Microbiol. Biotechnol. (2002), **12**(6), 1017–1021

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

In Vitro Bactericidal and Anticancer Activity of New Metabolite, ARK42, Isolated from *Aspergillus repens* K42

PARK, JE WON, BEOM SEOK SONG, DOJIN RYU[1], CHAN LEE[2], AND YOUNG BAE KIM*

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea
[1]Department of Food Science and Technology, University of Nebraska, Lincoln, NE 68583-0919, U.S.A.
[2]Department of Food Science and Technology, Chung-Ang University, Ansung 456-756, Korea

Received: October 9, 2002
Accepted: November 29, 2002

Abstract A novel antibacterial metabolite, ARK42, was isolated from a xerophilic fungal strain K42, and identified as *Aspergillus repens* based on its morphological characteristics. The metabolite exhibited antibacterial activities towards *Staphylococcus aureus*, *Bacillus cereus*, and *Pseudomonas aeruginosa*, with MICs of 25, 12.5, and 3.125 μg/ml, respectively, and killed *Pseudomonas aeruginosa* with minimal bactericidal concentration (MBC) of 12.5 μg/ml. Furthermore, anticancer activities were demonstrated against human colon cancer DLD-1 and lung cancer LXFL529 cells with an IC_{50} of 10 and 1 μg/ml, respectively.

Key words: *Aspergillus repens*, bactericidal activity, anticancer activity

The *Aspergillus glaucus* group (Anamorph, *Eurotium* Link: Fr) is generally prevalent in the mycoflora found in stored grains, because of its ability to grow at minimum moisture levels [13]. Some of these xerophiles, for example, *A. chevalieri* and *A. amsterlodami*, are considered to be mycotoxigenic [8], while other representatives, including *A. repens*, produce metabolites, such as anthraquinones and alkaloids [4, 22]. These metabolites are known to exhibit a variety of biological effects, such as inhibition of bacterial and fungal growth, mutagenicity, hepatoxicity, and toxicity in cockerels and rabbits [1, 2, 3, 5, 6, 17, 28, 29], and they have recently attracted worldwide interest as antitumor therapeutics [7, 13, 27, 30]. It has been suggested that the fungal anthraquinones and alkaloids may be responsible for such potent biological activities, and furthermore, none of the biologically active metabolites from the *Aspergillus glaucus* group have been found to be extractable, except by using organic solvents such as chloroform, dichloromethane, and ethyl acetate [2, 5, 22, 29, 30]. According to Bachmann *et al.* [5], all the fungal anthraquinone derivatives can be extracted with dichloromethane and chloroform. In addition, the alkaloid echinulin causing feed refusal by swine has been reported to be extractable with acetone and ethyl acetate [29]. Nevertheless, until now, there has been no report dealing with the biological activity of a water-soluble metabolite. In a recent screening by the current authors to discover biologically active metabolites from the xerophiles in stored rice, the fungal strain K42 was found to produce a new water-soluble antibacterial metabolite that was tentatively named ARK42. Accordingly, the current study was undertaken to investigate the taxonomy of the K42 strain, along with the isolation, physicochemical properties, and *in vitro* antibacterial and anticancer activities of ARK42.

The fungal strain K42 was originally isolated from a rice sample collected in Korea. CZ20S (Czapek agar with 20% sucrose: $NaNO_3$ 0.3%, K_2HPO_4 0.1%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, sucrose 20%, and agar 1.5%) and YE20S (Yeast extract agar with 20% sucrose: yeast extract 2%, sucrose 20%) media were used to identify strain K42 and for the production of ARK42, respectively [24]. The morphological characteristics of the strain K42 were determined with a culture incubated for two weeks on CZ20S at 25°C under an electron microscope (JEOL 8400 SEM, Peabody, MA, U.S.A.). Spectral and physicochemical data for ARK42 were obtained by the following instruments: UV, Kontron UVIKON 930; IR, Bio-rad FT-IR/RAMAN spectrophotometer; and Elemental analyzer, CE instruments Flash EA 1112 series. The microtiter-based MICs and MBCs (minimal bactericidal concentrations) of ARK42 against test microorganisms were determined using a Labsystems Bioscreen C reader, as recommended by the National Committee for Clinical

*Corresponding author
Phone: 82-2-3290-3416; Fax: 82-2-3290-3416;
E-mail: ybkim@korea.ac.kr

Laboratory Standards [19, 20]. The microorganisms tested in the current study were as follows: Gram (+) *Bacillus cereus* KCTC 1012, *B. subtilis* KCTC 1021 [16], *Listeria monocytogenes* ATCC 19118 [15], *Staphylococcus aureus* KCTC 1916 [11]; Gram (-) *Enterobacter cloacae* KCTC 1321B, *Escherichia coli* ATCC 43894 [12], *Klebsiella oxytoca* ATCC 10881, *Pseudomonas aeruginosa* ATCC 9027 [14], *P. aeruginosa* KCCM 11328, and *Salmonella typhimurium* KCTC 1925 [9]. The anticancer activity of ARK42 against human tumor cells was colorimetrically determined at 590 nm according to a sulforhodamine B assay [26]. Human colon cancer cells, DLD-1, and lung cancer cells, LXFL529, were obtained from the Korean Cell Line Bank (KCLB), Seoul, Korea. The concentration required to reduce cell viability by 50% (IC_{50}) was used as the index for anticancer activity.

The colonies of the strain K42 on CZ20S spread broadly and irregularly, attaining a diameter of 8 cm in 2 weeks at 25°C, were flat, and characterized by broad zones of light yellow to olive conidial heads. Reverse was colored in dark brown. The conidiophores were 170 to 200 μm in length and 6 μm in width (Fig. 1A). The vesicle produced at the top of the conidiophores was mostly subglobose and 22 to 32 μm in diameter. The conidia were globose, ornamented, and 4 to 6 μm in diameter. The asci were globose to subglobose with 8 spores and 13 μm in diameter. The ascospores were 3 to 6 μm in diameter, and subglobose with a convex smooth surface, without any equatorial ridges and furrows (Fig. 1B). Therefore, according to the taxonomic criteria of the genus *Aspergillus* established by Raper and Fennell [23], the strain was identified as *A. repens*. The isolation procedure of ARK42 is outlined in Fig. 2. After incubation at 25°C in YE20S as a static culture for 8 weeks, the broth was filtered with Whatman #42 (Whatman® Int. Ltd., England) and titrated to pH 7.

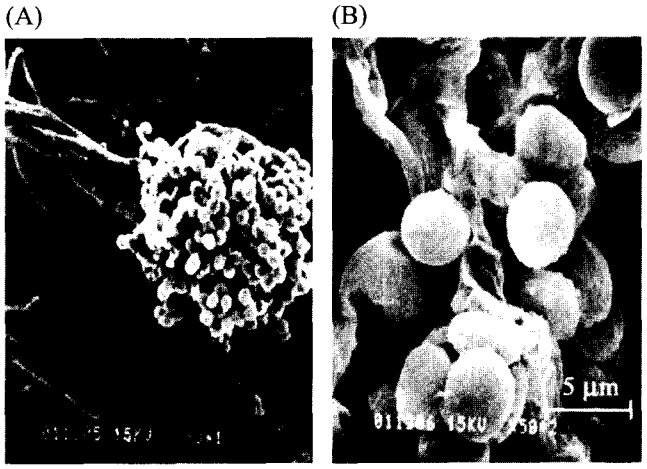

Fig. 1. Photographs of (A) conidiospores ×90 and (B) ascospores ×5,000 of strain K42 grown on CZ20S at 25°C for 2 weeks.

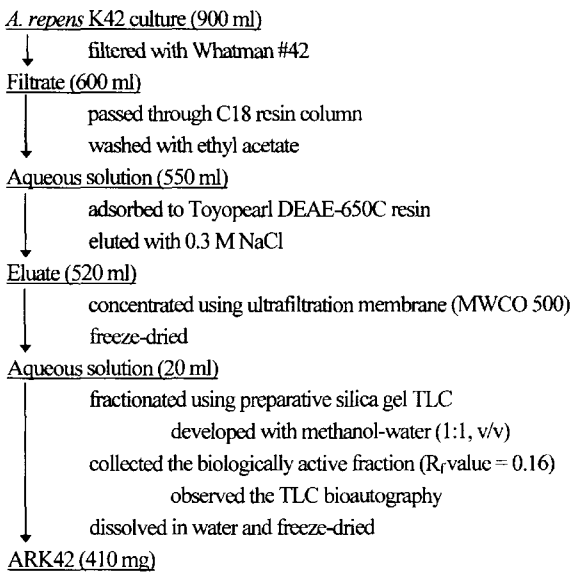

Fig. 2. Isolation procedures for ARK42 produced from *A. repens* K42.

The filtrate was passed through a C18 resin (Waters, Milford, MA, U.S.A.) column to remove the pigments, then washed with ethyl acetate. Next, the aqueous layer was applied to a Toyopearl® DEAE-650C (Supelco Inc., Bellefonte, PA, U.S.A.) resin, and eluted with 0.3 M NaCl. The eluate was then concentrated using an ultrafiltration membrane (MWCO 500, Millipore Co., Bedford, MA, U.S.A). After being freeze-dried, the concentrate was further purified using a preparative silica gel TLC (Kieselgel 60F254, 1

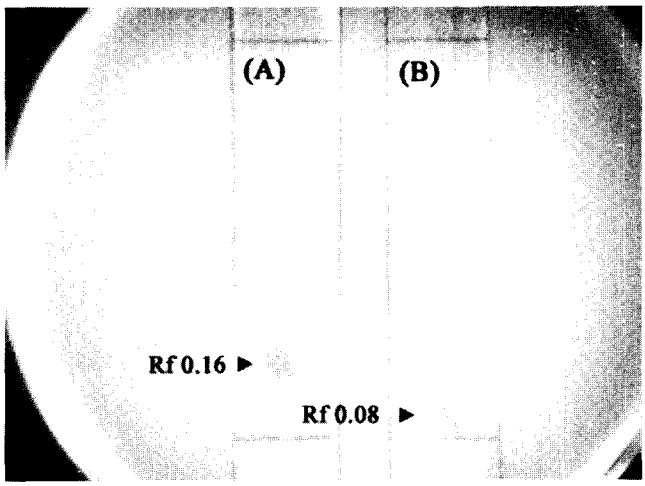

Fig. 3. TLC bioautography of ARK42 isolated from *A. repens* K42 culture.

Each TLC plate, (A) developed with methanol-water (1:1, v/v) and (B) with acetonitrile-water (1:1, v/v), was overlaid with a soft agar containing *P. aeruginosa* ATCC 9027 to detect the antibacterial fraction [9, 18]. The arrow indicates the inhibition zone observed after 24 h of incubation at 37°C.

Table 1. Physicochemical data for ARK42 isolated from *A. repens* K42 culture.

Appearance	Colorless amorphous powder
Nature	Acidic
Solubility : soluble	Water, DMSO
insoluble	Ethyl acetate, chloroform, diethyl ether
TLC, silica gel developed with methanol-water (1:1, v/v)	R_f 0.16
Elements analyzed (%)	C: 22.19, H: 4.18, O: 31.65
UV λ_{max} in water (nm)	214, 289
IR ν_{max} (cm^{-1})	3,417, 2,931, 1,653

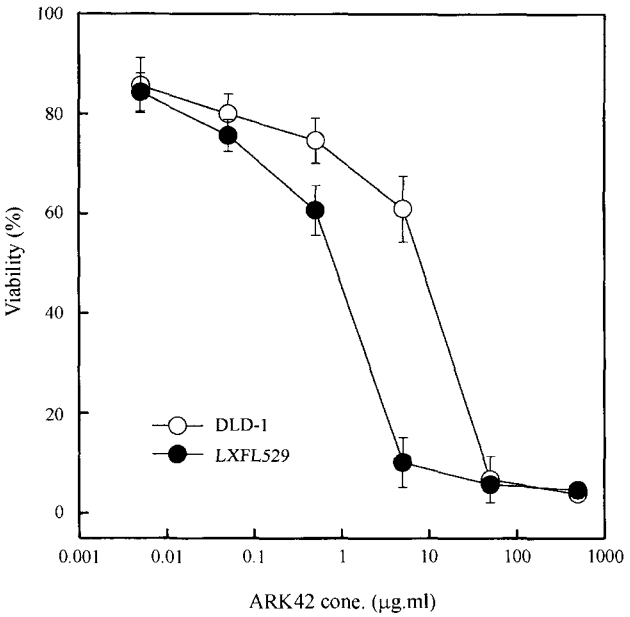

Fig. 4. Anticancer effects of ARK42 on human colon (DLD-1) and lung (LXFL529) cells.
The cells were incubated with ARK42 for 48 h. Growth inhibition was determined using a sulforhodamine B assay [19]. The data were calculated as viable percent, determined by A_{590} of treated cells over control cells ×100. The values given are the mean±SD of three separate experiments, each performed in quadruplicate.

μm thickness, Merck Co. Ltd., Whitehouse Station, NJ, U.S.A.), and the gel was developed with methanol-water (1:1, v/v) to give ARK42 (R_f value=0.16). The TLC bioautography of ARK42 is shown in Fig. 3. The active spot was cut from the silica plate, extracted with water, and finally freeze-dried to give ARK42 as a colorless amorphous powder. The powder was readily soluble in water and DMSO, yet insoluble in ethyl acetate, chloroform, and diethyl ether. Various physicochemical data for ARK42 are summarized in Table 1. UV absorption was exhibited at 214 and 289 nm, and IR absorption at 3,417, 2,931, and 1,653 cm^{-1}, due to phenolic hydroxyl and carbonyl groups, respectively. ARK42 showed antibacterial activities against *Staphylococcus aureus*, *Bacillus cereus*, and *Pseudomonas aeruginosa* with MICs of 25, 12.5, and 3.125 μg/ml, respectively (Table 2). It also showed bactericidal activity against *P. aeruginosa*, with an MBC of 12.5 μg/ml. As shown in Fig. 4, anticancer activities were observed against human colon cancer DLD-1 and lung cancer LXFL529 cells. The IC_{50} values of ARK42 against DLD-1 and LXFL529 was about 10 and 1 μg/ml.

In summary, we isolated *A. repens* K42 from stored rice. The strain was found to produce a biologically active metabolite, ARK42, which exhibited bactericidal activity against *P. aeruginosa*. Experiments with two human cancer cells also revealed anticancer activities. Based on the fact that ARK42 was adsorbed by a DEAE anion exchanger, it would appear that ARK42 contains carboxyl and/or carbonyl functional groups, which was coincident with its acidic nature in water and the IR spectrum results. Therefore, to the best of our knowledge, this is the first report on the isolation of a water-soluble metabolite, which exhibits bactericidal and anticancer activities, from the *A. glaucus* group. Further studies of ARK42 are currently underway to elucidate its structure using mass spectrometry

Table 2. *In vitro* antibacterial activity of ARK42 isolated from *A. repens* K42 culture.

	Bacteria	MIC (μg/ml)[a]	MBC (μg/ml)[b]
Gram-positive	*Bacillus cereus* KCTC 1012	12.5	50
	B. subtilis KCTC 1021	12.5	50
	Listeria monocytogenes ATCC 19118	25	-[c]
	Staphylococcus aureus KCTC 1916	25	-
Gram-negative	*Enterobacter cloacae* KCTC 1321B	>100	-
	Escherichia coli ATCC 43894	>100	-
	Klebsiellas oxytoca ATCC 10881	>100	-
	Pseudomonas aeruginosa ATCC 9027	6.25	12.5
	P. aeruginosa KCCM 11328	3.125	12.5
	Salmonella typhimurium KCTC 1925	>100	-

[a]The MIC was defined as the lowest concentration at which there was no sign of growth after 36 h of incubation.
[b]The MBC was determined by subculturing the broth from each well with no sign of growth after 36 h of incubation and from the control well (no addition of ARK42). The MBC was defined as the lowest concentration yielding CFU<0.1% of the control well.
[c]Not tested.

(MS) and nuclear magnetic resonance (NMR) spectrometry, and examine other biological activities such as its mutagenicity in the Ames/*Salmonella* microsome system [25].

Acknowledgment

This research was supported by the Post-doctoral fellowship program of the Brain Korea 21 project in 2002.

REFERENCES

1. Ali, M., N. Mohammed, M. A. Alnaqeeb, R. A. Hassan, and H. S. Ahmad. 1989. Toxicity of echinulin from *Aspergillus chevalieri* in rabbits. *Toxicol. Lett.* **48:** 235-241.
2. Anke, H., I. Kolthoum, H. Zähner, and H. Laatsch. 1980. Metabolic products of microorganisms. 185. The anthraquinones of the *Aspergillus glaucus* group. 1. Occurrence, isolation, identification and antimicrobial activity. *Arch. Microbiol.* **126:** 223-230.
3. Anke, H., I. Kolthoum, H. Zähner, and H. Laatsch. 1980. Metabolic products of microorganisms. 192. The anthraquinones of the *Aspergillus glaucus* group. 2. Biological activity. *Arch. Microbiol.* **126:** 231-236.
4. Ashley, J. N., H. Raistrick, and T. Richards. 1939. The crystalline colouring matters of species in the *Aspergillus glaucus* series. Part II. *Biochem. J.* **33:** 1291-1303.
5. Bachmann, M., J. Lûthy, and C. Schlatter. 1979. Toxicity and mutagenecity of molds of the *Aspergillus glaucus* group. Identification of physcion and three related anthraquinones as main toxic constituents from *Aspergillus chevalieri*. *J. Agric. Food Chem.* **27:** 1342-1347.
6. Bachmann, M., P. Blaser, J. Lüthy, and C. Schlatter. 1992. Toxicity and mutagenecity of anthraquinones from *Aspergillus chevalieri*. *J. Environ. Pathol. Toxicol. Oncol.* **11:** 49-52.
7. Choi, J.-Y., C.-S. Park, J. Choi, H. Rhim, and H.-J. Chun, 2001. Cytotoxic effect of urushiol on human ovarian cancer cells. *J. Microbiol. Biotechnol.* **11:** 399-405.
8. Frisvad, J. C. and U. Thrane. 1995. Mycotoxin production by food-borne fungi, pp. 251-260. *In* Samson, R. A., E. S. Hoekstra, J. C. Frisvad, and O. Filtenborg (eds.), *Introduction to Food-Borne Fungi*, Centraalbureau voor Schimmelcultures, The Netherlands.
9. Hahm, D.-H., M.-J. Yeom, E. H. Lee, I. Shim, H.-J. Lee, and H.-Y. Kim. 2001. Effect of scutellariae radix as a novel antibacterial herb on the *ppk* (polyphosphate kinase) mutant of *Salmonella typhimurium*. *J. Microbiol. Biotechnol.* **11:** 1061-1065.
10. Hostettman, K. 1998. Strategy for the biological and chemical evaluation of plant extracts. *Pure Appl. Chem.* **70:** 2122-2130.
11. Jeon, S.-M., H.-J. Kim, K.-B. Lee, J.-W. Kim, and M.-N. Kim. 2001. Antibacterial effect of the surface-modified biomedical polyurethane against *Staphylococcus aureus* and *Staphylococcus epidermidis*. *J. Microbiol. Biotechnol.* **11:** 259-265.
12. Jeon, Y.-J. and S.-K. Kim. 2001. Effect of antimicrobial activity by chitosan oligosaccharide N-conjugated with asparagines. *J. Microbiol. Biotechnol.* **11:** 281-286.
13. Kamei, H., T. Koide, T. Kojima, Y. Hashimoto, and M. Hasegawa. 1998. Inhibition of cell growth in culture by quinines. *Cancer Biotherapy Radiopharmaceut.* **13:** 185-188.
14. Kim, K.-Y., P. M. Davidson, and H.-J. Chung. 2000. Antomicrobial effectiveness of pine needle extract on foodborne illness bacteria. *J. Microbiol. Biotechnol.* **10:** 227-232.
15. Kim, S.-Y., Y.-M. Lee, S.-Y. Lee, Y.-S. Lee, J.-H. Kim, C. Ahn, B.-C. Kang, and G.-E. Ji. 2001. Synergistic effect of citric acid and Pediocin K1, a bacteriocin produced by *Pediococcus* sp. K1, on inhibition of *Listeria monocytogenes*. *J. Microbiol. Biotechnol.* **11:** 831-837.
16. Kim, Y.-H., W.-H. Yeo, Y.-S. Kim, S.-Y. Chae, and K.-S. Kim. 2000. Antiviral activity of antibiotic peptabiols, Chrysospemins B and D, produced by *Apiocrea* sp. 14T against TMV infection. *J. Microbiol. Biotechnol.* **10:** 522-528.
17. Krivobok, S., F. S. Murandi, R. Steiman, D. R. Marzin, and V. Betina. 1992. Mutagenecity of substituted anthraquinones in the Ames/*Salmonella* microsome system. *Mutation Res.* **279:** 1-8.
18. Mah, J.-H., K.-S. Kim, J.-H. Park, M.-W. Byun, Y.-B. Kim, and H.-J. Hwang. 2001. Bacteriocin with a broad antimicrobial spectrum, produced by *Bacillus* sp. isolated from Kimchi. *J. Microbiol. Biotechnol.* **11:** 577-584.
19. National Committee for Clinical Laboratory Standards. 1998. *Methods for determining bactericidal activity of antimicrobial agents - Approved guideline*, M26-A NCCLS, Wayne, PA, U.S.A.
20. National Committee for Clinical Laboratory Standards. 2000. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically - 5th edition: Approved standard* M7-A5 NCCLS, Wayne, PA, U.S.A.
21. Pitt, J. I. 1975. Xerophilic fungi and the spoilage of foods of plant origin, pp. 273-307. *In* R. B. Duckworth (ed.), *Water Relations of Foods*, Academic Press, London, U.K.
22. Podojil, M., P. Sedmera, J. Vokoun, V. Betina, H. Baráthová, Z. Ďuraèková, K. Horáková, and P. Nemec. 1979. *Eurotium (Aspergillus) repens* metabolites and their biological activity. *Folia Microbiol.* **23:** 438-443.
23. Raper, K. B. and D. I. Fennell. 1965. *The genus Aspergillus. Chap. X. Aspergillus glaucus group*, pp. 147-196. Robert E. Krieger Publishing Co., Huntington, NY, U.S.A.
24. Samson, R. A., E. S. Hoekstra, J. C. Frisvad, and O. Filtenborg. 1995. *Introduction to Food-Borne Fungi*, pp. 308-311. 4th ed. Centraalbureau voor Schimmelcultures, The Netherlands.
25. Shon, Y.-H., S.-Y. Kim, J.-S. Lee, J.-K. Lim, and K.-S. Nam. 2001. Antimutagenic effect of polysaccharides extracted from soybeans fermented with *Basidiomycetes* on 2-amino-

3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx). *J. Microbiol. Biotechnol.* **11:** 346-349.

26. Skehan, P., R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J. T. Warren, H. Bokesch, S. Kenney, and M. R. Boyd. 1990. New colorimetric assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **82:** 1107-1112.
27. Strausee, D. G. 1978. Anthracyclines. Modern tumor-inhibiting agents. *Folia Microbiol.* **23:** 152-161.
28. Umeda, M., T. Yamashita, M. Saito, S. Sekita, C. Takahashi, K. Yoshihira, S. Natori, H. Kurata, and S. Udagawa. 1974. Chemical and cytotoxicity survey on the metabolites of toxic fungi. *Japan. J. Exp. Med.* **44:** 83-96.
29. Vesonder, R. F., R. Lambert, D. T. Wicklow, and M. L. Biehl. 1988. *Eurotium* spp. and echinulin in feed refused by swine. *Appl. Environ. Microbiol.* **54:** 830-831.
30. Yim, H., Y. H. Lee, C. H. Lee, and S. K. Lee. 1999. Emodin, an anthraquinone derivative isolated from the rhizomes of *Rheum palmatum*, selectively inhibits the activity of casein kinase II as a competitive inhibitor. *Planta Med.* **65:** 9-13.