer according to the indicator of OD, % (exposure 24 h)*

Concentration of exometabolites, μg/ml	Viability of cells in the monolayer of the RD culture according to the OD indicator, *M*±m, % after incubation with exometabolites of different strains of *Streptomyces* sp.					
	Myt5	Myt7b	Myt7w	Lim9.2	Lim10	Conc18
2.5	69.6 ± 3.2	62.6 ± 4.3	59.6 ± 3.7	67.2 ± 1.9	62.8 ± 0.6	75.2 ± 6.1
25.0	36.3 ± 1.9	48.1 ± 1.2	31.0 ± 2.0	62.8 ± 4.2	63.7 ± 5.4	65.0 ± 2.5
50.0	26.9 ± 2.2	25.7 ± 1.1	27.5 ± 1.9	62.8 ± 2.7	62.8 ± 2.2	67.7 ± 2.7
100.0	25.7 ± 1.0	25.4 ± 0.9	25.4 ± 2.5	55.6 ± 3.0	61.1 ± 4.8	65.0 ± 1.9
250.0	25.1 ± 2.1	28.1 ± 2.2	29.8 ± 1.4	55.3 ± 4.1	51.3 ± 3.0	63.3 ± 3.4
500.0	18.1 ± 1.7	12.5 ± 1.0	15.3 ± 0.2	49.6 ± 1.2	51.7 ± 1.8	58.4 ± 2.8

Note: *as a percentage of viable cells to control with DMSO

Table 2. The influence of exometabolites of actinobacteria on the viability of cells in the monolayer of the Hep-2 culture according to the indicator of OD,% (exposure 24 h)*

Concentration of exometabolites, μg/ml	Viability of cells in the Hep-2 culture monolayer according to the OD indicator, *M*±*m*, % after incubation with exometabolites of different strains of *Streptomyces* sp.					
	Myt5	Myt7b	Myt7w	Lim9.2	Lim10	Conc18
2.5	94.3 ± 7.1	47.1 ± 1.5	99.7 ± 12.7	99.7 ± 10.3	89.7 ± 2.4	92.5 ± 6.1
25.0	62.1 ± 2.7	32.1 ± 1.7	62.9 ± 4.0	84.1 ± 2.4	77.4 ± 3.0	79.1 ± 4.3
50.0	41.0 ± 2.5	30.0 ± 2.2	62.1 ± 2.5	76.2 ± 3.1	60.5 ± 1.2	68.4 ± 2.6
100.0	38.6 ± 3.3	30.7 ± 3.0	33.6 ± 1.8	71.2 ± 3.2	64.9 ± 2.5	74.6 ± 3.5
250.0	31.1 ± 2.1	29.3 ± 2.3	31.4 ± 1.2	60.5 ± 1.6	60.5 ± 1.9	63.3 ± 1.5
500.0	31.4 ± 1.2	10.2 ± 0.9	23.6 ± 0.7	57.6 ± 2.9	56.5 ± 3.3	46.3 ± 4.2

Note: *as percentage of viable cells to DMSO control

toxic activity in both cell types at a concentration of 500.0 μg/ml, showed that their secondary metabolites included 89 compounds, of which 53 were identified. These compounds were detected singly (in one specimen) or in groups. Thus, groups of 2,5-diketopiperazines, germicidins, phenazines, and fatty acid derivatives were detected. The most represented group of metabolites are 2,5-diketopiperazines - cyclic dipeptides, most of which are unmodified or have minimal modifications. Diketopiperazines are synthesized by two different pathways, and the compounds detected in the culture liquid of the studied strain are most likely synthesized by tRNA-dependent cyclopeptide synthases. Some of these compounds have various biological activities and are even discovered for the first time for bacteria.

Other compounds are singletons, some of which (enterocin, 6-prenyltryptophol, medelamine A, and turbinaric acid) can provide the detected antitumor activity according to the cytotoxicity index of exometabolites of *Streptomyces* sp. Myt7b. In particular, its pronounced cytotoxic activity can be associated with four identified compounds - enterocin, 6-prenyltryptophol, medelamine A, and turbinaric acid, as they are the only compounds with such activity identified here. We cannot also exclude a role in the cytotoxic activity of *Streptomyces* sp. Myt7b of some unknown compound(s).

Enterocin is a rare example of a non-polyaromatic polyketide synthesized by type 2 PKS (type II polyketide synthetase). It has a wide range of biological activities, including cytotoxicity for HeLa and HepG2 cells (IC_{50} 0.41 and 0.83 mM, respectively) [8] (Fig.).

6-Prenyltryptophol (indole A), together with its analogs – indoles B and C, was discovered as a metabolite of *Streptomyces* sp. BL-49-58-005b, isolated from an unidentified marine hydrobiont. Indole

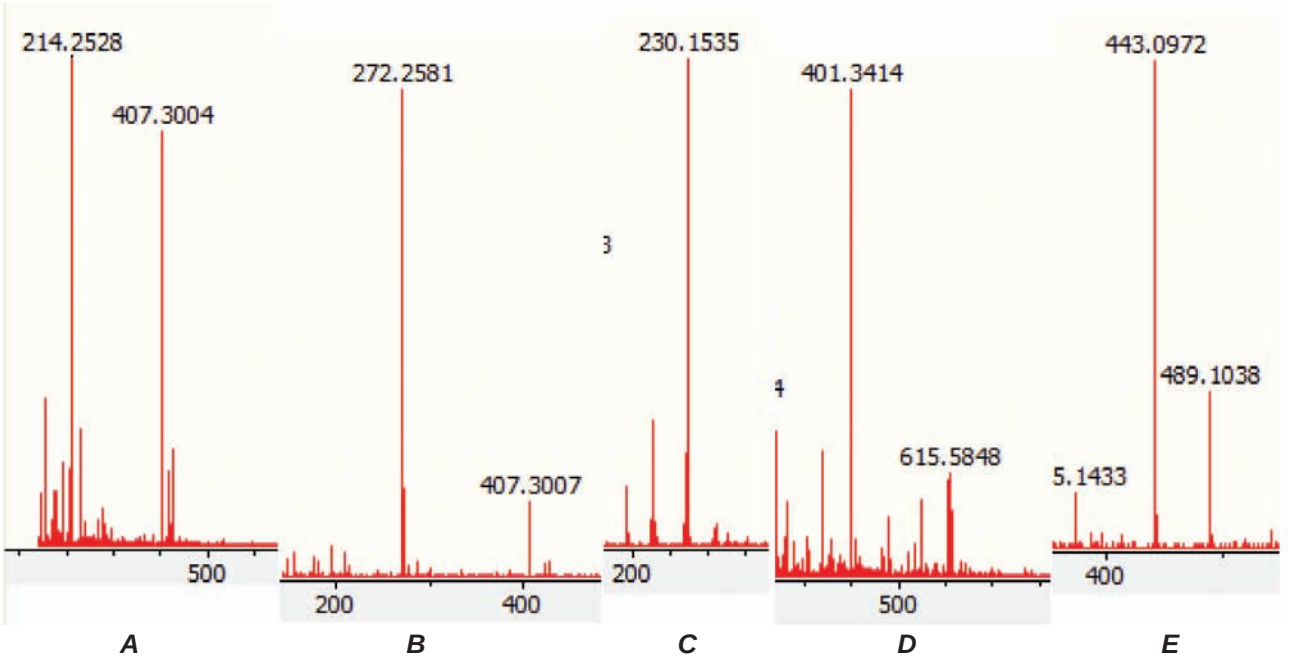

Fig. ESI-MS spectra of some of the identified compounds. ***A****: Medelamine A ([M+H]);* ***B****: Medelamine C ([M+H]);* ***C****: 6-Prenyltryptophol ([M+H]);* ***D****: Turbinaric acid ([M+H]); E: Enterocin ([M-H])*

A demonstrated activity against K-562 cells (IC_{50} 8.46 μM) [9]. In the exometabolome of *Streptomyces* sp. Myt7b indoles B and C were not detected.

Isomyristic acid derivatives, medelamines A and C, were discovered in the endophytic *Streptomyces* sp. YIM 66142. Medelamine A demonstrated cytotoxicity against rat kidney cells (IC_{50} 2.10 μg/ml), unlike medelamine C [10]. In the exometabolome of *Streptomyces* sp. Myt7b, in addition to protonated medelamine A, medelamine C was also detected, but no adduct was detected for it.

Turbinaric acid, which is a derivative of secosqualene, was isolated from the brown alga *Turbinaria ornata* and the zygomycete *Phycomyces blakesleeanus.* It is an inhibitor of squalene-2,3-epoxide cyclase and exhibits cytotoxic properties against mouse melanoma and human colon carcinoma cells at concentrations of 26.6 and 12.5 μg/ml, respectively [11].

Based on the above, it is possible to postulate the originality of the biosynthetic potential of the strain *Streptomyces* sp. Myt7b, in particular, its cytotoxic activity, which may be related to the identified compounds: enterocin, 6-prenyltryptophol, medelamine A and turbinaric acid.

Discussion

In this study, we tested the inhibitory ability against two types of cancer cells, RD and Hep-2, of six different strains of actinobacteria that were isolated from the Bay of Odesa. The results showed strong cytotoxic activity of exometabolites of *Streptomyces* sp. Myt7b, *Streptomyces* sp. Myt5, *Streptomyces* sp. Myt7w on RD and Hep-2 cells, the effect was dose-dependent. Exometabolites of strains of *Streptomyces* sp. Lim9.2, *Streptomyces* sp. Lim10, *Streptomyces* sp. Conc18 showed only a moderate effect and killed up to 37 to 48% of cancer cells at concentrations of 25.0-500.0 μg/ml. In general, exometabolites from strains of *Streptomyces* sp. Myt7b, *Streptomyces* sp. Myt5, *Streptomyces* sp. Myt7w were stronger in inhibiting cells than *Streptomyces* sp. Lim9.2, *Streptomyces* sp. Lim10 and *Streptomyces* sp. Conc18. It is important to note that *Streptomyces* sp. Myt7b showed the most promising results and caused up to 90.0% death of RD and Hep-2 cells at a concentration of 500.0 μg/ml. For the most promising strain, we performed a metabolomic analysis that revealed 89 secondary metabolites, of which 53 were identified, including enterocin, 6-prenyltryptophol, medelamine A, and turbinaric acid.

Thus, the results of research conducted on transplanted cultures of malignant cells RD and Hep-2 confirmed the data of metabolomic analysis of actinobacteria. In addition, the results of our research coincide with literature data, which indicate that compounds with cytostatic activity are found in representatives of many groups of prokaryotes - not only in Cyanobacteriota and Bacillota (*Bacillaceae*, *Thermoactinomycetaceae*) but also in Actinomycetota [12, 13].

Cytotoxic compounds of microorganisms of marine origin are extremely diverse in chemical structure. Among them are known alkaloids, terpenes, amino carbohydrates, polyketides, non-ribosomal peptides, and nucleoside compounds, etc. [14, 15]. Several metabolites of representatives of marine microbiota are unique in their structure. Non-ribosomal peptides with oxazole cycles in the structure of the molecule - mechercharmycine - were found in Bacillota of the genus *Thermoactinomyces*, and their ability to inhibit lung cancer was noted [16]. In the strain *Streptomyces* sp. KMM 9048 revealed specific variants of antitumor antibiotics of the aureolic acid group [17].

In a representative from the group of so-called "rare actinomycetes" discovered new cytotoxic bacteriocins from the group of thiopeptides – litoralimycins [18]. Representatives of the genus *Salinispora*, ind

igenous to the sea, have known polyketide compounds of the salinisporamide group, which are capable of antitumor action through specific inhibition of the proteasome [19]. Polyketides of the manumycin group, produced by marine representatives of the genus *Streptomyces*, have revealed an extremely rare mechanism of action based on the type of "molecular glue". They act by forming a covalent connection (bridge) between UBR7 ligase molecules and an abnormal variant of the TP53 protein characteristic of breast cancer cells, leading to tumor cell apoptosis [20].

Thus, experimental research of metabolites of Black Sea actinobacteria *Streptomyces* sp. Myt5, *Streptomyces* sp. Myt7b, *Streptomyces* sp. Myt7w, *Streptomyces* sp. Lim9.2 and *Streptomyces* sp. Lim10 on the model of transplanted cultures of human malignant cells – connective tissue – human rhabdomyosarcoma RD and glandular epithelial cells - adenocarcinoma of the human larynx Hep-2 showed high cytotoxic activity, the strain *Streptomyces* sp. Myt7b is a promising producer of antitumor compounds and can be recommended for more in-depth further studies *in vivo.*

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ЕКЗОМЕТАБОЛІТИ СТРЕПТОМІЦЕТІВ, ІЗОЛЬОВАНИХ ІЗ ОДЕСЬКОЇ ЗАТОКИ, ЩО ПРОЯВЛЯЮТЬ ТОКСИЧНУ ДІЮ ПРОТИ РАКОВИХ КЛІТИННИХ ЛІНІЙ ЛЮДИНИ

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Метою роботи було визначення цитотоксичної активності екзометаболітів морських актинобактерій на клітинних лініях аденокарциноми гортані людини Hep-2 і рабдоміосаркоми RD. Екзометаболіти 6 штамів *Streptomyces* sp. екстрагували етилацетатом і визначали їх склад за допомогою системи UltiMate™ 3000 BioRSUPLC, з'єднаної з масспектрометром. Цитотоксичну активність екзометаболітів по відношенню до ракових клітин визначали після 24 год інкубації методом мікроскопічного дослідження морфологічних змін клітин і дегенерації клітинного моношару.

Екзометаболіти штаму *Streptomyces* sp. Myt7b показали найвищу цитотоксичну активність і за концентрації 500,0 мкг/мл спричиняли загибель до 90,0% RD та Hep-2 клітин. В екзометаболомі штаму *Streptomyces* sp. Myt7b виявлено 53 ідентифіковані сполуки з потенційною цитотоксичною активністю, включаючи ентероцин, 6-пренілтриптофол, меделамін А та турбінарову кислоту. Зроблено висновок, що цей штам є перспективним продуцентом сполук із протипухлинною активністю.

Ключові слова: морські стрептоміцети, екзометаболіти, культури ракових клітин людини RD та Hep-2, цитотоксична активність.

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