Research Journal of Toxins, 2009
ISSN 1819-3560

Antimicrobial Properties of *Penicillium* Species Isolated from Agricultural Soils of Northern Iran

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Abstract: As a part of a research program that aimed to identify antibacterial and antifungal substances from fungus specimen of North Iranian soil samples, four *penicillium* species were identified as a source of secondary metabolites possessing antibiotic activities. These microorganisms were cultured in a liquid medium for 10 days. The antimicrobial disc assay activity of these extracts towards *Candida albicans, Bacillus subtillis*, *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* was performed. Supernatants and cell extracts of *P. viridicatum* Westling, *P. citrinum* Thom., *P. aurantiogriseum* Dierckx and *P. waksmanii* Zaleski showed distinguished antimicrobial activities. TLC assay of supernatants and cell extracts showed production of citrinin by *P. citrinum* and *P. aurantiogriseum* and penicillic acid by *P. viridicatum* and *P. aurantiogriseum*; while two unknown metabolites from *P. waksmanii* needed more examinations. These mycotoxins could be examined for other biological activities such as antineoplastic property.

Key words: Citrinin, penicillic acid, *P. aurantiogriseum*, *P. citrinum*, *P. viridicatum*, *P. waksmanii*

INTRODUCTION

Microorganisms have been traditionally used to produce a variety of important substances for the pharmaceutical and food industries. The discovery and development of antibiotics was one of the most significant advances in medicine in the 20th century (Uchida *et al*., 2006; Silva *et al*., 2004).

Despite of the huge expectative on synthetic molecules with effective antimicrobial properties, natural products are still a worth promise. Screening of novel strains are bringing about microorganisms, not yet assayed for their antibacterial activity that can produce innovative molecules or useful templates for new antibiotics development (Du *et al*., 2009; Takahashi *et al*., 2008).

A large number of fungal extracts and/or extracellular products have been found to have antimicrobial activity, mainly from the filamentous fungus *penicillium* sp. (Petit *et al*., 2009; Rancic *et al*., 2006). The filamentous fungi of the genus *Penicillium* that belong to Ascomycetes are recognized to be ubiquitous in the environment (Nakashima *et al*., 2008).

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Since, the discovery of penicillin, the micromycetes have been famous as producers of antibiotics and other secondary metabolites with biological activity (Sonjak *et al.*, 2005; Rancic *et al.*, 2006).

Previously unexplored environments have their own ecosphere, interactions and evolution that might contain new producer organisms (Bertinetti *et al.*, 2009). Agricultural fields in North of Iran hold a soil rich in nutritional compounds. There are various genuses of micromycetes which compete together in such habitats, so it is expected that fungus isolated from these areas should develop a sophisticated metabolism, most probably molecules of odd chemical structures possessing interesting biological properties (Montematini *et al.*, 2000). Those facts motivated the development of a research program which aimed to perform the isolation of antimicrobial substances from North Iranian soil fungi. The various species of *Penicillium* can colonise many different environments. They are common in soils, in foods, in drinks and in indoor air (Rundberget *et al.*, 2004; Leitao, 2009).

As a part of this program, twenty isolates of *penicillium* were investigated for antimicrobial as well as antifungal activities.

MATERIALS AND METHODS

Microorganisms

Penicillium species were isolated from a soil sample collected at agricultural soils of Northern Iran. Small portions of soil were serially diluted and plated on solid media containing (g L^{-1}): KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.5), Peptone (5.0), Dextrose (10.0) and Rose Bengal (0.033). Upon growth, individual colonies were transferred to other plates successively until the isolation of pure cultures (Esther *et al.*, 2007). *Penicillium* species was identified by microscopic observation of micro-structure (Pitt, 2000). The fungal strains were maintained on Potato Dextrose Agar (PDA) and refrigerated at 7°C (Esther *et al.*, 2007).

There were used the following Persian Type Culture Collection (PTCC) strains: *Candida albicans* PTCC 5027, *Bacillus subtillis* PTCC 1023, *Staphylococcus aureus* PTCC 1337, *Salmonella typhi* PTCC 1609 and *Escherichia coli* PTCC 1533 for antimicrobial assay.

Culture Condition

For metabolites production, *Penicillim* species were inoculated into twenty conical flasks, containing each, 500 mL of aqueous medium containing (g L): Dextrose (20.0), Peptone (5.0), KH_2PO_4 (1.0), $MgSO_4.7H_2O$ (0.5) and NaCl (5.0) and then incubated at the temperature of 28°C and shaking at 200 rpm. After 10 days, the mycelium was separated from the culture broth by filtration. The mycelia were extracted 3 times with 300 mL of methanol each. The extracts were dried by evaporation using a rotator vacuum distilling apparatus (Esther *et al.*, 2007).

Antimicrobial Assay

The antimicrobial activity of extracts was evaluated against *Candida albicans, Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* by qualitative disc test assay.

Two milligram of each extract were solubilized in 1 mL of chloroform. Fifty microliter of each solution were quantitatively loaded on a paper to make a final concentration of 100 μL $disc^{-1}$. The solvent was removed by dry air. The test plates were prepared with 20 mL of nutrient agar and sterilized in autoclave by 15 min at 121°C. The 0.4 mL of the diluted bacteria inoculum (500 μL of stock culture in 2.0 mL of nutrient broth let 18 h at 37°C were

transferred to 4.5 mL of saline solution) and homogenized using a vortex. A negative control was set using a disc impregnated with 50 μL of methanol and chloramphenicol (30 μg $disc^{-1}$) was used as positive control of antibacterial test; to antifungal test miconazol (50 μg $disc^{-1}$) was used. Experiments were run in duplicate. The result was ridden after incubation by 24 h at 37 μC (Esther *et al*., 2007).

Metabolites Assay

Thin Layer Chromatography (Kieselguhr 60 F254 TLC) was performed for identification of metabolites. Standard mycotoxins used for reference of R_f were citrinin and penicillic acid. 10 mL of each supernatant, methanol extract and 10 mL of standard solutions (1 mg mL^{-1}) were spotted on TLC plates. The elution systems used were as follows: chloroform-acetone- 2-propanol (85/15/20, v/v/v). The plates were developed under darkness and examined at daylight, 365 and 254 nm. Spots with R_f different from standard on either elution systems were not considered (Khaddor *et al*., 2007).

RESULTS

The initial antibacterial activity screening of soil isolates was made against six test microorganisms in agar medium. 80% of the investigated strains possessed antimicrobial activity at least against one of the test microorganisms (Table 1, 2).

Four strains showed high inhibition potential against *Candida albicans*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* over mm sterile zone.

It should be pointed out that from all *Penicillium* species isolated from agricultural soils of Northern Iran, *P. aurantiogriseum* (species No.1164) showed to be one of the most active isolates ($p<0.05$). At the same time, other isolates including: *P. viridicatum* (species No.1193), *P. citrinum* (species No.1154) and *P. waksmanii* (species No.1099) also showed considerable antimicrobial activities ($p<0.05$). The values of inhibition zones are shown in Table 1 and 2.

Identification of Mycotoxins

Two different toxins were identified in *P. aurantiogriseum* culture: penicillic acid and citrinin and their R_f were 0.77 and 0.85, respectively. Citrinin and penicillic acid were also

Table 1: Diameter of inhibition zones (mm) of tested supernatants on disc diffusion test

	Pathogenic microorganism				
Extract No.	*Candida albicans*	*Bacillus subtillis*	*Staphylococcus aureus*	*Salmonella typhi*	*Escherchia coli*
1102	10±0.02	10±0.02	10±0.02	10±0.02	10±0.02
1103	(a)	10±2	20±5	10±2	10±2
1053	(a)	10±5	10±2	10±2	10±2
1099	15±2	30±0.5	20±5	20±5	20±5
1130	(a)	10±0.02	10±0.02	(a)	(a)
1138	(a)	(a)	(a)	(a)	(a)
1139	(a)	10±2	10±2	10±2	10±2
1148	(a)	(a)	(a)	(a)	(a)
1154	15±2	20±5	10±2	10±2	10±2
1164	15±2	30±5	20±5	20±5	30±5
1193	15±2	30±5	15±2	15±2	15±2
1161	(a)	10±2	10±2	(a)	10±2
1146	(a)	(a)	(a)	(a)	(a)
1143	(a)	(a)	(a)	(a)	10±2
Control	20±5	35±5	35±5	35±5	35±5

Control: Miconazol (*C. albicans*); chloramphenicol (bacteria); (a): Absence of inhibition zone

Table 2: Diameter of inhibition zones (mm) of tested methanol extracts on disc diffusion test

	Pathogenic microorganism				
Extract No.	*Candida albicans*	*Bacillus subtillis*	*Staphylococcus aureus*	*Salmonella typhi*	*Escherchia coli*
1102	(a)	(a)	(a)	(a)	(a)
1103	(a)	10±0.05	(a)	(a)	(a)
1053	(a)	(a)	10±0.05	(a)	10±0.05
1099	(a)	(a)	(a)	(a)	30±0.5
1130	(a)	(a)	(a)	(a)	(a)
1138	(a)	(a)	(a)	(a)	(a)
1139	(a)	(a)	(a)	(a)	(a)
1148	(a)	(a)	(a)	(a)	(a)
1154	(a)	(a)	10±2	(a)	10±2
1164	15±2	20±5	20±5	20±5	20±5
1193	10±2	30±5	15±2	15±2	15±2
1161	(a)	(a)	10±0.05	(a)	10±0.05
1146	(a)	(a)	10±2	(a)	(a)
1143	(a)	(a)	10±2	(a)	10±2
1093	(a)	(a)	10±2	(a)	(a)
1098	(a)	10±2	10±2	10±2	10±2
1165	(a)	10±2	10±2	10±2	15±2
1151	(a)	10±2	10±2	(a)	(a)
1159	(a)	15±5	15±5	(a)	(a)
1129	(a)	15±2	15±2	(a)	(a)
Control	20±5	35±5	35±5	35±5	35±5

Control: Miconazol (*C. albicans*); chloramphenicol (bacteria); (a): Absence of inhibition zone

characterized in the cultures of *P. citrinum* and *P. viridicatum*, respectively. *P. waksmanii* could produce two metabolites with R_f 0.08 and 0.15 and these two metabolites were purified for further evaluation.

DISCUSSION

Microorganisms produce many bioactive compounds as secondary metabolites including antibiotics and cytotoxic compounds (Nakashima *et al*., 2008). To identify the biological activities of the metabolites produced by the *Penicillium* strains, isolated from North of Iran, we screened these species for antibiotic activities. This is the first report of the biological activity of *Penicillium* strains from soils of Northern Iran.

Some of the most well known metabolites are produced by species of *Penicillium*, the most famous and economically important being penicillins produced by *Penicillium chrysogenum* (Raper and Thom, 1949), mycophenolic acid produced by *Penicillium brevicompactum* (Bentley, 2000) and compactins produced by *Penicillium solitum* (Frisvad and Filtenborg, 1989). *Penicillium* strains are also well known because a large number of them produce mycotoxins (Frisvad *et al*., 2004).

Many of these mycotoxins such as citrinin and penicillic acid have also antimicrobial activity (Wang *et al*., 2004; Kang *et al*., 2007).

Among the *Penicillium* strains in our study, *P. aurantiogriseum* showed to be one of the most active isolates. These potency may be due to the simultaneous production of citrinin and penicillic acid in the cultures of this strain. There are few reports of the production of citrinin by *P. aurantiogriseum*, but Varnaite *et al*. (2006) also reported the production of citrinin by *P. aurantiogriseum*(Varnaite *et al*., 2006). Few reports are available about simultaneous production of citrinin and penicillic acid by *P. aurantiogriseum*.

Citrinin is the only mycotoxin produced by *P. citrinum*. *P. citrinum* is the major producer of this toxin, but production by *P. expansum* and *P. verrucosum* has also been

reliably reported. Literature citations indicate that at least 22 *Penicillium* species have been reported to produce citrinin, but the great majority of these are either regarded as synonyms, or require confirmation (Pitt and Leistner, 1988).

The antibacterial activity of *P. aurantiogriseum* and *P. viridicatum*, has been reported by Khaddor *et al.* (2007). They found that *P. aurantiogriseum* produces penicillic acid, terrestric acid and aurantiamine, while penicillic acid, terrestric acid, brevianamide A and xanthomegnin were produced by *P. viridicatum*.

We detected penicillic acid in the culture of *P. viridicatum*. The potential for production of penicillic acid by *P. viridicatum* also reported by Frisvad and Filtenborg (1983).

P. waksmanii is a ubiquitous anamorphic fungus, widespread at once in land and in various aquatic environments such as sewage sludge or algae (Petita *et al.*, 2004).

Actually, different metabolites have been isolated from cultures of *P. waksmanii*: two alkaloids (Kozlovskii *et al.*, 1997), four pyrones (pyrenocines A, B, D and E with significant cytotoxicity) and three sulfur-containing dioxopiperazines (Amagata *et al.*, 1998). Furthermore, Petita *et al.* (2004) has also detected griseofulvin in a marine strain of *Penicillium waksmanii* in 2004. *P. waksmanii* is an important producer of cellulase and hemicellulase as well (Han *et al.*, 2009).

Present finding about antimicrobial activity of terrestrial *Penicillium waksmanii* was not reported yet. The TLC analysis of metabolites from *Penicillium waksmanii* with dragendorff reagent detected that these two metabolites were not nitrogenous compounds like alkaloids and should be examined for further elucidations.

Malmstrom *et al.* (2000) have confirmed that *P. citrinum* (25 isolates) consistently produced citrinin and tanzawaic acid A, Varnaite *et al.* (2006) also reported the production of citrinin by *P. aurantiogriseum*.

Xin *et al.* (2005) also found a new cytotoxic compound from *P. aurantiogriseum*.

CONCLUSION

The aim of this study was to evaluate the biotechnological potential of North Iranian soil fungal species, as a source of antibacterial compounds that could be used as lead compounds to develop new antibiotics for clinical/therapeutic use.

As a conclusion, the selected species should further examine for the characterization of their metabolites and optimize for the culture conditions in large scale production.

ACKNOWLEDGMENT

The authors are grateful to Mazandaran University of Medical Sciences for financial support (grant No. 87-87).

REFERENCES

Amagata, T., K. Minoura and A. Numata, 1998. Cytotoxic metabolites produced by a fungal strain from a *Sargassum* algae. J. Antibiot., 51: 432-434.

Bentley, R., 2000. Mycophenolic acid: A one hundred year Odyssey from antibiotic to immunosuppressant. Chem. Rev., 100: 3801-3826.

Bertinetti, B.V., I.N. Pena and G.M. Cabrera, 2009. An antifungal tetrapeptide from the culture of *Penicillium* canescens. Chem. Biodiver., 6: 1178-1183.

Du, L., D. Li, T. Zhu, S. Cai, F. Wang, X. Xiao and Q. Gu, 2009. New alkaloids and diterpenes from a deep ocean sediment derived fungus *Penicillium* sp. Tetrahedron, 65: 1033-1039.

Esther, M.F., C.L. Mateus, M. Castro, Jacqueline and A. Takahashi, 2007. Antimicrobial properties of sclerotiorin, IsocHromophilone VI and pencolide, metabolites from Brazillian cerrado isolate of *Penicillium sclerotiorum* van beyma. Braz. J. Microbiol., 38: 785-789.

Frisvad, J.C. and O. Filtenborg, 1983. Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites. Applied Environ. Microbiol., 46: 1301-1310.

Frisvad, J.C. and O. Filtenborg, 1989. Terverticillate Penicillia: chemotaxonomy and mycotoxin production. Mycologia, 81: 837-861.

Frisvad, J.C., J. Smedsgaard, T.O. Larsen and R.A. Samson, 2004. Mycotoxins, drugs and other extrolites produced by species in *Penicillium* subgenus *Penicillium*. Stud. Mycol., 49: 201-241.

Han, L., J. Feng, C. Zhu and X. Zhang, 2009. Optimizing cellulase production of *Penicillium waksmanii* F10-2 with response surface methodology. Afr. J. Biotechnol., 8: 3879-3886.

Kang, S.W., C.H. Park, S.I. Hong and S.W. Kim, 2007. Production of penicillic acid by *Aspergillus sclerotiorum* CGF. Bioresour. Technol., 98: 191-197.

Khaddor, M., R. Saidi, A. Aidoun, A. Lamarti and T. Elaraki *et al*., 2007. Antibacterial effects and toxigenesis of *Penicillium aurantiogriseum* and *P. viridicatum*. Afr. J. Biotechnol., 6: 2314-2318.

Kozlovskii, A.G., N.G. Vinokurova, V.P. Zhelifonova and S.M. Ozerskaya, 1997. Alkaloid formation by Penicillia of the series fellutana and canescentia. Mikrobiologiia, 66: 429-433.

Leitao, A.L., 2009. Potential of *Penicillium* species in the bioremediation fields. Int. J. Environ. Res. Public Health, 6: 1393-1417.

Malmstrom, J., C. Christophersen and J. Frisvad, 2000. Secondary metabolites characteristic of *Penicillium citrinum*, *Penicillium steckii* and related species. Phytochemistry, 54: 301-309.

Montematini, A.C., M. Liotta, C.B. Venturi and L. Calegari, 2000. Antibacterial activity of *Penicillium* sp. strains isolated in extreme environments. Polar Biol., 23: 294-297.

Nakashima, T., S. Mayuzumi, S. Inaba, J.Y. Park and K. Anzai *et al*., 2008. Production of bioactive compounds based on phylogeny in the genus *Penicillium* preserved at NBRC. Biosci. Biotechnol. Biochem., 72: 3051-3054.

Petit, P., E.M. Lucas, L.M. Abreu, L.H. Pfenning and J.A. Takahashi, 2009. Novel antimicrobial secondary metabolites from a *Penicillium* sp. isolated from Brazilian cerrado soil. Electr. J. Boitechnol., 2: 1-9.

Petita, K.E., F. Mondeguerb, M.F. Roquebertc, J.F. Biarda and Y.F. Pouchusa, 2004. Detection of griseofulvin in a marine strain of *Penicillium waksmanii* by ion trap mass spectrometry. J. Microbiol. Methods, 58: 59-65.

Pitt, J.I., 2000. A Laboratory Guide to Common *Penicillium* species. 1st Edn., Food Science Australia, North Ryde, Australia.

Pitt, J.L. and L. Leistner, 1988. Toxigenic *Penicillium* Species. In: Mycotoxins and Animal Feedingstuffs I. The Toxigenic Fungi, Smith, J.E. (Ed.). CRC Press, Boca Raton, Florida.

Rancic, A., M. Sokovi, A. Karioti, J. Vukojevi and H. Skaltsa, 2006. Isolation and structural elucidation of two secondary metabolites from the filamentous fungus *Penicillium ochrochloron* with antimicrobial activity. Environ. Toxicol. Pharmacol., 22: 80-84.

Raper, K.B. and C. Thom, 1949. Manual of the Penicillia. William and Wilkins Co., Baltimore, USA.

Rundberget, T., I. Skaar and A. Flaoyen, 2004. The presence of *Penicillium* and *Penicillium* mycotoxins in food wastes. Int. J. Food. Microbiol., 90: 181-188.

Silva, M.G., N.A J.C. Furtad, M.T.V. Pupo, M.J. Fonseca and S. Said, 2004. Antibacterial activity from *Penicillium corylophilum* Dierckx. Microbiol. Res., 159: 317-322.

Sonjak, S., J.C. Frisvad and N.G. Cimerman, 2005. Comparison of secondary metabolite production by *Penicillium* crustosum strains, isolated from Arctic and other various ecological niches. FEMS Microbiol. Ecol., 53: 51-60.

Takahashi, J.A., M.C. Castro, G.G. Souza, E. Lucas and A. Bracarense *et al*., 2008. Isolation and screening of fungal species isolated from Brazilian Cerrado soil for antibacterial activity against *E. coli*, *S. aureus*, *S. typhimurium*, *S. pyogenes* and *L. monocytogens*. J. Mycol. Med., 18: 198-204.

Uchida, R., R. Imasato, Y. Yamaguchi, R. Masuma, K. Shiomi, H. Tomoda and S. Omura, 2006. Yaequinolones, new insecticidal antibiotics produced by *Penicillium* sp. FKI-2140. J. Antibiot., 59: 646-651.

Varnaite, R., V. Raudoniene and A. Lugauskas, 2006. Chromatographic characteristics of secondary metabolites of micromycetes detected on vegetables and grains. Ecologica, 3: 48-53.

Wang, J.J., C.L. Lee and T.M. Pan, 2004. Modified mutation method for screening low citrinin producing strains of *Monascus purpurus* on rice culture. J. Agric. Food. Chem., 52: 6977-6982.

Xin, Z.H., W.M. Zhu, O.O. Gu, Y.C. Fang, L. Duan and C.B. Cui, 2005. A new cytotoxic compound from *Penicillium auratiogriseum*, symbiotic or epiphytic fungus of sponge *Mycale plumose*. Chin. Chem. Lett., 16: 1227-1229.