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PHYSICAL-CHEMICAL AND TOXIGENIC CHARACTERISTICS OF THE NEW METABOLITES FROM *ULOCLADIUM CONSORTIALE* 960

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Fungi as producers of biologically active metabolites were and remain of particular interest of the investigators. As the same time, despite the large number of different investigations and numerous scientific publications a major part of described species aren't studied at that aspect. The data concerning biosynthetic potential of fungi of Ulocladium genus are practically absent in literature. However the screening carried out by us early showed that U. consortiale 960 possess the wide spectrum of antibiotic activity. **Aim.** *This investigation became the follow-up of our previous studies and its main task was isolation, purification of U. consortiale 960 active metabolite(s) and obtaining of them in crystalline state for possible determination of structure.* **Methods.** *For obtaining the biologically active metabolites column chromatography and recrystallization were used; characterisation of obtained substances were made by physical-chemical and microbiological methods.* **Results.** *As a result of work done two compounds in crystalline form with different spectrum of antibiotic activity against indicator test cultures were obtained. Neither of obtained compounds showed dermatocidal and toxigenic activity in rabbit skin test. From the obtained spectral characteristics of substance UC960 it may be concluded that it belongs to aromatic compounds with benzene ring, has double linkage, methyl- and carboxyl groups.* **Conclusions.** *Obtained data showed that antibiotic nature of strain 960 is determined by the variety of biologically active metabolites with different biological and physical-chemical properties. The presented results and the analysis of literature data show that U. consortiale 960 possibly can produce the new for Ulocladium genus biologically active metabolites, which require further investigations.*

Keywords: Ulocladium, Ulocladium consortiale, antibiotic activity, metabolites.

Fungi as producers of biologically active metabolites such as ferments, antibiotics, toxins, vitamins, growth factors and others were and remain of particular interest of the investigators. As the same time despite the large number of different investigations and numerous scientific publications a major part of described species are not studied at that aspect. Considering this fact active search of new biologically active metabolites are of one of the priority directions of not only fundamental but applied science and filamentous fungi are one of the most perspective groups of producers.

The comprehensive screening of biological activity of more than 300 fungal strains belonging to genera *Aspergillus, Penicillium, Paecilomyces, Alternaria, Ulocladium, Gliocladium, Nectria, Eupenicillium, Bipolaris, Tritirahium, Myrothecium, Beauvenaria, Acremonium, Botriodiplodia, Cephaliophora* had been carried out by us earlier. These strains were isolated from different ecological niches including extreme such as radionuclide polluted soil and Chernobyl zone "yellow" forest leaf litter, saline black soil, mountain regions

of West Pamir and others [1-3]. The results of screening showed different ability of studied fungi to synthesize biological active metabolites. Our attention was attracted to strains of the species which biological potential is poorly known and there are no any data in available literature.

The situation described above concerns fungi of *Ulocladium* Preuss genus which phylogenetically related to *Alternaria* Nees [4, 5]. These fungi genera are mostly found as common saprobes on plant materials, in soil and airs as well as they are known as plant pathogens [6, 7]. Certain unidentified strains are capable to growth on such technogenic substrate as polypropylene, rubber, fluorine plastics [8, 9], possess pectinase, cellulase, glucanase, and non-specific lipase activities [10, 11] and can cause diseases in humans [12]. Fungi of *Ulocladium* genus are used as biocontrol agents against plant pathogenic fungi of *Botrytis* genus which cause diseases of number of important agricultural plants [11, 13], as well as experimental models in investigations of microgravitation influence on growth and development of filamentous fungi under conditions of minimal quantities of nutrients [14].

In the available scientific literature there are almost no information concerning biosynthetic potential of *Ulocladium* genus with the exception of one article by B. Andersen and M. Hollensted (2008) in which the describe the results of systemic investigations of metabolite production by 52 strains of different *Ulocladium* species [15]. The authors had been shown that majority of studied *U. arborescens*, *U. chartarum*, *U. cucurbitae*, *U. dauci*, *U. multiforme* and *U. oudemansii* strains can produce infectopyrones and derivatives of altertoxin I while *U. atrum* strains – curvularin and dehydrocurvularin.

It was also reported earlier that unidentified *Ulocladium* strains can synthesize piperine, terpestacin and tenuazonic acid [16], *U. botrytis* and *U. chartarum* starins –ulocladol [17, 18], and one *U. consortiale* strain formed infectopyrone [19]. *Ulocladium* sp. BPS7 isolated from anthropogenic extreme environment (carwash) produced 10-undecen-1-ol [20]. This compound has previously been identified as a constituent of the plant *Machilus zuihoensis* [21]; the essential oils of this plant are known to possess antimicrobial activity.

Among 10 of studied by us strains *U. consortiale* 960 was found to be the most active [2, 22]. It possessed the wide spectrum of antibiotic activity against Gram positive bacteria (*Bacillus subtilis*, *B. licheniformis*, *Staphylococcus aureus*, *Micrococcus varians*, *M. flavus*), Gram negative bacteria (*Escherichia coli*, *Pseudomonas syringae*, *Pectobacterium carotovorum*) and yeasts (*Candida albicans*, *Kluyveromyces marxianus*, *Trichosporon cutaneum*). At the same time, it showed no activity against filamentous test fungi (*Botrytis cinerea*, *Rhizoctonia solani*, *Phoma betae*, *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *Fusarium lactis*, *F. oxysporum*). Thus, this investigation became the follow-up of our previous studies and its main task was the isolation, the purification of *U. consortiale* 960 active metabolite(s) and the obtaining of them in crystalline state for possible determination of their structure.

Materials and Methods. In investigations we used cultural filtrate of *U. consortiale* 960 obtained after static cultivation during 14 days at 26 °C on liquid modified Czapek-Dox medium containing (g/l): glucose – 20.0; $NaNO_3$ – 1.0; KH_2PO_4 – 1.0; KCl – 0.5; $MgSO_4 \times 7H_2O$ – 0.5; $FeSO_4 \times 7H_2O$ – 0.01 in distilled water; pH 5.2 [23]. The conventional multistep method

we applied for isolation of active metabolites involved fitting of extraction methods (combined chromatography), actually extraction and accumulation of obtained substances, primary purification of extract from protein, lipid and pigment foreign impurities, fractionation by column chromatography and recrystallization of active substances.

Fitting the extraction conditions of active metabolites using chloroform was carried out proceeding from the data of combined chromatography by Schevchik [24]. After three times extraction in the 1:4 ratio during 15 minutes and evaporation in a rotary vacuum concentrator (Reanal, Hungary) to 1/10 part by volume obtained residue was purified from impurities of protein nature by precipitation with lead acetate; from non-polar lipid impurities – by liquid-liquid disproportionation in acetonitrile-n-butanol-hexane system; from pigments – by column chromatography with activated carbon BAU-1 as sorbent and methanol as mobile phase. All of these procedures were followed by dehydratation with anhydrous sodium sulphate [25]. Obtained partially purified extract was evaporated and fractionated by column chromatography.

For column chromatography silica gel L of II grade of activity by Brockman, 100-160 μm particle size (Lachema, Czech Republic) was used as sorbent. As mobile phase single solvents and their systems were used in order of increasing polarity: n-hexane → n-hexane-chloroform (5:1) → chloroform → chloroform-acetone (5:1) → chloroform-acetone (9:1) → acetone → acetone-acetonitrile (5:1) → acetonitrile → water. Volume of collected fractions was 50 ml and flow rate – 0.5 ml/min. The contents of dry substances were measured by weighing of 5 ml of each fraction after drying under 105 ºC to constant weight.

In obtained fractions the presence of active metabolites was checked by thin layer chromatography (TLC) and their activity against indicator species (*Bacillus subtilis* 617 and *Kluyveromyces marxianus* 899) was determined by agar disc diffusion assay methods (10.0 μl/disk) [26]. For TLC 10 μl of specimen was applied to Silufol UV254 plates, (Kavallier, Czech Republic); as mobile phase solvent systems of benzol-ethyl acetate-methanol (5:5:1) and toluol-isopropanol-formic acid (6:3:1) were used. Visualization of spots on the plates was carried out in iodine saturated developing tank [27]. Combined active fractions were evaporated *in vacuo* at 50 ºC and active substances underwent the recrystallization from benzol. Such methodological approach allowed obtaining of homogeneous substance which visualized on chromatogram as single spot.

Physical-chemical and spectrum characteristics were measured by conventional methods. So, the elemental analysis was determined by Cheronis sodium fusion method [28-30], spectrum characteristics were obtained by spectrophotometers Specord and UR-10 (Germany) in UV-, visible light and IR-spectral ranges [31].

Rabbit skin test was used for assess of toxicity of obtained crystallized preparation [32]. The animals, their general state and skin health were under constantly observation for 5 days.

Results. Selection of chloroform according the data of combined chromatography by Schevchik as suitable extractant suggests that active agent can be moderately-polar. Initial preparation possessed high antibiotic activity against Gram positive test bacteria, moderate activity against yeast test cultures and

low activity against Gram negative test bacteria. No activity was showed against filamentous test fungi [22].

Fig. 1 shows the column chromatography elution profile of *U. consortiale* 960 metabolites. Column was fed with solvent systems in the next order: fraction 10 – n-hexane; fractions 20-50 – n-hexane-chloroform (5:1-1:1); fractions 61-64 – chloroform; fractions 71-73 – chloroform-methanol (9:1), fractions 74-82 – chloroform-methanol (4:1). As can be seem from Fig.1 only fractions 71-73 and 81-82 exhibited activity against test cultures.

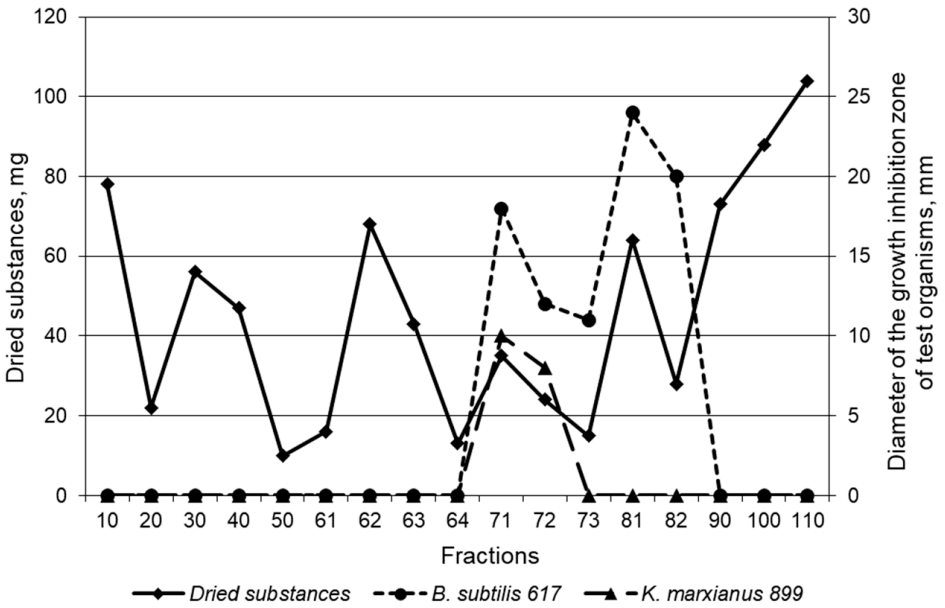
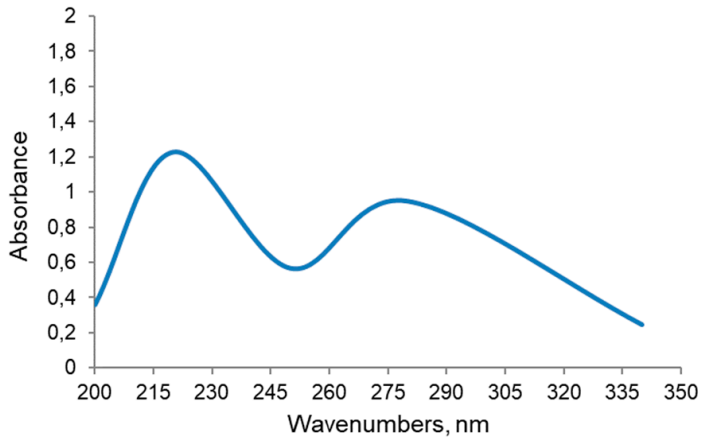


Fig. 1. Elution profile of preparation from *U. consortiale* 960

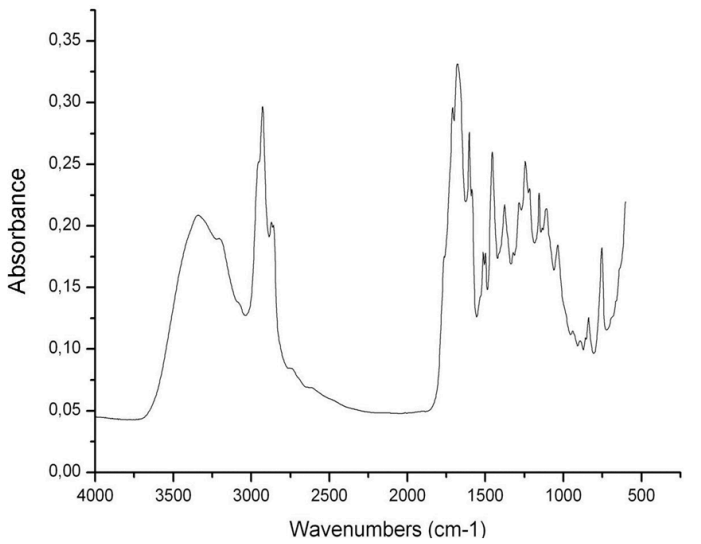
Fractions 71-73 (chloroform-methanol, 9:1) showed the activity against *B. subtilis* 617 and *K. marxianus* 899, possessed identical chromatographic mobility and was represented by three spots with Rf 0.36; 0.57 and 0.82 in toluol-chloroform-acetone (10:10:3) system. Obtained after evaporation preparation displayed antibiotic activity against studied test cultures but didn't show any reaction in rabbit skin test.

Rechromatography of combined 71-73 fractions with using solvent systems chloroform-methanol (98:2) → chloroform-methanol (95:5) → chloroform-methanol (9:1) → chloroform-methanol (4:1) fixed the output of active substance in fractions 1-2, which possessed identical parameters of chromatographic mobility and biological activity. For additional purification active substance underwent recrystallization from benzole and assigned conventional name UC960.

Measurement of UC960 spectrum characteristic in UV- and visible light spectral ranges (fig. 2) displayed the presence of two distinct absorption maxima in UV-rang at wavelengths 220 and 282 nm. When these data were compared with tabular data of absorption maxima of main classes of organic compounds we suggested that molecule of UC960 may contain α,β-unsaturated carboxylic acid, nitrogen, acyclic diene and mononuclear benzol derivate [33].



a



b

Fig. 2. Absorption spectrum of UC960 preparation: UV-absorption; b) IR-absorption

In visible light spectral range there was no absorption maxima on which may also pointed the absence of coloration of sample. Absorption maxima in IR-spectral range testify that structure of molecule contains double linkages, carboxylic acid derivate and conjugated with benzene ring methyl group. However, the elemental analysis of studied substance showed the lack of nitrogen, sulphur and halogens, but the presence of main elements of organic compounds – carbon, hydrogen and oxygen. So, on the base of obtained data we can conclude that obtained substance belong to aromatic compounds with benzene ring, double linkage, methyl and carboxyl groups. Determination of exact chemical structure needs additional investigations.

Fractions 81-82 (chloroform-methanol, 5:1) possessed antibiotic activity only against *B. subtilis* 617 and showed two spots on thin layer plate with Rf 0.55 and 0.90 (solvent system: benzol-chloroform-methanol, 5:2:1). Obtained after evaporation preparation also showed the absence of reaction in rabbit skin test.

Discussion. This stage of our investigation was dedicated to isolation of biologically active metabolites of *U. consortiale* 960 in purified form, which in previous studies possessed the antibacterial, antifungal and phytotoxic activities [2, 22]. As a result of work done two compounds in crystalline form with different spectrum of antibiotic activity against indicator test cultures were obtained.

On the base of studied physical-chemical and spectral characteristics of UC960 compound we can state the presence of certain chemical groups in its structure but on this step it is impossible to describe the whole structure of the molecule. Comparison of our results with literature data concerning biologically active metabolites of *Ulocladium* genus allows to suggest that obtained metabolite looks like infectopyrone, the potential mycotoxin first isolated from *Alternaria infectoria* [19]. UC960 contains in its structure the same groups but unlike infectopyrone aromatic part of the molecule is represented by benzene but not pyrone ring. Interestingly, that the fact of biosynthesis of infectopyrone by *U. consortiale* was mentioned in the above article in which these compounds were characterized as potential mycotoxins. In our case neither of obtained by us compounds showed even hyperemia in rabbit skin test and thus they didn't possess dermatocidal activity and toxigenic properties.

Presented above results testify that antibiotic potential of *U. consortiale* 960 as well as other *Ulocladium* species needs more in-depth study. Obtained data showed that antibiotic properties of strain 960 depend on whole complex of biologically active metabolites differing from each other by biological and physical-chemical properties. The necessity of further investigation of *U. consortiale* metabolites is no doubt because they can answer the questions concerning the chemical nature of these substances and mechanisms of their biological activity.

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ФІЗИКО-ХІМІЧНІ ТА ТОКСИГЕННІ ВЛАСТИВОСТІ НОВИХ МЕТАБОЛІТІВ *ULOCLADIUM CONSORTIALE* 960

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Резюме

Інтерес вчених до грибів-продуцентів біологічно активних метаболітів постійно зростає. Але, незважаючи на завелику кількість проведених та опублікованих досліджень, багато раніше визначених видів залишаються поза увагою вчених. Так, в літературі практично немає відомостей про біосинтетичний потенціал грибів р. *Ulocladium*, тоді як здійснений нами скринінг показав антибіотичну активність широкого спектра дії у штаму *U. consortiale* 960. **Метою** цієї роботи було виділення, очищення та отримання в кристалічному вигляді активного метаболіту(ів) гриба *U. consortiale* 960. **Методи.** Для отримання біологічно активних метаболітів застосовували екстракцію з подальшим розділенням за допомогою колонкової хроматографії та перекристалізації; для охарактеризування властивостей отриманих препаратів використовували фізико-хімічні і мікробіологічні тести. **Результати.** Було отримано в кристалічному вигляді дві сполуки, які проявляли антибіотичну активність щодо індикаторних тест-культур різного спектру дії. Жодна з отриманих сполук не мала дерматоцидної дії в шкірній пробі на кролику і не проявляла токсигенних властивостей. Спектральні характеристики сполуки UC960 з широким спектром антибіотичної дії вказують на ароматичний характер цієї сполуки, що має в структурі бензольне кільце, подвійний зв'язок, метильну та карбоксильну групи. **Висновки.** Отримані дані показали, що антибіотичні властивості досліджуваного штаму обумовлені комплексом біологічно активних метаболітів, що вирізняються біологічними та фізико-хімічними властивостями. Представлені результати та порівняльний аналіз літературних джерел допускають можливість біосинтезу штамом *U. consortiale* 960 нових для р. *Ulocladium* біологічно активних метаболітів, що потребує додаткових досліджень.

Ключові слова: Ulocladium, *Ulocladium consortiale*, антибіотична активність, метаболіти.

ФИЗИКО-ХИМИЧЕСКИЕ И ТОКСИГЕННЫЕ СВОЙСТВА НОВЫХ МЕТАБОЛИТОВ *ULOCLADIUM CONSORTIALE* 960

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Резюме

Интерес к грибам как продуцентам биологически активных метаболитов не угасает и по сей день. Но, несмотря на огромное количество проведенных исследований и опубликованных работ, значительная часть ранее описанных видов не достаточно изучена в этом направлении. В литературе практически отсутствуют сведения о биосинтетическом потенциале грибов р. *Ulocladium*, в то же время проведенный нами скрининг показал антибиотическую активность широкого спектра действия штамма *U. consortiale* 960. **Целью** данной работы предусматривалось выделение, очистка и

получение в кристаллическом виде активного метаболита(ов) гриба *U. consortiale* 960. **Методы.** Для получения биологически активных метаболитов использовали экстракцию с последующей колоночной хроматографией и перекристаллизацией; для описания свойств полученных препаратов использовали физико-химические и микробиологические тесты. **Результаты.** В результате работы в кристаллическом виде были получены два соединения, которые проявляли антибиотическую активность разного спектра действия по отношению к индикаторным тест-культурам. Ни одно из выделенных соединений в кожной пробе на кролике не проявляло дерматоцидного действия и токсигенных свойств. Спектральные характеристики соединения UC960 с широким спектром антибиотической активности указали на ароматический характер этого соединения, в структуре которого присутствуют бензольное кольцо, двойная связь, метильная и карбоксильная группы. **Выводы.** Полученные данные показали, что антибиотические свойства исследованного штамма обусловлены целым комплексом биологически активных метаболитов, которые отличаются биологическими и физико-химическими свойствами. Представленные результаты и анализ литературных данных показывают возможность биосинтеза штаммом *U. consortiale* 960 новых для р. *Ulocladium* биологически активных метаболитов, что требует дальнейших исследований.

Ключевые слова: Ulocladium, *Ulocladium consortiale*, антибиотическая активность, метаболиты.

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