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Identification of biological secondary metabolites in three *Penicillium* species, *P. goditanum*, *P. moldavicum*, and *P. corylophilum*

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ABSTRACT

Microorganisms are important components of soil. Some soil filamentous fungi such as *Penicilium* produce many bioactive small molecules, or secondary metabolites, that range from beneficial bioactive compounds to harmful toxins. In this study, the metabolites of three *penicillium* species (*P. goditanum*, *P. moldavicum* and *P. corylophilum*) were extracted by adding ethyl acetate to liquid cultures. The metabolites were determined using gas chromatography and mass spectrometry. The results obtained from the GC-MS analysis showed that *Penicillium* species are sources of bioactive compounds. We have identified different groups of compounds, such as alkaloids, alkenes, sesquiterpens, fatty acids, and essential oils. Among them, 1,3,8-p-Menthatriene, 2-methylenecyclohexane, anthracene, neoisolongifolene, [14] annulene and thioxanthene in *P. goditanum*, isocyclocitral, coumarin-6-ol and 2, 4, 6-Trimethoxystyrene in *P. moldavicum* as well as asarone in *P. corylophilum* were major compounds. Moreover, several compounds, such as oxalic acid, dibenzothiophene, hexadecanoic acid, and alkane hydrocarbons, were identified in all species.

Keywords: biological compounds, fungi, GC-MS.

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Introduction

Microorganisms such as fungi are important components of soil (1). Secondary metabolites are produced by all organisms; however, they are known mostly in plants, insects, fungi, algae, and prokaryotes (2). Filamentous fungi produce many bioactive small molecules, or secondary metabolites, that range from beneficial bioactive compounds (antibiotics, anticancer, anti-infective, antimicrobial, and antioxidant) to harmful toxins (3). Some of them contribute to plant pathogens or animal diseases (4). In particular, secondary metabolites often have complex structures that are not involved in the complete life cycle or perpetuity of the producer (5), but these molecules play an important role in the biology of fungi by functioning as defense compounds (6, 7) or signaling molecules (8, 9) in ecological interactions. Moreover, secondary metabolites are the cause of the virulence that some fungi impose on their host plants. Another advantage of secondary metabolites is the survival of the produced organism in its ecological niche (4). *Penicillium* species are of the ascomycetes fungal genus, are asexual(10), and are found in soil, foods, and air (11). This genus is the source of major antibiotics and mycotoxins (12). Some *Penicillium* species are capable of breaking down various types of xenobiotic compounds and are used in bioremediation (13). Many studies have been performed on the production and characterization of metabolites in *Penicillium* species (10, 12, 14). *Penicillium* subgenus *Penicillium* was the first anamorphic genus to be studied using secondary metabolites. The species of this genus produce many secondary metabolites, such as terpenes, polyketides, and amino acids (12). Many volatile compounds are produced in the species distinctively. The discovery of bioactive secondary metabolites is an interest of both pharmaceutical and agrochemical industries.

In this paper, we identify some secondary metabolites in three *Penicillium* species (*P. goditanum*, *P. moldavicum,* and *P. corylophilum*). We also consider the biological activity of compounds based on past studies.

Materials and Methods

Growth condition

Penicillium species were isolated from soil and prepared in the plant pathology laboratory. After the species were identified by the identification key, spores were grown in a liquid culture of potato dextrose broth (PDB) and incubated at 25ºC in a shaker for 14 days at 130 rpm.

Extraction and isolation of metabolites

The metabolites were determined using gas chromatography and extracted for GC analysis using the method of Siddiquee *et al*. (2012) with some modifications (15). The extraction was performed by adding 10 ml ethyl acetate to 50 ml liquid culture in an Erlenmeyer flask after the infiltration of the culture. The mixture was incubated at 4ºC for 10 min and then shook for 10 min at 130 rpm. The supernatant (metabolites and ethyl acetate) was separated from the liquid culture and evaporated to dryness with a rotary evaporator at 45ºC. The residue was dissolved in 1 ml methanol, filtered through a 0.2 µm syringe filter, and stored at 4ºC for 24 h before being used for GC-MS.

Gas chromatography and mass spectrometry conditions

An Agilent technologies 7890 A gas chromatograph connected to a 5975 Cinert MSD was used to identify the secondary metabolites from the crude extract of Penicillium species. An HP-5MS fused silica capillary column (Hewlett-Packard, 30 m * 0.25 mm i.d., 0.25 μm film thickness, cross-linked to 5% phenyl methyl siloxane stationary phase) was used. The entire system was controlled by Chemstation software (Hewlett-Packard, version A.01.01). Electron impact mass spectra were recorded at 70 eV. Ultra-high purity He (99.999%) was used as the carrier gas at a flow rate of 1 mL/min. The injection volume was 1 μL, and all injections were performed in splitless mode. Injector and detector temperatures were 250°C and 280°C, respectively. Column oven temperature was initially set at 50°C for 5 min, then increased to 260°C (ramp, 4°C/ min) and held for 5 min.

Results

Each point in a total ion chromatogram consists of an entire mass spectrum. Response for that point is determined by simply summing the abundances. Qualitative analyses of abundance and retention time of compounds from *P. goditanum, P. moldavicum, P. corylophilum* are listed, respectively, in Tables 1, 2, and 3. Different groups of compounds, including alkaloids, alkenes, sesquiterpenes, fatty acids, and essential oils, were identified in this study.

Table 1. Characteristics of the peaks obtained by GC-MS analysis in *P. goditanum*

Compounds	RT, min	Abundance %	Compounds	RT, min	Abundance %
1,2-Cyclopentanedione	7.287	78	Tetradecane	24.353	96
2,4-Nonadiyne	9.487	7	Hexadecane	30.338	95
Decane	9.825	90	Azulene	32.627	60
Phthalan	10.649	9	Anthracene	34.835	92
1,3,8-p-Menthatriene	13.166	9	Neoisolongifolene	35.413	35
Undecane	13.824	91	Octadecane	35.722	96
6-Methyltricosane	16.262	17	Thioxanthene	37.771	90
Dodecane	17.589	97	Hexadecanoic acid	38.881	68
Oxalic acid	20.158	40	Phenanthrene	41.055	96
Phosphoramidous difluoride	22.859	9	[14]Annulene	41.587	50
2-Methylenecyclohexane	24.184	53	Dibenzothiophene	36.712	96

Table 2. Characteristics of the peaks obtained by GC-MS analysis in *P. moldavicum*

Compounds	RT, min	Abundance %	Compounds	RT, min	Abundance %
Decane	9.825	91			
1-Allylazetidine	24.004	7	Octadecane	35.722	97
Tetradecane	24.353	96	Acridine	36.695	42
Pentafluoropropionic acid	26.533	10	1,2-Benzenedicarboxylic acid	37.416	86
Oxalic acid	28.976	53	Hexadecanoic acid	38.881	97
1-Nonadecene	30.128	49	Coumarin-6-ol	39.138	64
Hexadecane	30.344	98	Phenanthrene	40.180	46
2,3,5,6-Tetrafluoroaniline	31.677	43	2-Methyl-4,6-quinolinediol	41.559	32
1H-Purin-2-amine	32.615	9	9,15-Octadecadienoic acid		
2,4,6-Trimethoxystyrene	33.715	8	1-Methyl-4-(p-tolyl) aminocytosine	42.766	99
Isocyclocitral	33.714	14		43.378	38
Vanillin methyl ether	33.891	72			

Table 3. Characteristics of the peaks obtained by GC-MS analysis in *P. corylophilum*

Compounds	RT, min	Abundance %	Compounds	RT, min	Abundance %
Decane	9.825	72	Octadecane	35.722	97
Dodecane	17.595	97	Tridecane	35.951	43
p-Fluoro-N,N-dimethylaniline	21.680	53	Dibenzothiophene	36.712	94
Oxalic acid	23.220	53	Hexadecane	38.281	91
Nonadecane	23.403	47	Anthracene	38.343	89
Tetradecane	24.358	96	Hexadecanoic acid	881/38	98
1H-Azepine	24.736	7	Acridin-9-amine	39.184	86
3,4-Methylenedioxyanisole	27.534	72	Eicosane	40.614	91
Pyrimidin-4-one	29.869	7	Phenanthrene	41.049	93
Hexadecane	30.338	98	4-Phenyl-8-aminoisoquinoline	43.779	47
Asarone	33.994	50			
Diphenylethyne	34.830	62			

Discussion

Many compounds are identified in the present study. Some of them are biological compounds with antimicrobial activities. We aimed to explain the biological activity of some secondary metabolites of three *Penicillium* species based on previous studies.

Major compounds produced only in *P. goditanum*

[14] Annulene (Cyclotetradecaheptaene) is an aromatic compound. Derivatives of this compound are effective inhibitors of fungal growth (16).

1,3,8-p-Menthatriene is an essential oil with antimicrobial activity (17). Oils have moderate to strong antimicrobial activity against some fungi and bacteria (18). Essential oils such as 1,3,8-p-Menthatriene are able to inhibit the growth of *Staphylococcus aureus* (MIC=15µg/ml) and *Listeria monocytogenesis* (MIC=137µg/ml) (19). Anthracene is one of a chemical group that is called polycyclic aromatic hydrocarbons (PAHs) (20). PAHs are found naturally in the environment and can also be man-made (21). Most PAHs, such as anthracene, are used to make dyes, plastics, and pesticides (22). Some fungi such as *Rhizoctonia solani* have been tested for anthracene metabolism (23). Some *Penicillium* species metabolize these PAHs poorly (24). Many soil fungi, such as *zigomysetes* and *melanconiales,* are able to degrade anthracene (20).

Thioxanthene is a psychotropic drug with antimicrobial activity (25). There are several reports that some thioxanthene derivatives are biological active compounds. Yunnikova and Voronina (1996) studied the antimicrobial activity of a series of xanthene and thioxanthene derivatives. They reported that this compound has a bacteriostatic affect against *Staphylococcus aureus* (26). The structure-activity relationships of a series of thioxanthene isomers have been reported in a multidrug resistant (MDR) human breast cancer cell line (27).

Neoisolongifolene is a sesquiterpenoid that was discovered in *Gliocladium roseum* in previous studies (28). Bukvicki *et al*. (2012) identified this compound from the ethyl acetate extract of *Porella cordaeana*. They reported that many compounds of *Porella cordaeana* extract such as neoisolongifolene have antimicrobial activity against some strains of yeast and bacteria (29).

Major compounds produced only in *P. moldavicum*

According to previous studies, the range of volatile compounds such as Isocyclocitral in spoilage fungi in vitro and on grain has been evaluated by electronic nose technology (30).

2,4,6-Trimethoxystyrene alkaloid is a bioactive compound that is quite toxic for brine shrimp, but has only weak cytotoxic activity. This alkaloid has been reported to be the major bioactive compound in *Duguetia panamensis* and *D. colombiana* (31).

Coumarin-6-ol is one of the coumarin compounds. Some coumarin compounds have antimicrobial activity against gram-positive and gram-negative bacteria, yeasts, mold, and plant pathogenic fungi. Moreover, natural coumarin compounds (extracted from plants grown in Finland) have weak antibacterial and antifungal activities, except the inhibitory effect they show against *Fusarium culmorum* (32).

Major compounds produced only in *P. corylophilum*

Asarone is an essential oil with antimicrobial activity (33, 34). This compound has an inhibition rate of 100% against *Phytophthora cactorum* and moderate activity against *Cryponectria parasitica* and *Fusarium circinatum* (34).

Common compounds identified in *Penicillium* species

Oxalic acid is an organic acid that many fungi are able to produce in nature (36). The production of oxalic acid by fungi is important for various physiologic and biogeochemical processes (36). It seems to be a pathogenicity factor for *Sclerotinia sclerotiorum* (37). This compound also has antimicrobial activity (38, 39). In the present study, oxalic acid was characterized in all of the species.

Dibenzothiophene (DBT) is an organic sulphur (40) and a source of carbon, sulfur, and energy for many bacteria such as *Brevibacterium* and *Pseudomonas*. This compound was produced in *P. goditanum* and *P. corylophilum*.

Many fatty acids have been known to have potent antibacterial, antifungal, and antiviral activities (41). Hexadecanoic acid, or palmitic acid, is a saturated fatty acid with antifungal activity against *Alernaria solani*, *Aspergillus niger*, *Aspergillus terreus*, *Cucumerinum lagenarium*, *Emericella nidulans,* and *Fusarium oxysporum* (42). This compound also has bactericidal properties against gram-negative and gram-positive organisms (43). Moreover, hexadecanoic acid has some biological activities such as antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic, and hemolytic activities (38, 39). This compound was produced in all of the species except *P. corylophilum.*

Alkane hydrocarbons such as tridecan, pentadecane, octadecane, nonadecane, heptadecane, dodecane, eicosane, decane, and hexadecane were produced in all of the species. These compounds have antimicrobial activity (15).

The results of this study showed that *Penicillium* species produce many important secondary metabolites with high biological activities. Based on the significance of employing bioactive compounds in pharmacy to produce drugs for the treatment of many diseases, the purification of compounds produced by *Penicillium* species can be useful. Because of the production of biologic toxins against plant pathogens, using these compounds and applying them in agriculture are other advantages of biological secondary metabolites.

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