J. Microbiol. Biotechnol. (2009), **19**(2), 204–207
doi: 10.4014/jmb.0810.566
First published online 30 December 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Antibacterial and Synergistic Activity of Isocryptomerin Isolated from *Selaginella tamariscina*

Lee, Juneyoung[1], Yunjung Choi[1], Eun-Rhan Woo[2], and Dong Gun Lee[1]*

[1]*School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Korea*
[2]*College of Pharmacy, Chosun University, Gwangju 501-759, Korea*

Received: October 9, 2008 / Accepted: November 14, 2008

We investigated novel antibacterial and synergistic activities of isocryptomerin isolated from *Selaginella tamariscina*. Isocryptomerin showed potent antibacterial activity against Gram-positive and Gram-negative bacterial strains including clinical isolates of antibiotic-resistant species such as methicillin-resistant *Staphylococcus aureus* (MRSA). Additionally, we further investigated the synergistic activity of isocryptomerin with a conventional antibiotic against MRSA. The result indicated that isocryptomerin had considerable synergistic activity in combination with cefotaxime. In summary, the present study suggests that isocryptomerin may have potential as a novel therapeutic agent for treatment of infectious diseases by not only human pathogenic bacteria but also multidrug-resistant bacteria.

Keywords: Isocryptomerin, antibacterial activity, synergistic activity

The success of infectious disease chemotherapy has been dimmed ever since the dawn of the antimicrobial drug era because of antibiotic resistance [18, 25]. Today, because of the emergence of resistant organisms [8, 22], numerous diseases are becoming increasingly difficult to treat, including microorganisms responsible for severe infections in hospitalized patients, foodborne pathogens, and sexually transmitted pathogens, some of which are now resistant to most available antimicrobial drugs [20, 21, 26]. The clinical impact of resistance is immense, characterized by increased cost, morbidity, and mortality [3, 10], hence the importance of developing new antimicrobial agents [2].

Isocryptomerin is one of the biflavonoids isolated from *Selaginella tamariscina*, which belongs to *Selaginellaceae*, a traditional medicinal plant for therapeutic agents in the Orient. Previously, isocryptomerin was reported to suppress lymphocyte proliferation and showed cytotoxicity against human tumor cell lines [17, 23]. Although several biological properties of isocryptomerin were studied, its antimicrobial activity and potential as therapeutic agents in human microbial infections have not been studied.

In this study, we suggest isocryptomerin as a latent drug for bacterial diseases by investigating its novel antibacterial and synergistic activities with a conventional antibiotic.

Plant Material, Extraction, and Isolation

Dried whole plants of *Selaginella tamariscina* Spring, which belongs to the *Selaginellaceae* family, were obtained from herbal drug stores in Gwangju. The plants were authenticated by the Department of Pharmacognosy, Chosun University. Voucher specimens (853-16) were deposited in the Herbarium of the College of Pharmacy, Chosun University. The whole plant of *Selaginella tamariscina* (0.6 kg) was extracted three times with MeOH at room temperature and evaporated to dryness under reduced pressure to obtain 50.54 g of residue. The methanol extract was suspended in water and then partitioned by dichloromethane, ethyl acetate, and *n*-butanol in turn. The EtOAc fraction (1.74 g) was subjected to column chromatography over a silica gel (Merck 43–60 and 63–200 μm; Germany; 200 g) eluting with a $CHCl_3$-MeOH-H_2O (12:1: 0.1→8:1: 0.1→5:1: 0.1→2:1: 0.1→1:1: 0.1→MeOH only) gradient system. Fractions were combined based on their TLC pattern to yield subfractions designated as E1-E10. Subfraction E3 (30.49 mg) was purified by column chromatography over a Sephadex LH 20 eluting with a MeOH to give isocryptomerin (3.75 mg). The physical and chemical data, including MS, ^{1}H NMR, ^{13}C NMR, and HSQC, of isocryptomerin were identical to those reported previously [17, 23, 15, 13]. The chemical structure of isocryptomerin is shown in Fig. 1.

Antibacterial Activity of Isocryptomerin

Bacillus subtilis (KCTC 1918) was obtained from the Korean Collection for Type Cultures (KCTC) of the Korea Research Institute of Bioscience and Biotechnology

*Corresponding author
Phone: +82-53-950-5373; Fax: +82-53-955-5522;
E-mail: dglee222@knu.ac.kr

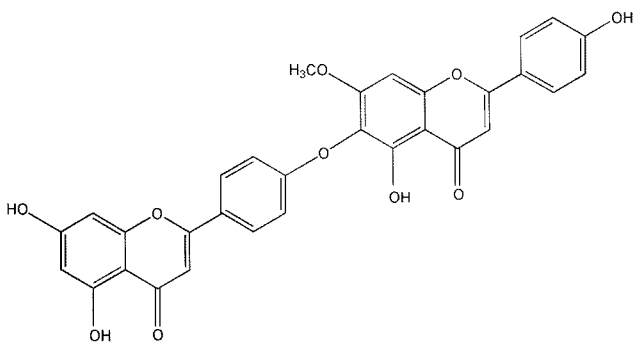

Fig. 1. Chemical structure of isocryptomerin isolated from *Selaginella tamariscina.*

(KRIBB), Daejeon, Korea. *Escherichia coli* O-157 (ATCC 43895) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, U.S.A.). Methicillin-resistant *Staphylococcus aureus* (MRSA) was clinically isolated from patients of the Kyungpook National University Hospital, Daegu, Korea. The MRSA isolate was screened for the presence of the *mec*A gene using PCR [6]. Bacterial cells (2×10^7 cells/ml) were inoculated into a Mueller-Hinton broth [12, 16] and 0.1 ml/well was dispensed onto 96-well microtiter plates. The bacteria numbers were calculated by measurement of turbidity with a spectrophotometer (DU530; Beckman, Fullerton, CA, U.S.A.) [19]. MICs (minimum inhibitory concentrations) were determined by a serial 2-fold dilution of test compounds, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [7]. After 24 h of incubation at 37°C, the minimal concentration of compound required to prevent the growth of a given test organism was determined and was defined as the MIC. The growth was assayed with a microtiter ELISA Reader (Molecular Devices Emax, CA, U.S.A.) by monitoring absorption at 620 nm [24]. In the present study, propionic acid and nisin were used as positive controls and purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Propionic acid is an antibacterial agent that is widely used as a food preservative agent [5]. Nisin is used in processed cheese production to extend the shelf life by suppressing Gram-positive spoilage and pathogenic bacteria [1]. The result showed that isocryptomerin, in MIC values of 10–20 μg/ml, had remarkable antibacterial activity against Gram-positive and Gram-negative bacteria including the clinical isolates of antibiotic-resistant bacteria like MRSA, responsible for difficult-to-treat infections in humans (Table 1). This result suggests that isocryptomerin can be applied as a therapeutic model for treating bacterial infectious diseases.

Table 1. Antibacterial activity of isocryptomerin.

Bacterial strains	MIC (μg/ml)		
	Isocryptomerin	Propionic acid	Nisin
B. subtilis	20	40	60
MRSA	10	40	40
E. coli O-157	20	40	ND[a]

[a]Not determined.

Synergistic Activity of Isocryptomerin with Cefotaxime

Nowadays, abuse of conventional antibiotics to treat microbial infections has led to the emergence of antibiotics-resistant pathogens. Drug efflux transporters as MDR (multidrug-resistant) pumps are capable of antibiotic resistance, because they selectively pump toxic molecules out to the extracellular condition by keeping toxic molecules to a minimum in an intracellular condition [11]. Above all, we examined sensitivities of antibiotics to MRSA. We used ampicillin, cefotaxime, chloramphenicol, kanamycin, and vancomycin as positive controls. These antibiotics were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Specifically, MRSA used in this study was not sensitive to several known antibiotics such as cefotaxime and kanamycin (Table 2). One strategy employed to overcome these resistance mechanisms is the use of combination of antibiotics, such as β-lactams together with β-lactamase inhibitors [9]. Cefotaxime, one kind of commercial β-lactam antibiotics, is known to inhibit the synthesis of the bacterial cell wall by binding to the reactive Ser62 of the D-alanyl-D-alanine carboxypeptidase/transpeptidase, which catalyzes the final step in the cross-linking of the bacterial cell wall peptidoglycan [14].

The combination effect of isocryptomerin (2.3 μg) and cefotaxime (10 μg) was investigated against MRSA and the effects were evaluated by using the fractional inhibitory concentration index (FICI). FICI was calculated using the following formula: FICI=($MIC_{Drug\ A}$ in combination/$MIC_{Drug\ A}$ alone)+($MIC_{Drug\ B}$ in combination/$MIC_{Drug\ B}$ alone). Exponential phased cells of MRSA cultured in LB medium were seeded on 96-well microtiter plates at a density of 2×10^7 cells (100 μl per well). Isocryptomerin and cefotaxime were added to the well, and the cell suspension was incubated for 12 h at 37°C. The activity was determined by measuring the absorbance at 620 nm using a microtiter ELISA Reader (Molecular Devices Emax, CA, U.S.A.). The FICI was

Table 2. Antibacterial activity of isocryptomerin or antibiotics against MRSA.

Bacterial strains	MIC (μg/ml)					
	Isocryptomerin	Ampicilln	Cefotaxime	Chloramphenicol	Kanamycin	Vancomycin
MRSA	10	1.25	40	<0.625	320	<0.625

Table 3. Combinational activity of isocryptomerin and cefotaxime

Bacterial strains	FICI[a]	Interactive category
MRSA	0.48	Synergy

[a]The fractional inhibitory concentration index (FICI) was calculated by the formula: FICI=($MIC_{Drug\ A}$ in combination/$MIC_{Drug\ A}$ alone)+($MIC_{Drug\ B}$ in combination/$MIC_{Drug\ B}$ alone).

calculated from both the FICI of isocryptomerin and cefotaxime. The FICI<0.5, 0.5≤FICI≤0.75, 0.76≤FICI≤1, 1<FICI≤4, and >4 were defined as synergy, partial synergy, additive effect, indifference, and antagonism, respectively [4]. The result exhibited that the combination of isocryptomerin with cefotaxime significantly improved the antibacterial activity compared with when tested alone. Based on the calculations, the combination of isocryptomerin and cefotaxime exhibited synergy, and showed the FICI value of 0.48 (Table 3). This may be explained on the assumption that cefotaxime can inhibit the synthesis of the bacterial cell wall and, in succession, isocryptomerin exerts its antibacterial activity effectively against the weakened bacterial cells. This result also indicates that isocryptomerin may be used with established antibiotics for treating multidrug-resistant bacterial strains.

In summary, this is the first study to investigate the antibacterial properties of isocryptomerin including its synergistic effects with existing antibiotics. Although the exact mechanism(s) that isocryptomerin exerts its antibacterial activity must be further investigated, the primary significance of this study is the suggestion of the potential of isocryptomerin as an adjuvant for antibacterial therapy for the treatment of infectious diseases caused by drug-resistant bacteria. According to the results, it can also be expected that isocryptomerin may be the basis for the development of new antibacterial agents having synergic activity with conventional β-lactam antibiotics.

Acknowledgement

J. Lee and Y. Choi contributed equally to this work and should be considered co-first authors.

References

1. Bauer, R. and L. M. Dicks. 2005. Mode of action of lipid II-targeting lantibiotics. *Int. J. Food Microbiol.* **101:** 201–216.
2. Bush, K. 2004. Antibacterial drug discovery in the 21st century. *Clin. Microbiol. Infect.* **10 Suppl 4:** 10–17.
3. Cassell, G. H. 1997. Emergent antibiotic resistance: Health risks and economic impact. *FEMS Immunol. Med. Microbiol.* **18:** 271–274.
4. Cavalieri, S. J., J. R. Biehle, and W. E. Sanders Jr. 1995. Synergistic activities of clarithromycin and antituberculous drugs against multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* **39:** 1542–1545.
5. Cha, D. S. and M. S. Chinnan. 2004. Biopolymer-based antimicrobial packaging: A review. *Crit. Rev. Food Sci. Nutr.* **44:** 223–237.
6. Cha, H. Y., D. C. Moon, C. H. Choi, J. Y. Oh, Y. S. Jeong, Y. C. Lee, *et al.* 2005. Prevalence of the ST239 clone of methicillin-resistant *Staphylococcus aureus* and differences in antimicrobial susceptibilities of ST239 and ST5 clones identified in a Korean hospital. *J. Clin. Microbiol.* **43:** 3610–3614.
7. Clinical and Laboratory Standards Institute. 2005. *Performance Standards for Antimicrobial Susceptibility Testing.* Fifteenth Informational Supplement, Approved standard MS 100-S15. CLSI, Wayne, PA.
8. Cookson, B. 2005. Clinical significance of emergence of bacterial antimicrobial resistance in the hospital environment. *J. Appl. Microbiol.* **99:** 989–996.
9. Hemaiswarya, S., A. K. Kruthiventi, and M. Doble. 2008. Synergism between natural products and antibiotics against infectious diseases. *Phytomedicine* **15:** 639–652.
10. Howard, D. H., R. D. Scott, R. Packard, and D. Jones. 2003. The global impact of drug resistance. *Clin. Infect. Dis.* **36 (Suppl 1):** S4–S10.
11. Jung, H. J. and D. G. Lee. 2008. Synergistic antibacterial effect between silybin and N,N'-dicyclohexylcarbodiimide in clinical *Pseudomonas aeruginosa* isolates. *J. Microbiol.* **46:** 462–467.
12. Jung, H. J., K.-S. Jeong, and D. G. Lee. 2008. Effective antibacterial action of Tat (47–58) by increased uptake into bacterial cells in the presence of trypsin. *J. Microbiol. Biotechnol.* **18:** 990–996.
13. Kim, H. P., H. Park, K. H. Son, H. W. Chang, and S. S. Kang. 2008. Biochemical pharmacology of biflavonoids: Implications for anti-inflammatory action. *Arch. Pharm. Res.* **31:** 265–273.
14. Kuzin, A. P., H. Liu, J. A. Kelly, and J. R. Knox. 1995. Binding of cephalothin and cefotaxime to D-ala-D-ala-peptidase reveals a functional basis of a natural mutation in a low-affinity penicillin-binding protein and in extended-spectrum beta-lactamases. *Biochemistry* **34:** 9532–9540.
15. Lee, C. W., H. J. Choi, H. S. Kim, D. H. Kim, I. S. Chang, H. T. Moon, S. Y. Lee, W. K. Oh, and E. R. Woo. 2008. Biflavonoids isolated from *Selaginella tamariscina* regulate the expression of matrix metalloproteinase in human skin fibroblasts. *Bioorg. Med. Chem.* **16:** 732–738.
16. Lee, J. H., H. Y. Yang, H. S. Lee, and S. K. Hong. 2008. Chemical composition and antimicrobial activity of essential oil from cones of *Pinus koraiensis*. *J. Microbiol. Biotechnol.* **18:** 497–502.
17. Lee, S. J., J. H. Choi, K. H. Son, H. W. Chang, S. S. Kang, and H. P. Kim. 1995. Suppression of mouse lymphocyte proliferation *in vitro* by naturally-occurring biflavonoids. *Life Sci.* **57:** 551–558.
18. MacKenzie, F. M., M. J. Struelens, K. J. Towner, and I. M. Gould. 2005. Report of the Consensus Conference on Antibiotic Resistance; Prevention and Control (ARPAC). *Clin. Microbiol. Infect.* **11:** 938–954.
19. Park, K. H., J. S. Kim, Y. R. Lee, Y. J. Moon, H. Hur, Y. H. Choi, *et al.* 2007. Low-density lipoprotein protects *Vibrio vulnificus*-induced lethality through blocking lipopolysaccharide action. *Exp. Mol. Med.* **39:** 673–678.
20. Paterson, D. L. 2006. The epidemiological profile of infections with multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* species. *Clin. Infect. Dis.* **43(Suppl 2):** S43–S48.

21. Rice, L. B. 2006. Antimicrobial resistance in Gram-positive bacteria. *Am. J. Infect. Control* **34(5 Suppl 1):** S11–S19.
22. Roos, K. L. 2004. Emerging antimicrobial-resistant infections. *Arch. Neurol.* **61:** 1512–1514.
23. Silva, G. L., H. Chai, M. P. Gupta, N. R. Farnsworth, G. A. Cordell, J. M. Pezzuto, C. W. Beecher, and A. D. Kinghorn. 1995. Cytotoxic biflavonoids from *Selaginella willdenowii*. *Phytochemistry* **40:** 129–134.
24. Sung, W. S., H. J. Jung, I.-S. Lee, H. S. Kim, and D. G. Lee. 2006. Antimicrobial effect of furaneol against human pathogenic bacteria and fungi. *J. Microbiol. Biotechnol.* **16:** 349–354.
25. Weber, J. T. and P. Courvalin. 2005. An emptying quiver: Antimicrobial drugs and resistance. *Emerg. Infect. Dis.* **11:** 791–793.
26. White, D. G., S. Zhao, S. Simjee, D. D. Wagner, and P. F. McDermott. 2002. Antimicrobial resistance of foodborne pathogens. *Microbes Infect.* **4:** 405–412.