OPEM
www.opem.org
Oriental Pharmacy and Experimental Medicine 2004 **4(4)**, 261-266

Phytochemical isoflavones against diabetic foot bacteria

Kaushiki Mazumdar[1], Noton Kumar Dutta[1], Sujata G Dastidar[1], Noboru Motohashi[2] and Yoshiaki Shirataki[3,*]

[1]Division of Microbiology, Department of Pharmaceutical Technology, Jadavpur University, Calcutta 700 032 India; [2]Meiji Pharmaceutical University, Kiyose, Tokyo, Japan; [3]Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama, Japan

SUMMARY

Wound swabs and pus samples were collected from diabetic foot ulcers, and control pus samples from non-diabetic cases. In 144 diabetic cases screened, *Pseudomonas aeruginosa* was isolated from 78 cases, in which 10.59% of the isolates were multidrug resistant (MDR), whereas the 60 control cases were not MDR. The isolated bacteria were decreasingly resistant to 6 clinically administrated antimicrobics such as ceftazidime, gentamicin, ciprofloxacin, tobramycin, piperacillin and amikacin. Therefore, it is demanded that new and more effective antimicrobials of phytochemical origins are sought after.

Among 11 isoflavones (**YS11-YS21**) isolated from *Sophora* and *Euchresta* (Leguminosae; pea plant family), 2 (**YS19** and **YS21**) prominently exhibited the high antibacterial activity both *in vitro* and *in vivo*. By the preliminary results, the object of this paper is to evaluate the *in vitro* antibacterial effect of **YS19** and **YS21** on the clinically isolated bactera of *Ps. Aeruginosa* in hospitals. All the isolates were sensitive to **YS19** and **YS21** and for both, minimum inhibitory concentration (MIC) values ranged from 2 to 50 μg/mL. The MIC_{90} values of **YS19** and **YS21** were 50 μg/mL. It is suggested that these isoflavones might consist a basis phytochemical prevention and therapy for diabetic foot infections caused by pseudomonads.

Key words: Isoflavones; Diabetic foot bacteria

INTRODUCTION

Foot infections, common in melituric diabetes, have been found to be decisive in the mortality of patients suffering from the disease. *Pseudomonas aeruginosa*, pathogenic bacteria, is one of the most commonly isolated pathogens from the foot infections of diabetic patients (Chincholikar and Pal, 2002). Recently, it is advised to gradually prohibit the clinical administration of antibiotics such as cefatrizidine, gentamicin, ciprofloxacin, tobramycin, piperacillin and amikacin against the pathogenic (disease-causing) bacteria due to emergence of multidrug resistant (MDR) bacteria strains (Chincholikar and Pal, 2002). Thus, antimicrobials without toxic side effects are sought after. From pre-historic ages to today, some herbal extracts have been exhibiting antimicrobial activities. For example, *Cinchona* has been administrated for treatment of malaria (Hora and Nair, 1944). Some coumarin derivatives exhibited inhibitory activities against many pathogenic bacteria, also including *Helicobacter pylori* (Kawase *et al.*,2001). Prenylflavanones, derived from common

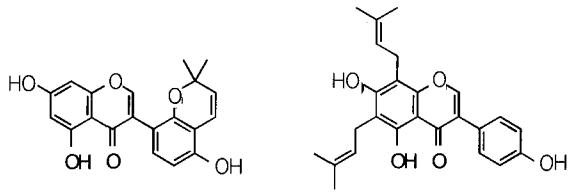
*Correspondence: Dr Yoshiaki Shirataki, Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama, 350-0295 Japan. Tel & Fax: +81-49-271-7053; E-mail: shiratak@josai.ac.jp

leguminous plant extracts, have been tested for antibacterial activities. Such bioactive components of natural plant source have shown promising antimicrobial activity both *in vitro* (Shirataki *et al.*, 2001) and *in vivo* (Dastidar *et al.*, 2001). Additionally, the natural components have also revealed selective tumor cytotoxicity, anti-human immunodeficiency virus (HIV) properties, radial generation and free radical oxygen-scavenging activity (Shirataki *et al.*, 2001). Proceeding in this line, 11 isoflavones (**YS11-YS21**) isolated from *Sophora* and *Euchresta* genera (Leguminosae; pea plant family) were studied for *in vitro* antimicrobial activities (MIC determined by agar dilution method) against 214 kinds of both Gram-positive and Gram-negative bacterial strains. Of the isoflavones, **YS19**(sophoraisoflavone A) and **YS21** (6,8-diprenylgenistein) were antimicrobial with MIC 25-100 μg/mL against most of the strains (Dastidar *et al.*, 2004). Two isoflavones (**YS19** and **YS21**) were shown to provide significant protection to Swiss white mice (each 20 g), challenged with 50 Median Lethal Dose (LD) of a virulent strain of *Salmonella typhimurium* at doses of 30 and 60 μg/mouse. However, there were no toxic side effects from either of the 2 isoflavones on animal tissue (Dastidar *et al.*, 2004). It was observed that all the strains of *Pseudomonas* spp. tested were inhibited by the two isoflavones at concentrations of 25-200 μg/mL when tested by agar dilution method (Dastidar *et al.*, 2004). Hence, the present study was designed to examine whether the clinical bacteria strains of drug-resistant *Pseudomonas aeruginosa* isolated from diabetic foot ulcers were susceptible to these isoflavones or not. Of 144 screened diabetic patients with the typical diabetic foot ulcers, *Pseudomonas aeruginosa* were isolated from 78 cases. The isolated strains were tested for antibiotic-sensitivity by using a disc-diffusion method with the conventional antibiotics. Further, the sensitivity of the MDR-strains with respect to **YS19** and **YS21**was also tested by a broth dilution method.

MATERIALS AND METHODS

Isoflavones

The preparation and structure elucidation of isoflavones (**YS11-YS21**) were previously described (Shirataki *et al.*, 1988; Shirataki *et al.*, 1991 Dastidar *et al.*, 2001; Shirataki *et al.*, 2001; Dastidar *et al.*, 2004). The purification of eleven isoflavones (**YS11-YS21**) was performed by TLC (Merck: Silica gel 60F254; solvent: benzene: EtOAc = 1 : 1) and HPLC (Waters: 600-MI-UV system; column: symmetry C_{18} 5 μm, 4.6 mm × 150 mm; mobile phase: MeOH : H_2O = 4 : 1; flow rate: 0.4 mL/min; pressure: 600 psi; detector: UV254 nm; temperature: 23℃). All isoflavones were preserved at 4℃ (Fig. 1).



YS19 **YS21**

Fig. 1. Structures of **YS19**(sophoraisoflavone A) and **YS21**(6, 8-dipremylgenistein).

Antibiotics

Six discs with antibiotics (ceftazidime, gentamicin, ciprofloxacin, tobramycin, piperacillin and amikacin) were obtained from Hi Media, India. The concentrations of the antibiotics per disc were ceftazidime (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), tobramycin (10 μg), piperacillin (100 μg) and amikacin (30 μg), respectively.

Media

Liquid media were peptone water [PW; 1.0% bacteriological peptone (Oxoid) plus 0.5% NaCl (Analar)], nutrient broth (NB; Oxoid) and Mueller-Hinton broth (MHB; Oxoid). Solid media were MacConkey's agar (Hi Media), nutrient agar

(NA), blood agar (BA; prepared by adding 10% defibrinated sheep blood to molten NA), and Mueller Hinton agar (MHA); NA and MHA were solidified forms of NB and MHB, respectively, using 1.5% agar (Oxoid No.3).

Isolation of bacterial strains

In total of 144 clinical samples obtained from different hospitals in Calcutta, India, all the samples were screened. Wound swabs and pus samples were collected from the ulcers. The control pus samples were collected from 60 non-diabetic patients (surgical wounds). The samples were cultured onto MacConkey's agar and BA. The plates were examined after overnight incubation at 37℃ to quantify the growth of organisms. The colony count was performed and the organisms were identified by the conventional methods (Collee *et al.*, 1996; Koneman *et al.*, 1997) /standard phenotypic tests such as pigment production, growth on cetrimide agar, ability to grow at 42℃, gelatinase production and oxidative metabolism on Hugh-Leifson O/F medium. *Pseudomonas aeruginosa* ATCC 27853 was used as the sensitive control and was obtained from Central Drugs Laboratory of Calcutta, India.

Determination of antibiotic susceptibility pattern of the isolates

Antibiotic sensitivity testing was performed by Kirby Bauer technique on MHA and the interpretation of results was performed according to NCCLS guidelines (NCCLS, 2003).

Detection of *in vitro* antibacterial activity of YS19 and YS21 by broth dilution method

The MIC of **YS19** and **YS21** against the clinical isolates were accurately determined by broth dilution method (NCCLS, 2004). For this detection, 0.1 mL of standardized suspension of strain [10^6 colony forming units (cfu)/mL] was added to each tube containing isoflavone at concentrations of 0 (control), 2, 5, 10, 25, 50 and 100 μg/mL in MHB. These were incubated at 37℃ for 24 h, and examined for the visible growth after gentle vortexing for the tubes. The lowest concentration of an isoflavone in a tube which failed to show the visible macroscopic growth was considered as its MIC. The MIC determination was performed in duplicate for each strain, and the experiment was repeated when necessary.

Determination on the action mode of YS19 and YS21

A highly sensitive control (reference) strain of *Pseudomonas* spp. (*Pseudomonas aeruginosa* ATCC 27853) was grown in NB overnight, 2 mL of which was added to 4 mL of fresh NB and incubated for 2 hours to obtain a logarithmic phase culture. At this stage, the cfu counts of the cultures were determined. **YS19** and **YS21** were added separately into different broths at higher concentrations than the MIC. The cfu counts were again determined after 2, 4, 6 and 18 h, respectively.

RESULTS

Anti-biogramme pattern of the isolates

In 144 diabetic cases screened, pathogenic *Pseudomonas aeruginosa* could be isolated from 78 diabetic patients. 10.59% of the 78 diabetic patients with *Pseudomonas aeruginosa* were found to be MDR, whereas *Pseudomonas aeruginosa* from the control (60 cases) of non-diabetics were not MDR. Each MDR strain of *Pseudomonas aeruginosa* was resistant to the routinely used antibiotics and followed by ceftazidime(96.04%), gentamicin (72.62%), ciprofloxacin(60.39%), tobramycin(59.34%), piperacillin (55.28%) and amikacin (37.57%), respectively. By this order, amikacin was the most effective *in vitro* (Fig. 2). The ranges of inhibition zones of each antibiotic were as follows: ceftazidime (22-29 mm), gentamicin (16-21 mm),

ciprofloxacin (25-33 mm), tobramycin (19-25 mm), piperacillin (25-33 mm) and amikacin (18-26 mm), respectively. *Pseudomonas aeruginosa* ATCC 27853 was sensitive to all the antibiotics.

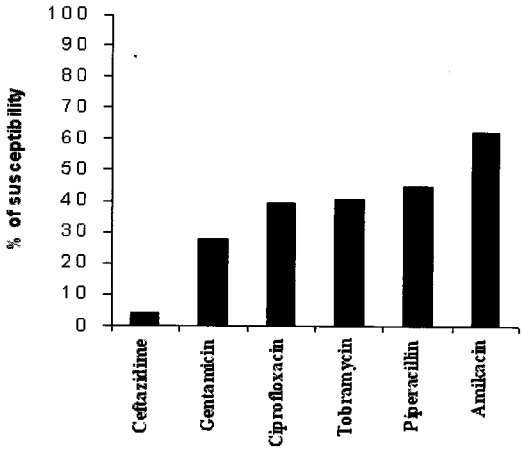


Fig. 2. Susceptibility pattern of *Pseudomonas* spp. (n = 79).

Inhibition on the growth of *Pseudomonas aeruginosa in vitro* by YS19 and YS21

All the 78 clinical isolates as well as the control (reference) strain of *Ps. aeruginosa* were inhibited by both **YS19** and **YS21** within 50 μg/mL concentrations, when tested by broth dilution method.

In case of **YS19**, 30 strains were inhibited at concentrations of 2-10 μg/mL, while the remaining 49 strains stopped growing at 25-50 μg/mL concentrations. In case of **YS21**, 31 out of 79 strains stopped growing at administrations of 2-10 μg/mL concentrations. The rest (48 strains) were inhibited at concentrations of 25-50 μg/mL (Table 1). The MIC values for a given isolate were either identical or within ± 1 dilution with respect to different isolates.

Bacteriostatic action of YS19 and YS21

The MICs of both **YS19** and **YS21** against *Pseudomonas aeruginosa* ATCC 27853 were found to be 10 μg/mL. At the logarithmic growth phase of the cultures, when the cfu counts of the strains were 5.5×10^8 for **YS19** and 3.0×10^8 for **YS21**, 25 μg/mL of the respective agents was added. Subsequently, the cfu of the cultures were determined. For **YS19**, the cfu was 1.0×10^6 after 2 hours, 2.0×10^5 after 4 hours, 4.0×10^4 after 6 hours and 1.5×10^4 at the end of 18 hours (Fig. 3). Similar bacteriostatic action was exhibited by **YS21** (Fig. 3).

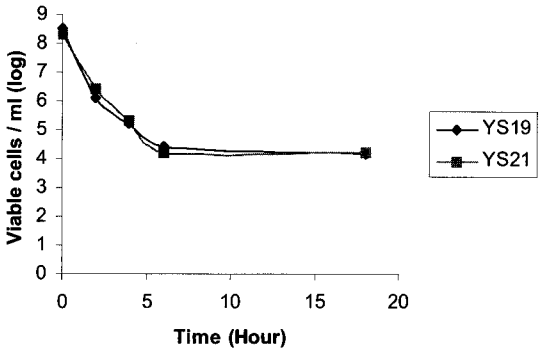


Fig. 3. Bacteriostatic activities of **YS19** and **YS21** on *Pseudomonas aeruginosa* ATCC 27853.

Table 1. Inhibitory spectrum of **YS19** and **YS21** with respect to *Pseudomonas* spp.

Agent	Bacterial strain	No. tested	Minimum inhibitory concentration(μg/mL)					
			2	5	10	25	50	100
YS19	*Pseudomonas* spp.	79	4	10	16	30	19	-
YS21	*Pseudomonas* spp.	79	2	12	17	32	16	-

DISCUSSION

Diabetic foot ulcer is the most common complication among diabetic patients requiring hospitalization. It is also the most common cause of non-traumatic lower extremity amputations. Physicians have an important role in the prevention, early diagnosis and management of diabetic foot complications. One of common pathogen isolated from diabetic foot ulcers is *Pseudomonas aeruginosa* (Chincholikar and Pal, 2002). A battery of powerful antimicrobics is prevalent against *Pseudomonas aeruginosa*. However, most of these are being rendered ineffective with time, due to the emergence of MDR in the *Pseudomonas* spp.

Over the last 20 years, there has been considerable progress in the care of the diabetic foot. The increased limb survival rate of patients attending multi-disciplinary clinics results from advances in the care of neuropathic foot, neuro-ischaemic foot and from management of infection. However, significant concerns still remain, requiring a call to action (Edmonds, 2003).

The last two decades have not really witnessed the discovery of any true antibacterial drug. Significantly newer agents might be basically the earlier molecules having new biomolecule alterations in the structure. Consequently, many researchers have focused their attention towards the diverse herbal extracts with antimicrobial actions.

The present study deals with 2 potentially antibacterial isoflavones **YS19** and **YS21**. The two isoflavones had antibacterial efficacy both *in vitro* and *in vivo*. Their *in vitro* MIC ranged from 25-200 μg/mL. Intraperitoneal injection of two isoflavones (**YS19** and **YS21**) at a dose of 30 μg mouse to Swiss mice challenged with Median Lethal Dose (LD 50) of *Salmonella typhimurium* NCTC 74 manifested significant protection to the mice ($p<0.001$). It was observed that two isoflavones were totally non-toxic to the animal tissue. Strains of *Pseudomonas*, which is not a very susceptible organism, were also inhibited by **YS19** and **YS21** at 25-200 μg/mL concentrations (Dastidar *et al.*, 2004). Hence, **YS19** and **YS21** were employed against the clinical isolates of *Pseudomonas* spp. from diabetic foot ulcers to study their effectiveness in this respect. Two isoflavones (**YS19** and **YS21**) could inhibit all isolates at concentrations of 2-50 μg/mL (Table 1). Even the MDR strains (10.59%) were susceptible to **YS19** and **YS21.** Both isoflavones (**YS19** and **YS21**) were bacteriostatic in action against *Pseudomonas aeruginosa* ATCC 27853 (Fig. 3).

The World Health Organization (WHO) has emphasized the need for better utilisation of indigenous medicine, based on local medicinal plants available. Owing to the realization of the limitations of the antibiotics, two isoflavones (**YS19** and **YS21**) from natural sources are being considered safer. An upsurge in the use of products based on plants is expected, especially in the field of health-related functional plant products (Schuster, 2001). Therefore, the present study opens up a new vista in the treatment of diabetic foot ulcers caused by high-pathogenic *Pseudomonas aeruginosa*.

ACKNOWLEDGEMENTS

We are grateful to All India Council for Technical Education (AICTE), Government of India for providing financial support for carrying out the work. HPLC separation of this study was supported in part by a Grant from the Apparatus Fund 2002 of Josai University.

REFERENCES

Chincholikar DA, Pal RB. (2002) Study of fungal and bacterial infections of the diabetic foot. *Indian J. Pathol. Microbiol.* **45,** 15-22.

Collee JG, Fraser AG, Marmion BP, Simmons A. (1996) (Eds.) Mackie and McCartney Practical

Medical Microbiology. 14th Ed. (Churchill Livingstone, London).

Dastidar SG, Mahapatra SK, Ganguly K, Chakrabarty AN, Shirataki Y, Motohashi N. (2001) Antimicrobial activity of prenylflavonones. *In vivo* **15,** 519-524.

Dastidar SG, Manna A, Kumar KA, Mazumdar K, Dutta NK, Chakrabarty AN, Motohashi N, Shirataki Y. (2004) Studies on the antibacterial potentiality of isoflavones. *Int. J. Antimicrob. Agents* **23,** 99-102.

Edmonds ME. (2004) The Diabetic foot, *Diabetes Metab. Res. Rev.* **20**(suppl 1), S9-S12.

Hora SL, Nair KK. (1944) Pollution of streams and conservation of fisheries. *Proc. Natl. Inst. Sci. India* **10,** 147-166.

Kawase M, Varu B, Shah A, Motohashi N, Tani S, Saito S, Debnath S, Mahapatra S, Dastidar SG, Chakrabarty AN. (2001) Antimicrobial activity of new coumarin derivatives. *Arzneim-Forsch/Drug Res.* **51,** 67-71.

Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC Jr. (1997) (Eds.) Colour Atlas and Textbook of Diagnostic Microbiology. 5th ed. (Lippincott, USA).

National Committee for Clinical Laboratory Standards. (1993) Methods for Dilution in Antimicrobial Susceptibility Tests. Approved Standard M2-A5. NCCLS, Villanova, PA .

National Committee for Clinical Laboratory Standards. (2003) Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard, M2-A8. NCCLS, Wayne, PA.

Schuster BG. (2001) A new integrated program for natural product development and the value of an ethnomedical approach. *J. Altern. Complement. Med.* **7**(suppl 1), S61-S72.

Shirataki Y, Motohashi N, Tani S, Sakagami H, Satoh K, Nakashima H, Mahapatra SK, Ganguly K, Dastidar SG, Chakrabarty AN. (2001) *In vitro* biological activity of prenylflavonones. *Anticancer Res.* **21,** 275-280.

Shirataki Y, Noguchi M, Yokoe I, Tomimori T, Komatsu M. (1991) Sophoraflavonones H, I and J flavonostilbenes from *Sophora moorcroftiana. Chem. Pharm. Bull.* **39,** 1568-1572.

Shirataki Y, Yokoe I, Noguchi M, Tomimori T, Komatsu M. (1988) Studies on the constituents of *Sophora* spp. *Chem. Pharm. Bull.* **36,** 2220-2225.