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Screening, identification, and antibiotic activity of secondary metabolites of *Penicillium sp.* LPB2019K3-2 isolated from endemic amphipods of Lake Baikal

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ABSTRACT

This study aimed to assess the influence of nutrient media content on the production of antibiotics and the ability of water fungi isolated from lake Baikal to synthesize novel natural products. Interest in this topic stems from the high demand for new drugs, and studies are carried out via the screening of new natural products with biological activity produced by unstudied or extremophilic microorganisms. For this study, a strain of *Penicillium* sp. was isolated from endemic Baikal phytophagous amphipod species. Here, we identified natural products using the following classical assays: biotechnological cultivation, MALDI identification of the strain, natural product extraction, antimicrobial activity determination, and modern methods such as HPLC-MS for the dereplication and description of natural products. It was found that many detected metabolites were not included in the most extensive database. Most of the identified metabolites were characterized by their biological activity and demonstrated antibiotic activity against model Gram-positive and Gram-negative bacteria. The isolated strain of water fungus produced penicolinate B, meleagrin A, austinoneol A, andrastin A, and other natural products. Additionally, we show that the synthesis of low-molecular-weight natural products depends on the composition of the microbiological nutrient media used for cultivation. Thus, although the golden age of antibiotics ended many years ago and microscopic fungi are well studied producers of known antibiotics, the water fungi of the Lake Baikal ecosystem possess great potential in the search for new

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natural products for the development of new drugs. These natural products can become new pharmaceuticals and can be used in therapy to treat new diseases such as SARS, MERS, H5N1, etc.

1 Introduction

The chemistry of low-molecular-weight natural products is a well-known branch of pharmaceutical chemistry and is a historically effective method for translating the biotechnological potential of molecules found and extracted from different organisms such as plants, animals, fungi, or microorganisms (Newman and Cragg 2020). Microorganisms produce thousands of secondary metabolites with biological activity. Among the most identified natural products, most of them are produced by actinobacteria (65-70%), non-filamentous bacteria (10-15%), and microscopic fungi (20%). The biological activity of microbial natural products is often characterized by antibiotic activities (Demain 2014).

Nowadays, humankind needs a source of new natural products with biological activity. This is due to the increased number of diseases and the resistance of many pathogenic microorganisms to existing antibiotics (Hasan et al. 2015; Aslam et al. 2018). As the antibiotic crisis continues to grow, it will lead to the creation of new pharmaceutical ingredients using new assays, techniques, and substances. Additionally, the antibiotic crisis can be partially solved by intensifying the screening of new natural products, antibiotics, and their producers (Talebi et al. 2019; Jorgensen et al. 2022; Bhomwick et al. 2022).

The aquatic environment requires special attention because of the huge variety of free-living and symbiotic microorganisms and their secondary metabolism products (Aleruchi et al. 2018; Dat et al. 2021; Jamal and Sathianeson 2022). The biotechnological potential of aquatic microorganisms is great, and this is demonstrated by their ability to synthesize enzymes, antibiotics, terpenes, carbohydrates, and other organic molecules with biological activity (Imhoff 2016; Hitora et al. 2021).

The first antibiotic obtained from the aquatic fungus *Acremonium chrysogenum* was cephalosporin (Hu and Zhu 2016). Advances in the chemistry and biotechnology of cephalosporin C led to the synthesis of cephalosporin based drugs, which are used in regular medical practice to prevent the pathogenic spread and *Staphylococcus* infection. The discovery of penicillin led to studies on microorganisms as a great source of antibiotics, which, consequently, resulted in the development of biotechnological methods and the synthesis of biologically active natural products (Dembitsky 2014; Zhu et al. 2014; Richter et al. 2014; Gonçalves and Romano 2018). In the past 5-6 decades, interest in fungi has decreased (Kavanagh and Sheehan 2018; Yadav et al. 2019; Agrawal et al. 2022). There are significant barriers to the development of pharmaceutical studies with fungi, as these microorganisms are described as having an extremely low cultivation efficiency and high pathogenicity (Gupta et al. 2020). Such limitations restrict the discovery of antibacterial compounds that are synthesized by various unknown fungi. Studies on microorganisms that typically inhabit shallow water or soils led to the isolation of similar microorganisms with equal and well-studied biosynthetic capabilities (Hamza et al. 2015; Keller 2019). However, the studies performed in recent years testify to the intensification of studies using fungi for pharmaceutical biotechnology (Guo et al. 2022; Chen et al. 2022; Smith et al. 2023).

The commercial interest of pharmaceutical companies and the increase in of amount of clinical infections has led to the discovery of natural products produced by aquatic fungi. These compounds can be used to treat various diseases such as cardiovascular disease, diabetes, cancer, etc.(Grossart and Rojas-Jimenez 2016; Das et al. 2022; Miri et al. 2022). These compound-producing fungi were isolated from seawater, freshwater, and deep-sea organisms such as corals, macroalgae thickets, and other aquatic organisms (Frisvad 2015; Durand et al. 2019; Fernandes et al. 2022; Rad et al., 2022).

Lake Baikal is characterized as having high biotechnological and biomedical potential due to its diverse and well-studied flora, fauna, and associated microorganisms (Axenov-Gribanov, 2016a, 2016b, 2020; Protasov 2017; Shishlyannikova et al. 2017; Sukhanova et al. 2017; Zemskaya 2020; Voitsekhovskaia et al. 2020; Lipko and Belykh 2021). Amphipods (crustaceans) represent the main group of aquatic organisms in Lake Baikal, as they are well distributed and reflect the highest diversity in the lake (Rabosky 2022).

Until now, no studies have been conducted on the pharmaceutical properties of the aquatic fungi of Baikal. This study aimed to assess the influence of the nutrient media content on the production of biologically active natural antibiotic products and to estimate the Baikal *Penicillium* sp. strain's ability to synthesize novel natural products.

2 Materials and Methods

2.1 Animal samples and isolation and identification of strain *Penicillium* sp. LPB2019K3-2

The strain *Penicillium* sp. LPB2019K3-2 was isolated from the endemic species of the amphipod *Eulimnogammarus cyaneus*. *E. cyaneus* is a relatively small (adult body size: 11-15 mm) phytophagous species that is widespread around the shoreline of

Lake Baikal (Jakob et al. 2017; Takhteev 2019;). The amphipods were collected from Listvyanka village (N 51.867936, E 104.829715, South Baikal) using a benthic dragnet. The amphipods were then washed with 70 % ethanol and sterile distilled water. Then, animals were homogenized manually in 20 % sterile glycerol at a ratio of 1:10. One liter of water from the sampling point was collected as a negative control. This water was filtered through a syringe bacterial filter with a 0.45 µm pore diameter.

Strain of *Penicillium* sp. were isolated and cultured on solid nutrient medium mannitol–soya flour (MS) agar (D-mannitol, 20 g/L; soy flour, 20 g/L; agar, 20 g/L; pH 7.2) at 28 °C (Zhao et al. 2019). The culturing medium was supplemented with the antibiotic phosphomycin (100 µg/mL). Homogenates were diluted from 10 to 1000 times in sterile 1 % saline solution, and the prepared dilutions were plated on MS medium; each dilution was replicated thrice. The prepared Petri dishes were incubated for 14 days at 28 °C and were checked every 24 h to determine the appearance of fungal colonies. Fungi were recognized based on the morphology of the colonies and aerial mycelium (Suhaib et al. 2011).

The isolated strain was identified using the MALDI BIOTYPER system (Massachusetts, USA). For this, 12-18 h colonies were used (Sogawa et al. 2011). The direct load method was implemented using α-cyano-4-hydroxycinnamic acid. Triplicate identification was performed until samples of the strain were in the "Green zone" (high-reliable identification) (Ferreira et al. 2010).

2.2 Cultivation of strain and extraction of secondary metabolites

The isolated and identified fungal strain was cultured to evaluate the primary synthesis of secondary metabolites. Cultivation was performed in a selected liquid media such as HMP-broth (HMP-base, 21g/L; NaCL, 9 g/L) or TSB (casein peptone/pancreatic 17 g/L; K_2HPO_4 2.5 g/L; glucose, 2.5 g/L; NaCL 5 g/L; soy peptone3 g/L). Cultivation was performed in 100 mL of liquid nutrient media for 7 days at 28 °C (UI Hassan et al. 2019).

After 7 days of cultivation, liquid cultures were centrifuged at 3000 rpm for 10 minutes. Metabolites were extracted from the supernatant with ethyl acetate (Sigma Aldrich, Darmstadt, Germany) in equal proportion. Crude extracts from cell biomass were obtained using an acetone: methanol mixture (1:1 ratio). Natural products were extracted according to the general manual for the isolation of natural products (Nahar and Sarker 2012). The resultant extracts were evaporated and dissolved in 500 µL of methanol (Sigma Aldrich, Darmstadt, Germany).

2.3 Estimation of antibiotic activity

Three test cultures, namely *Bacillus subtilis* ATCC 66337, *Pseudomonas putida* KT 2440, and *Saccharomyces cerevisiae* BY4742, were selected to test the antibiotic activity of the crude extracts. The antibiotic activity of the crude extracts was qualitatively analyzed using the disk diffusion method (Surabhi et al. 2018). To assess antimicrobial activity, 30 µl of the extract was loaded onto 5 mm paper disks and dried at room temperature. Then, the disks were transferred onto solid LB and YPD media with inoculated test cultures. Experimental Petri plates were incubated for 12–24 h at 37 °C until growth inhibition zones appeared.

2.4 Screening and identification of secondary metabolites

To screen and identify the secondary metabolites in the fungi cultures, we used the modern and often used HPLC-MS method and further dereplication analysis. The HPLC-MS method allowed us to perform a separation of the crude extract for detailed chemical analysis or analysis of natural products. The dereplication analysis of natural products allowed us to estimate the chemical composition of the primary and secondary metabolites using a database of natural products.

For HPLC-MS analysis, samples were chromatographically separated using the UHPLC system (Ultimate 3000, Dionex, Sunnyvale, USA). The C18 UPLC column (ACQUITY UPLC BEH 100 mm x 2.1 mm, 1.7 µm 130 Å, Waters, USA) was used in this study. A linear gradient of acetonitrile in water was used. The time of analysis was 20 min, with a flow rate of 0.5 mL/min. The solvents were supplemented by 0.1 % ammonium formate. After an initial assessment, the samples were analyzed with mass spectrometry (ultra-high resolution, Orbitrap XL, Thermo Fisher Scientific, USA). Mass detection was performed in positive mode with the detection range set to 160–2500. Data were collected and analyzed using Xcalibur software v.4.4. (Thermo Fisher Scientific, USA). The dereplication of natural products and screening for known compounds was performed using the Dictionary of Natural Products database ver.2019 (CRC Press, Boca Raton, USA), and the following search parameters were used: accurate molecular mass, absorption spectra, and biological source of compound isolation (Whittle et al. 2003). Compounds were considered to be similar when the difference in the accurate mass was less than 10 ppm and when the absorption spectrum and biological source of the compound isolation were identical.

2.5 Reactives and chemicals

All chemicals used in this study for analytics and extraction procedures were characterized as "pharmacopeial grade" and manufactured by Sigma Aldrich (Darmstadt, Germany), MP biomedicals (Eschwege, Germany), and BD (Heidelberg, Germany). For mass spectrometry (both LCMS and MALDY), we used ultra-pure chemicals with the grades "for mass spectrometry", "HPLC", and "molecular biology grade". The chemicals used to prepare the nutrient media and for the cultivation of *Penicillium* sp.

were characterized as "microbiological grade", except soy flour. The soy flour was bought at a local market.

2.6 Statistical analysis

The experiments to estimate antimicrobial activity were performed three times to standardize the cultivation parameters and to reduce the risk of research being performed with a wild and nonstable strain. Only qualitative analysis was performed during the current study. For mass spectrometric analysis, we used a combined sample pooled from the above-mentioned three extracts.

3 Results

3.1 Estimation of antibiotic activity

In this study, the strain *Penicillium* sp. LPB2019K3-2 was isolated from endemic species of the amphipod *E. cyaneus*. This strain of fungus has been found in 80% of the amphipod *E. cyaneus* from Listvyanka village. Being detritivorous, phytophagous, and necrophagous, amphipods of this species undoubtedly have associations with microorganisms. The latter may provide defense against various pathogenic agents ingested by amphipods. Due to the disease caused by bacterial infection with *E.cyaneus* being unknown, it can be described by its close association with aquatic fungi. The disease caused by fungal infection associated with *E. cyaneus* has never been mentioned in the literature.

In addition to the studied strain, in this study, we isolated another 14 morphologically different strains of fungi. However, due to the fast sporulation and hazardous experiments carried out with microscopic sporulating fungi, here, we perform an analysis to characterize the biotechnological potential of only one strain—*Penicillium* sp. LPB2019K3-2.

3.2 Estimation of antibiotic activity

The chosen strain is characterized by the presence of activity against Gram-positive and Gram-negative bacteria. According to classical assays of microorganism cultivation (Zhao et al. 2019), the strain of *Penicillium* sp. LPB2019K3-2 was cultivated in liquid nutrient media using an orbital shaker to produce secondary and bioactive metabolites (Nahar and Sarker 2012). The extracted natural products were tested using the model and nonpathogenic test cultures of microorganisms (Surabhi et al. 2018).

Table 1 demonstrates the antibacterial activity of the strain *Penicillium* sp. LPB2019K3-2 cultivated in the liquid nutrient media TSB and HMP. The results of the study revealed that the fungal strain *Penicillium* sp. LPB2019K3-2 growing in TSB liquid medium was able to synthesize natural products extracellularly, and inhibiting the growth of the bacterial test cultures *B. subtilis* and *P. putida*. Moreover, we found that the extracts of cellular biomass of the strain *Penicillium* sp. LPB2019K3-2 cultivated in TSB medium were active only against the Gram-positive bacteria *B. subtilis*. Antibiotic activity against *S. cerevisiae* was not observed. It was revealed that the strain cultivated in HMP nutrient medium also showed similar activity to that of the strain cultivated in the TSB medium. Cultivation of the strain *Penicillium* sp. LPB2019K3-2 in the tested nutrient media did not lead to the synthesis of natural products with activity against *S. cerevisiae*.

3.3 Screening and identification of secondary metabolites

Liquid chromatography and high-resolution mass spectrometry were used to assess the composition of the metabolites produced by the Baikal strain of *Penicillium* sp. Figure 1 presents chromatograms of the cell-free liquid culture and cellular biomass of strain *Penicillium* sp. LPB2019K3-2 cultivated in TSB and HMP liquid nutrient media.

The analysis of the cell-free liquid culture and cellular biomass extracts of the strain *Penicillium* sp. LPB2019K3-2 cultivated in a TSB liquid medium allowed for the identification of 8 out of 88 detected compounds known for the genus *Penicillium*. Cultivation of the strain in the HMP nutrient medium revealed 8 out of 58 detected compounds. Forty-six detected natural products had no hits in the used database and were characterized as having masses from m/z226.1672 to m/z 1305.2320 in the amphiphilic and nonpolar parts of the chromatograms. The identification and a brief description of the secondary metabolites are presented in Table 2.

Table 1 Antibiotic activity of strain *Penicillium* sp. LPB2019K3-2 cultivated in liquid nutrient media TSB and HMP

Medium	Crude extract	*B. subtilis*	*P. putida*	*S. cerevisiae*
TSB	Cell-free liquid culture	+	+	-
	Cellular biomass	+	-	-
HMP	Cell-free liquid culture	+	-	-
	Cellular biomass	+	+	-

"+" and "-"represent the presence or absence of antibiotic activity; TSB and HMP—nutrient media.

Table 2 Natural products identified within the Dictionary of Natural Products (CRC Press) from crude extracts of strain *Penicillium* sp. LPB2019K3-2 cultivated in liquid nutrient media

No	Retention time (min)	Natural products	Detected mass	Accurate mass	Δ ([M] (ppm)	Bioactivity	TSB medium		HMP medium	
							Cellular biomass	Cell-free liquid culture	Cellular biomass	Cell-free liquid culture
1	2.3	Penicolinate B	398.22681	398.220558	15.7	Antimalarial agent. Exhibits antibacterial and antifungal props	-	-	-	+
2	3.0	TryptamineNb-[2-(Methoxycarbonyl)acetyl	260.1154	260.116093	3.0	-	+	+	+	-
3	4.0	Cyclo(leucyltyrosyl) (3S,6S)-form	276.1467	276.147393	2.4	Inhibits biofilm formation of *Staphylococcus epidermidis*	-	+	+	-
4	4.3	Cyclo(4-hydroxyprolylleucyl); (3S,7R,8aR)-form	226.13092	226.131743	3.6	-	-	-	+	-
5	5.4	Cyclo(phenylalanylprolyl) (3S,8aS)-form	244.1202	244.121178	4.1	Shows a broad spectrum of antibacterial and gastrointestinal cell maturation-enhancing activity	-	+	+	-
6	7.1	Meleagrin A	433.1737	433.175005	2.9	Shows structural similarity to tremorgenic mycotoxins. Closely relatedtoNeoxaline	-	+	+	+
7	8.01	Territrem C	512.2044	512.204635	0.5	Strongly inhibits acetyl cholinesterase. Tremorgenic toxin	-	+	-	-
8	9.7	1,3,8-Trihydroxy-6-propylanthraquinone2'S-Hydroxy	314.0819	314.07904	9.1	-	-	+	-	-
9	11.8	Austinoneol A	414.20325	414.20424	2.4	-	+	-	-	-
10	11.9	Andrastin A	486.26044	486.261755	2.7	Protein farnesyltransferase (PFTase) inhibitor Mycotoxin	-	-	+	+
11	13.6	Predecaturin E	477.28498	477.287909	6.1	-	-	-	+	-
12	18.39	Citriquinone A	336.15891	336.15729	4.8	Antibacterialagent	+	-	-	-

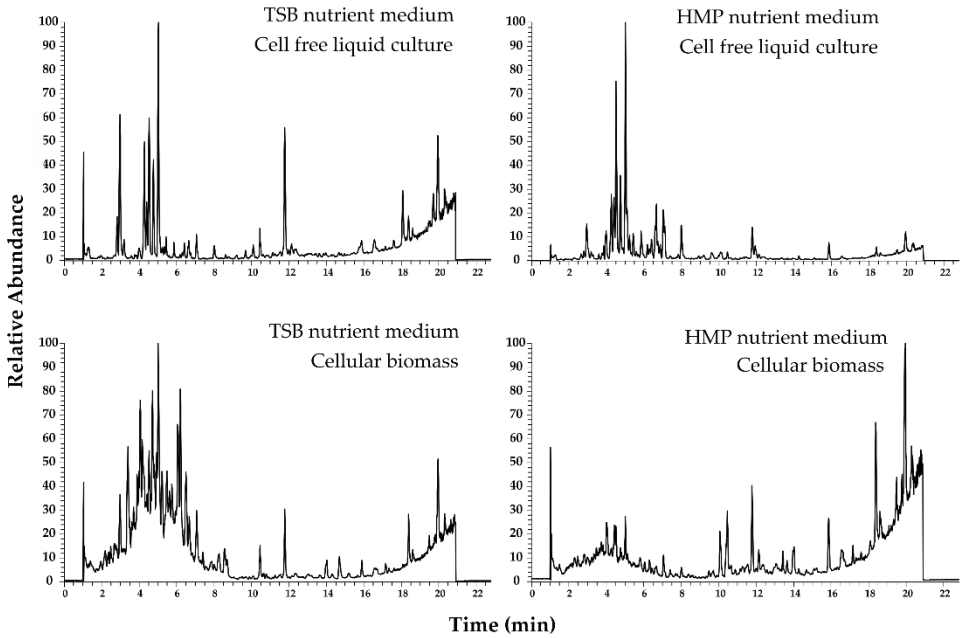

Figure 1 Chromatograms of cell-free liquid culture of strain *Penicillium* sp. LPB2019K3-2 cultivated in the TSB and HMP nutrient media

4 Discussion

The results of the current research describe the first study highlighting the antibiotic potential of the Baikal strain of *Penicillium* sp. isolated from the endemic amphipod *E. cyaneus*. In this study, *Penicillium* sp. was isolated and used, this strain was often (80%) found in the amphipod *E. cyaneus*. The results of the study found that the isolated strain was characterized by its antimicrobial activity. The strain inhibited the growth of Gram-positive *B. subtilis* and Gram-negative *P. putida*. By superposing the data from Tables 1 and 2, it can be assumed that activity against *P. putida* could be induced by the natural product cyclo (phenylalanylprolyl) (3S, 8aS)-form. Based on library data and published biological activity, this natural product demonstrates a broad spectrum of antibacterial and gastrointestinal cell maturation-enhancing activity (Bertinetti et al. 2009; Santos et al. 2019). Additionally, at least two natural products namely tryptamine Nb-[2-(Methoxycarbonyl) acetyl and meleagrin A are responsible for the activity against *B. subtilis*. Meleagrin A, known as a mycotoxin and antimicrobial agent, is produced by marine *Penicillium* sp. (Nielsen et al. 1999). However, the toxicity of meleagrin A does not explain the absence of activity against *S. cerevisiae*, as demonstrated in another study (Scopel 2013; Varga et al. 2015; Hamed et al. 2020). Thus, despite the presence of bioactive natural products in the list of identified metabolites (Table 2), there is a strong possibility that here, we have a low concentration of meleagrin A. Additionally, another (new) molecule could be or its non-active monomer could be responsible for the observed activity. Furthermore, the analysis of natural products revealed a low number of natural products that can be identified with high reliability based on the analyzed parameters, such as the accurate mass, UV spectrum, and biological source.

Accordingly, similar to previous studies of Baikal Lake microorganisms, there is no doubt that further studies on the secondary metabolites and metabolic pathways of this strain have great potential. Such potential is confirmed by the presence of a great number of new nonidentified natural products.

Nowadays, biologically active compounds produced by various microorganisms isolated from unusual habitats are receiving more attention (Gonçalves et al. 2013; Devi 2014; Durvasula and Rao 2018; Kumaravel et al. 2018; Zhang et al. 2018). The ecosystem of Lake Baikal and its inhabitants are no exception. Similarly, previous studies performed on microorganisms demonstrated the extent of antimicrobial and enzymatic activity. Studies performed on actinobacteria isolated from the Baikal-endemic mollusk *Benedictia baicalensis* revealed the new molecules Baikalomycins A–C, which demonstrated varying degrees of anticancer activity (Voitsekhovskaia et al. 2020). Other natural products found in Baikal microorganisms isolated from Baikal amphipods are Perquinolines A–C. Although these natural products did not show any prominent activity in the assays employed, the biosynthetic pathway leading to the formation of these compounds represents an unprecedented assembly of the tetrahydroisoquinoline core structure (Rebets et al. 2019). The findings that have been published to date suggest that there is no doubt that Baikal invertebrates are associated with microorganisms. However, the roles of the above-mentioned microorganisms in the life of amphipods is unknown. We can state with confidence that the

above-mentioned associations demonstrate the highest levels of biological organization and adaptation to aggressive environments.

Thus, representatives of *Penicillium* sp. can adapt to new, sometimes extreme, environments and specific conditions (Shukla et al. 2020; Ibrar et al. 2020), such as the ecosystem of Lake Baikal. The environmental peculiarities of Lake Baikal (low temperature, high water transparency, penetrating UV radiation, and high oxygen content) create specific conditions for speciation, the maintenance of the high level of biodiversity (Timoshkin 2009), and the adaptation of organisms.

Lake Baikal is a home to more than 2500 species of aquatic organisms (Berkin et al. 2009). Studies performed on the microorganisms in Lake Baikal have led to a comprehensive understanding of the role of microorganisms in ecosystems. However, only a few studies describe the biosynthetic potential of Baikal microorganisms. This reveals the importance of using modern molecular biotechnology methods and of creating new ways to study the biosynthetic and biomedical potential of microorganisms.

The extreme conditions mentioned here may help fungi of the genus *Penicillium* to produce specific and bioactive natural products. These compounds can play an ecological role in animals' lives and their symbionts. Moreover, the novel secondary metabolites detected in crude extracts of the studied *Penicillium* fungi can help us in the search for new drug candidates and can be used in the field of biopharmacy.

Conclusion

Thus, the study of microorganisms symbiotic to those that are endemic to Lake Baikal may result in the discovery of a new era of the chemistry of natural products in response to the increase in adaptation potential to different stress factors. Additionally, although the golden age of antibiotics ended many years ago and microscopic fungi are well-studied producers of known antibiotics, the water fungi of the Lake Baikal ecosystem possess great potential in the search for new natural compounds for the development of new drugs to act as therapies for new diseases such as SARS, MERS, H5N1, etc.

Conflicts of Interest

The authors declare no conflicts of interest.

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