UDC 582.282.195.2:579.222
DOI: 10.17721/1728.2748.2023.94.5-9

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EFFECT OF EXTRACTION SOLVENTS ON THE PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY IN *XYLARIA POLYMORPHA* AND *XYLARIA LONGIPES* STRAINS

Background. *Fungi of the genus Xylaria are known for their wide range of secondary metabolites, including antioxidant polyphenolic compounds. Due to their critical role as chain-breaking antioxidants, phenolic compounds have been studied extensively in plants and fungi. The objective of this study was to assess and compare the total phenolic content and antioxidant capacities of the biomass of different strains of two common representatives of this genus in Ukraine – Xylaria polymorpha and Xylaria longipes – by applying various solvents for extraction.*

Methods. *A vegetative mycelium was cultivated in submerged conditions and extracted with ethanol, methanol, and ethyl acetate. Total phenolic content in extracts was determined using the Folin–Ciocalteu method with gallic acid as the standard. Antioxidant activity was assessed by determining the DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging spectrophotometric assay. The correlation between the total phenolic content and antioxidant capacity of the extracts was assessed using the Pearson correlation coefficient for each organic solvent.*

Results. *Methanol was the most effective solvent in yielding the highest quantities of phenolic compounds. Among all the strains studied, X. polymorpha IBK 2736 exhibited the maximum yield of phenolic compounds at 21,64 ± 0,03 mg GAE/g. Conversely, when ethyl acetate was used as the solvent, the phenolic yield from the biomass of the same strain was significantly lower at 0,68 ± 0,14 mg GAE/g, as well as for other strains in our investigation. Furthermore, the methanol extract of X. longipes IBK 2726 demonstrated the highest antioxidant activity, reaching 88,99 ± 0,07 %, while the ethyl acetate extract of X. longipes IBK 2718 exhibited the lowest antioxidant activity with a value of 41,28 ± 0,33 %.*

Conclusions. *The results indicated that the amount of extracted phenolic compounds was greatly influenced by the choice of solvent. Methanol was found to be the most effective solvent for extracting these compounds from studied strains, outperforming ethanol and ethyl acetate. Moreover, methanol extracts displayed a strong antioxidant capacity, and the correlation analysis confirmed the relationship between it and the phenolic content present in them. Overall, all the strains investigated showed significant antioxidant potential, highlighting the importance of further studies of the chemical properties of their antioxidant components.*

Keywords: *polyphenols, antioxidant activity, Xylaria, Ascomycota, submerged cultivation, biomass.*

Background

The worldwide exploration of fungi as a source of novel active bioproducts is ongoing and remains relevant because of their potential in biotechnological applications. Fungi of the genus *Xylaria* Hill ex Schrank are among those that researchers are continuously screening for new bioactive compounds since these produce a range of secondary metabolites with different properties (Chen et al., 2018; Song et al., 2014). Among them, phenolic constituents are of particular interest because of their remarkable potential for free radicals scavenging and thus reducing oxidative stress (Mathew, Abraham, & Zakaria, 2015). Phenolic derivatives obtained from the fruiting bodies of xylariaceous species include tyrosol from *Xylaria longipes* Nitschke (Schneider, Anke, & Sterner, 1996), xylarinols from *Xylaria polymorpha* (Pers.) Grev. (Lee et al., 2009), globoscin from *Xylaria globosa* (Pers.) Mont. (Adeboya et al., 2010) etc.

Moreover, it has been reported that the content of polysaccharides and phenolic compounds in plants and fungi correlates with antioxidant activity (Cheung, Cheung, & Ooi, 2003; Guo et al., 2012; Kaur, & Kapoor, 2002). Fungi proved to be essential among sources of various natural antioxidants. Extracts prepared from the fruiting bodies and mycelium of medicinal mushroom species, such as *Ganoderma lucidum* (Curtis) P. Karst, *Hericium erinaceus* (Bull.) Pers, *Inonotus obliquus* (Fr.) Pilát exhibited significant scavenging activity against the ABTS radical cation, DPPH, and superoxide radical anion (Lee et al., 2008; Mustafin et al., 2022). Thus, it is important to consider the effect of the total phenolic content on the antioxidant activity of fungal extracts. To broaden the spectrum of such compounds, a comprehensive screening of new productive fungal species and strains remains relevant.

The aim of our study was to determine and compare the content of phenolic compounds in different strains of two widespread representatives of the genus *Xylaria* in Ukraine – *X. polymorpha* and *X. longipes*. The strains for this study were selected as promising for biomass accumulation based on preliminary culture and morphological investigation (Atamanchuk, & Bisko, 2022; Atamanchuk, 2023). In addition, the free radicals scavenging potential of studied strains was of particular focus. To ensure a more extensive screening of strains for total phenolic content and antioxidant activity, different solvents were used for the extraction.

Methods

Fungal strains, inoculum preparation and cultivating conditions. All the strains were isolated into pure culture from the entostromatal tissue of fruiting bodies of *X. polymorpha* and *X. longipes*, collected in different regions of Ukraine and preserved in the IBK Mushroom Culture Collection (Bisko et al., 2023). For the experiments, fungal strains were first grown in Petri dishes for 7 days at 25 ± 1 °C on a glucose-yeast-peptone agar medium (GYPA), containing (g/l): glucose, 25; peptone, 3; yeast extract, 3; $MgSO_4$, 0,25; KH_2PO_4, 1; K_2HPO_4, 1; agar-agar, 21. The obtained mycelium was homogenized and sterilely

inoculated in 250 ml Erlenmeyer flasks (in 6 duplicates), containing 100 ml of GYP medium (10 % v/v). Incubation was carried out for 9 days in submerged conditions on a laboratory shaker with the agitation speed of 120 rpm, at 25 ± 1 °C, in darkness. Finally, mycelial biomass was harvested by filtration and dried at 60 °C until constant weight.

Extracts preparation. Extraction was carried out using three types of solvents: ethyl acetate (98 %), ethanol (96 %) and methanol (98 %). For the extraction, previously powdered mycelial biomass was poured with each of the solvents in a ratio of 1:5 (w/v), stirred, and stored for 24 h at room temperature (20 ± 1 ºC). Then, the extracts were centrifuged for 15 min at 3000 g, aftwerwards the supernatant was separated and evaporated using a vacuum rotary evaporator at 40 °C. The residues were dissolved in the same solvent in a ratio of 1:1 (w/v) and then stored at 4 °C before further analysis.

Determination of antioxidant activity. The antioxidant capacity of studied extracts was determined by 2,2–Diphenyl–1–picrylhydrazyl (DPPH) scavenging assay (Liu et al., 2007). A volume of 0,1 ml of the extract was mixed with 2,9 ml of 120 µM DPPH (Alfa aesar®) solution in methanol. The mixture was incubated for 30 min in the dark at 37 ºC. The absorbance was measured at 517 nm using UV–Visible spectrophotometer (Jenway® 6850). The DPPH radical scavenging activities were expressed as the percentage inhibition of free radical production or antioxidant index (AI %) using the following formula:

$$AI(\%) = ((A_0 - A_1)/A_0) \times 100,$$

where A_0 is the absorbance of the control reaction (containing all reagents except the test compound), A_1 is the absorbance of the test compound.

Determination of total phenol content (TPC). Total phenol content was estimated using Folin–Ciocalteu (FC) reagent–based assay (Elfahri et al., 2016). A volume of 0,5 ml extract and 0,5 ml of FC (Sigma–Aldrich®) reagent was mixed. After 3 min, 10 ml of 7,5 % sodium carbonate solution and 5 ml of distilled water was added. The final mixture was shaken and incubated in the dark at room temperature for 30 minutes. The absorbance was recorded at 750 nm, using a UV–Visible spectrophotometer (Jenway® 6850). This procedure was also repeated with aliquots of 1–500 µg/ml gallic acid solutions which were used as a standard for the calibration curve. The total phenolic value of the samples was obtained from the regression equation $y = 0{,}0025x + 0{,}0982$ with $R^2 = 0{,}9852$. The content of total phenolics was estimated as gallic acid equivalents (GAE) and then converted into mg/g of dry weight.

Statistical Analysis. All experiments (apart from cultivation) were performed in triplicate. The data were recorded as means ± SD (standard deviation) and analyzed with Excel statistical functions using the Microsoft Office XP software. Correlations were obtained by Pearson correlation coefficient in bivariate correlations. Differences at $p \leq 0{,}05$ were considered to be significant.

Results

Total phenolic content (TPC). The concentration of phenolic compounds in the extracts, measured in milligrams of gallic acid equivalent per gram of dry weight of biomass (mg GAE/ g dw), varied depending on the extraction solvent used. Among the solvents tested, methanol extracts of both fungi exhibited the highest content of total phenolic compounds (TPC), followed by ethanol extracts. Ethyl acetate extraction, on the other hand, resulted in a significantly lower amount of phenols. The methanol extract of *X. polymorpha* IBK 2736 had the highest TPC value among all the studied strains, reaching 21,64 ± 0,03 mg GAE/g dw. A similar TPC value of 20,40 ± 0,02 mg GAE/g dw was obtained when using methanol to extract biomass from another *X. polymorpha* strain – IBK 2720. In comparison, the TPC values for *X. longipes* were lower, with strain IBK 2718 and IBK 2726 yielding 14,81 ± 0,29 mg GAE/g dw and 16,60 ± 0,04 mg GAE/g dw, respectively.

As shown in Fig. 1, with ethanol the TPC values for *X. polymorpha* IBK 2720 and *X. longipes* IBK 2718 were approximately two and a half times lower than those obtained through methanol extraction. For the other two studied strains, TPC values were also lower than in methanol extraction, but the reduction was not as significant. Among the solvents tested, ethyl acetate performed the least effectively in extracting phenolic compounds. The amount of TPC in the extracts from *X. longipes* IBK 2726 was the highest – 1,83 ± 0,06 mg GAE/g dw. Following closely were *X. polymorpha* IBK 2720 and IBK 2726, with similar values of 0,72 ± 0,21 and 0,68 ± 0,14 mg GAE/g dw, respectively (Fig. 1). The higher ($p < 0{,}05$) yield of TPC with methanol extraction can be explained by solubility principle, suggesting that the polarity of the phenolic compounds in biomass of studied strains is similar to those in methanol.

The findings indicated that the methanol and ethanol extracts of the studied strains contain phenolic compounds comparable to those found in certain medicinal plants and mushrooms, known for their antioxidant properties. In the research conducted by (Proestos et al., 2006), authors observed a range of total phenolic content varying from 2,9 mg/g (for *Humulus lupulus* L. leaves) to 28,2 mg/g (for *Geranium purpureum* Vill. leaves) in greek aromatic plants. Other researchers have also reported similar values to ours but expressed in terms of 100 g of dry matter. Specifically, the phenolic content ranged from 34,5 mg/100 g in *Praecitrullus vulgaris var. fistulosius* (Stocks) Pangalo to 253,5 mg/100 g in *Chenopodium album* L. (Kaur, & Kapoor, 2002). When comparing the results obtained from similar studies on other fungi, it was observed that the choice of solvent had varying effects on the extraction of phenolic compounds depending on the species. For instance, in case of *Pleurotus ostreatus* (Jacq.) P. Kumm, using ethanol resulted in the extraction of 102,78 mg/100 g phenols, while using methanol yielded 100,45 mg/100 g (Garcia, Ontero, Arellano, 2020). In contrast, for *Lentinula edodes* (Berk.) Pegler, the total phenol content obtained by these authors was 81,83 mg/100 g with ethanol and 78,92 mg/100 g with methanol.

To evaluate our findings concerning the phenolic compounds present in representatives of the genus *Xylaria*, we made a comparison with the research conducted by (Liu et al., 2007). Their study focused on the antioxidant activity and phenolic content of an endophytic *Xylaria* sp. from *Ginkgo biloba* L. The authors reported phenol values ranging from 9,71 to 54,51 mg GAE/g of dry weight. They also found that methanol extraction yielded the highest phenolic values, while hexane extraction resulted in the lowest values. The results of 18,36 ± 0,4 mg/g were obtained by other researchers, who used methanol to extract phenols from *Xylaria feejeensis* (Berk.) Fr. (Rebbapragada, & Kalyanaraman, 2016). However, it's worth noting that our extraction method and experimental approach for determining the total phenol content differed from the mentioned studies. Additionally, we investigated distinct species and strains, which could account for the variations in the obtained results.

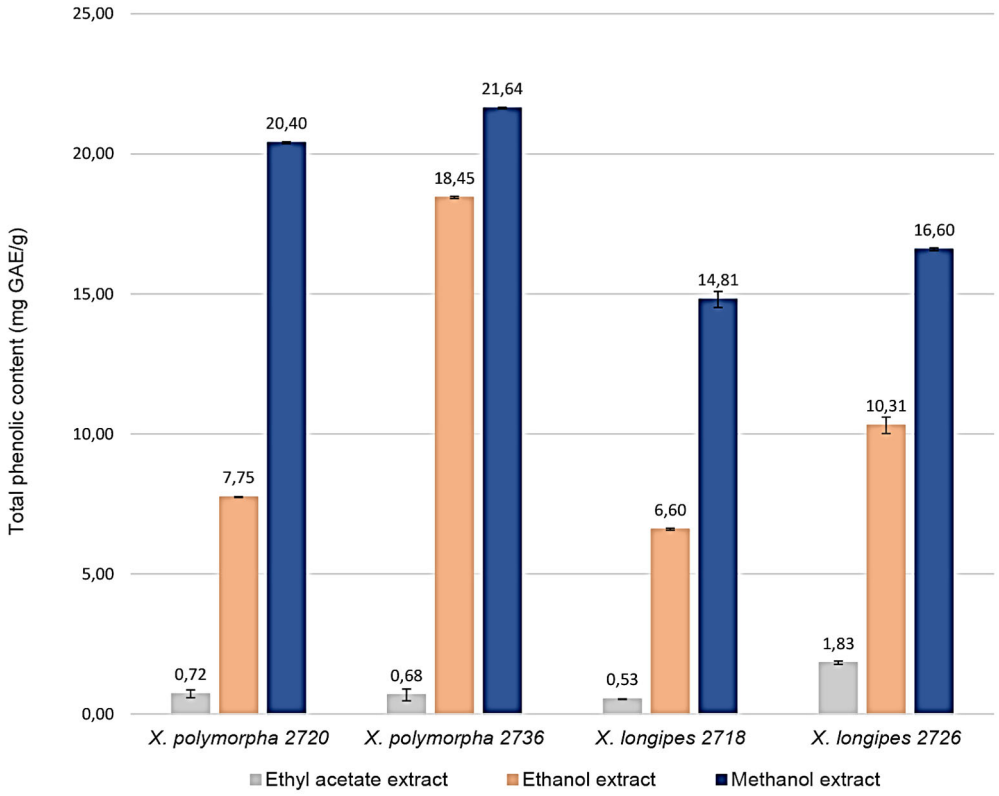

Fig. 1. Total phenolic content of the extracts of biomass of *X. polymorpha* and *X. longipes* strains obtained with different solvents. The results are the mean of triplicate, expressed as mg GAE/g dry weight and error bars indicate standard deviation

Scavenging activity of DPPH radical. Nowadays there is considerable progress in understanding and researching free radicals, especially reactive oxygen species (ROS), and the development of antioxidants. Different types of free radical scavengers, including natural, sem-synthetic, and synthetic, have received significant attention as a chain–breaking antioxidants that safeguard aerobic organisms from oxidative stress (Villaño et al., 2007). Polyphenolic compounds are of particular interest as free radical scavengers, because these antioxidants are effective in preventing lipid peroxidation due to the presence of a hydroxyl group, which enables them to donate hydrogen and provide a protective effect (Ali Al-Mamary, & Moussa, 2021). The DPPH method applied in this study is based on scavenging of a 2,2–Diphenyl-1-picrylhydrazyl through the addition of a radical species or antioxidant leading to the decolorization of DPPH solution. The extent of color change directly corresponds to the concentration and potency of the antioxidants. A significant decrease in the absorbance of the reaction mixture indicates that the tested compound has a strong free radical scavenging activity.

Figure 2 presents the scavenging activity of DPPH radicals for different extracts in our study. Methanol extracts of *X. longipes* strains, possessing high phenolic contents, exhibited strong DPPH radical scavenging activity. On the other hand, *X. polymorpha* strains showed higher activity in ethanol extracts, reaching 84,24 ± 0,07 % and 86,55 ± 0,20 % for strains IBK 2736 and IBK 2720, respectively. The lowest values were recorded for ethyl acetate extracts: from 41,28 ± 0,33 to 60,83 ± 0,17 % (Fig. 2).

The extracts derived from the investigated *X. polymorpha* and *X. longipes* strains demonstrated higher free radical-scavenging capacity when compared to numerous plants and fungi studied by the same method. For example, out of 36 studied plants, 22 ethanol extracts exhibited free radical–scavenging activity ranging from 12,8 % to 69,5 %, with only 3 extracts showing up to 80 % and higher (Kaur, & Kapoor, 2002). In the study conducted by (Liu et al., 2007) mentioned above, even though the extracts of the endophytic *Xylaria* sp. had a substantially higher phenol content, their antioxidant activity was notably lower compared to the results obtained in our research. The authors reported values of 66,29 % for the methanol extract and 29,66 % for the ethanol extract, which were lower than the values observed in our study for analogical solvents. The methanolic and ethyl acetate extracts of *X. feejeensis* HMJAU22039 demonstrated antioxidant activity of 73,86 % and 69,24 %, respectively (Rebbapragada, & Kalyanaraman, 2016), which are similar to our findings for *X. polymorpha* strains.

It is known that the antioxidant activity is well correlated with the content of phenolic compounds, so it was decided to determine the interconnection between the obtained values of phenolic compounds with the DPPH scavenging activity in obtained fungal extracts. For this purpose, the Pearson correlation coefficient was calculated for each organic solvent. The results revealed a highly significant negative correlation (Pearson coefficient -0,97) for methanol extracts, suggesting that phenolic compounds may play a crucial role in their antioxidant activity.

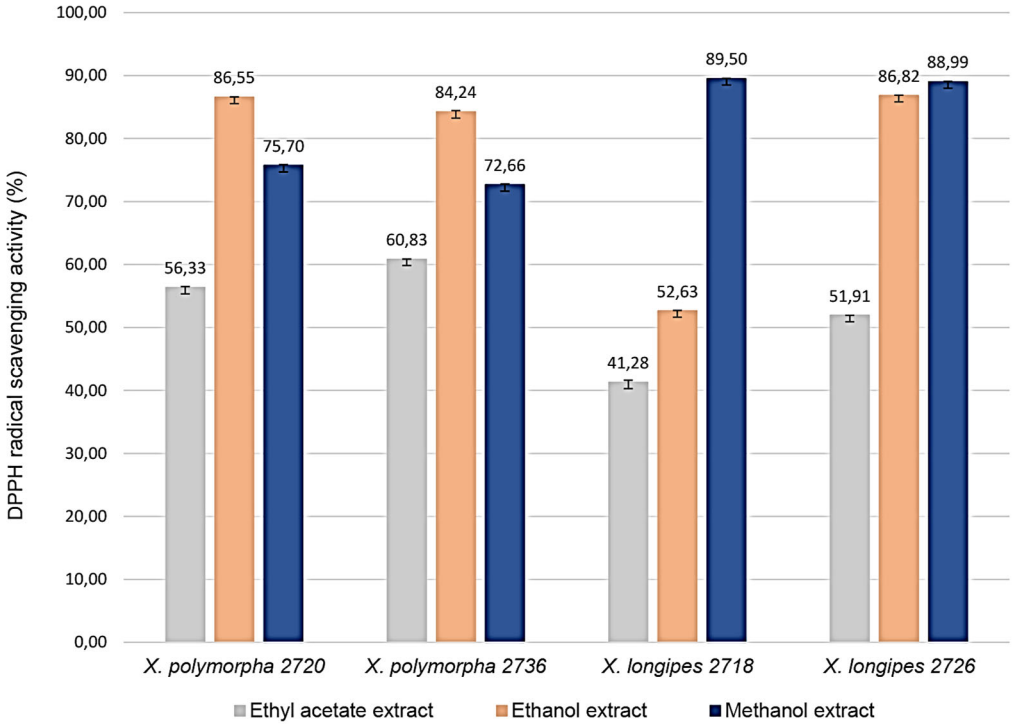

Fig. 2. Free radical–scavenging capacity of the extracts of biomass of *X. polymorpha* and *X. longipes* strains obtained with different solvents, measured in DPPH assay. The results are the mean of triplicate and error bars indicate standard deviation

On the other hand, in the ethanol and ethyl acetate extracts, there was no significant correlation between free radical scavenging activity and total phenolic content, with correlation coefficients of 0,46 and 0,07, respectively. This lack of correlation could be attributed to the presence of other chemical components in these extracts, such as sugars or ascorbic acid. The presence of compounds with a high hydrogen–donating capacity to scavenge DPPH radicals might influence the antioxidant capacity independently of phenolic content (Saeed, Khan, & Shabbir, 2012).

Moreover, it is important to note that the Folin–Ciocalteu method may yield different responses depending on the specific phenolic compounds present, as different phenols can have distinct reactions in this method. This implies a possible variation in the antioxidant response of phenolic compounds based on their chemical structures. Nevertheless, these techniques allow us to assess and compare the potential of fungal species and strains and select the most productive strains for further detailed analyses.

Discussion and conclusions

The current study provided useful and efficient quantitative analysis of total phenol contents in *X. polymorpha* and *X. longipes* strains from the IBK Mushroom Culture Collection under submerged liquid cultivation conditions. The results demonstrated that depending on the solvent for the biomass extraction, different yields of phenolic compounds were recovered. From each studied strain the highest amount of phenols was yielded using methanol, proving it is an optimal solvent for their extraction compared to ethanol and ethyl acetate. Moreover, methanol extracts were more effective in antioxidant capacity, and the correlation analysis supported the connection between these properties and the phenolic content present in them. Having revealed a high antioxidant potential in the studied fungal strains, further detailed study of the chemical characteristics of their antioxidant components remains relevant.

Authors' contribution: Alisa Atamanchuk – conceptualization, methodology, data validation, formal analysis, writing – original draft preparation; Nina Bisko – conceptualization, writing – review and editing, supervision.

Acknowledgments, sources of funding. The authors would like to acknowledge K. O. Romanenko, PhD (Department of Phytohormonology, M.G. Kholodny Institute of Botay) for essential technical support and access to the laboratory equipment.

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Отримано редакцією журналу / Received: 28.08.23
Прорецензовано / Revised: 28.08.23
Схвалено до друку / Accepted: 29.09.23

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ВПЛИВ РОЗЧИННИКІВ ДЛЯ ЕКСТРАКЦІЇ НА ВМІСТ ФЕНОЛІВ ТА АНТИОКСИДАНТНУ ЗДАТНІСТЬ БІОМАСИ ШТАМІВ *XYLARIA POLYMORPHA* ТА *XYLARIA LONGIPES*

В с т у п . ***Гриби роду Xylaria є відомими продуцентами широкого спектра вторинних метаболітів, включаючи поліфенольні сполуки з антиоксидантними властивостями. Дослідження актуальні, оскільки зосереджені на одержанні фенольних сполук із грибів та рослин, зважаючи на важливі захисні функції антиоксидантів проти окиснювального стресу. Мета роботи – оцінити і порівняти загальний вміст фенолів та антиоксидантну активність у біомасі різних штамів двох поширених в Україні представників роду Xylaria – Xylaria polymorpha та Xylaria longipes, використовуючи різні розчинники для екстракції.***

М е т о д и . ***Вегетативний міцелій досліджуваних штамів культивували за глибинних умов та екстрагували етанолом, метанолом та етилацетатом. Загальний вміст фенольних сполук у екстрактах визначали за методом Фоліна-Чокальтеу з використанням галової кислоти як стандарту. Антиоксидантну активність оцінювали за допомогою спектрофотометричного аналізу поглинання вільних радикалів DPPH (2,2–дифеніл–1–пікрилгідразилу). Кореляцію між загальним вмістом фенолів та антиоксидантною активністю екстрактів оцінювали за допомогою коефіцієнта кореляції Пірсона для кожного органічного розчинника.***

Р е з у л ь т а т и . ***Під час екстракції біомаси метанолом було зафіксовано найвищий вміст фенольних сполук серед усіх штамів, із максимальним значенням 21,64 ± 0,03 мг еквівалента галової кислоти/г сухої ваги (мг ГКЕ/г) для штаму X. polymorpha IBK 2736. Використання етилацетату призвело до значно нижчого виходу фенольних сполук із біомаси цього ж штаму – 0,68 ± 0,14 мг ГКЕ/г, а також усіх інших штамів у проведеному дослідженні. Аналогічно, значно вищі показники антиоксидантної активності спостерігались під час екстракції біомаси метанолом, найнижчі – етилацетатом. Найвища антиоксидантна активність була виявлена в метанольному екстракті X. longipes IBK 2726 – 88,99 ± 0,07 %, тоді як найнижча була зафіксована в етилацетатному екстракті X. longipes IBK 2718, зі значенням 41,28 ± 0,33 %.***

В и с н о в к и . ***Вибір розчинника істотно вплинув на кількість екстрагованих фенольних сполук. Метанол виявився найбільш ефективним для екстракції фенолів із біомаси досліджених штамів видів Xylaria порівняно з етанолом та етилацетатом. Метанольні екстракти також проявили високу антиоксидантну активність, кореляційний аналіз підтвердив зв'язок між антиоксидантною здатністю та вмістом фенольних сполук. Усі досліджені штами проявили значний антиоксидантний потенціал, що вказує на важливість подальших досліджень хімічних характеристик їхніх антиоксидантних компонентів.***

К л ю ч о в і с л о в а : ***фенольні сполуки, антиоксидантна активність, Xylaria, Ascomycota, глибинне культивування, біомаса.***

Автори заявляють про відсутність конфлікту інтересів. Спонсори не брали участі в розробленні дослідження; у зборі, аналізі чи інтерпретації даних; у написанні рукопису; в рішенні про публікацію результатів.

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; in the decision to publish the results.