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Broadspectrim, A Novel Antibacterial from *Streptomyces* sp.

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Abstract: Rapid emergence of antibacterial resistance is well documented as a serious problem worldwide. This situation shows that the potencies of prevalent antibiotics are decreasing steadily. This situation implies the need for searching new antimicrobials to replace with invalidated ones or use in antibiotic rotation programs. In a four years study, from 1,300 soil Actinomycete isolates collected from different localities of Kerman, Hormozgan, Sistan and Baloochestan, south and south east Provinces of Iran, *Streptomyces* sp. isolate No. 419 showed widest antibacterial activity. The active principle named as Broadspectrim. It showed antibacterial activity against wide range of G+ and G- bacteria as *Bacillus anthracis, Bacillus subtilis, Citrobacter diversus, Citrobacter freundii, Corynebacterium diphtheriae, Enterobacter* sp., *Escherichia coli, Klebsiella pneumoniae, Micrococcus luteum, Proteus vulgaris, Proteus rettgeri, Proteus mirabilis, Proteus morganii, Pseudomonas fluorescens, Pseudomonas aeruginosa, Pseudomonas syringae* pv *syringae, Salmonella* Para Typhi A, B, C, D, *Salmonella typhi, Sarcinia* sp., *S. marcescens, Shigella dysentery, S. flexneri, S. sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae, Vibrio cholera* Eltor (INABA) and *Xanthomonas* sp. but *Shigella flexneri* and *Staphylococcus albus* were resistant to it.

Key words: Antibacterial, broadspectrim, bacterial resistance, *Streptomyces* sp.

INTRODUCTION

Over the past 20 years, there has been a lot of interest in the investigation of natural materials as sources of new antibacterial agents[1]. It has been said that researchers have so far discovered approximately over 10,000 biologically active compounds of microbial origin. Roughly two-thirds of these are Actinomycete products and more than a few have been utilized as important chemotherapeutics[2]. According to many reports bacterial resistances are spreading throughout the world[3-10]. Fridkin *et al.*[11] reported antimicrobial resistance increasing in all health-care-associated pathogens. They examined changes in resistance dissemination during 1996–1999 in 23 hospitals and noticed significant increase in prevalence of resistance. The prevalence of antimicrobial resistance in urinary pathogens has been well demonstrated by several workers. Gupta *et al.*[12] studying acute cystitis in women, reported prevalence of multi-resistant *E. coli* in more that 20% of 4342 urine isolates to ampicillin, cephalothin and sulfamethoxazole in each year of their study. They noticed that the prevalence of resistance to trimethoprim and trimethoprim-sulfamethoxazole rose from 9% in 1992 to more than 18% in 1996 among *E. coli* isolates. In a 4-year period, Manges *et al.*[13] examined 255 *E. coli* isolates of three geographically diverse communities, California, Michigan and Minnesota and noticed that a single clonal group of *E. coli* accounted for nearly half of community-acquired urinary tract infections in women that were caused by *E. coli* strains with resistance to trimethoprim-sulfamethoxazole. Schroeder *et al.*[14] by investigating 752 *E. coli* isolates found that approximately 40% of human isolates were resistant to trimethoprim-sulfamethoxazole. In poultry industry, prevalence of resistance in *E. coli* is uprising too. Blanco *et al.*[15] reported that from 468 avian *E. coli* strains isolated in Spain, 67% showed resistance to trimethoprim+sulfamethoxazole and 13% to 24% showed resistance to fluoroquinolones.

An alternative to combat the problem of microbial resistance is development of new antibacterials for substitution with ineffective ones. Because of the side effects and the resistance that pathogenic micro-organisms build against the common antibiotics, special attention should be focused on new antimicrobials from *Streptomyces*[16]. *Streptomyces* have been much studied as potential producers of antibiotics[17-19].

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At the present study in a four year survey 1,300 pure Actinomycete soil isolates were collected and assayed against wide range of human and plant pathogenic bacteria. *Streptomyces* isolate No. 419 showed the widest spectrum of antibacterial activity and the active principle was named as Broadspectrim.

MATERIALS AND METHODS

Culture media: A synthetic medium, Casein glycerol (or starch) agar (CGA) was used for screening and isolating of Actinomycetes which composed of: glycerol or soluble starch, 10 gr; casein, 0.3 gr; KNO_3, 2 gr; NaCl, 2 gr; K_2HPO_4, 2 gr; $MgSO_4.7H_2O$, 0.05 gr; $CaCO_3$, 0.02 gr; $FeSO_4.7H_2O$, 0.01 gr and agar, 18 gr in 1 L of distilled H_2O (pH 7.2). In submerged cultures, Agar was excluded (CG medium). Actinomycetes colonies with different morphologies were selected and transferred to CGA slants for further studies[20, 21].

Tested bacteria: The following bacteria were prepared for bioassay and screening procedures: *Bacillus anthracis*, *Citrobacter freundii*, *Corynebacterium diphtheriae, Proteus vulgaris*, *Proteus mirabilis*, *Proteus morganii*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Salmonella* Para Typhi B, *Shigella sonnei* and *Staphylococcus epidermidis* from Medical Diagnostic and Research Laboratory of Kashani Hospital, Kerman, Iran; *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Serratia marcescens*, *Shigella dysentery*, *Shigella flexneri*, *Staphylococcus aureus*, *Staphylococcus albus*, *Streptococcus pneumoniae* and *Vibrio cholera* Eltor (INABA) from Institute of Pasteur, Tehran, Iran; *Bacillus subtilis*, *Citrobacter diversus* and *Micrococcus luteum*, *Sarcinia* sp. from Pathology Research Laboratory of College of Medicine, University of Medical Sciences, Kerman, Iran; *Enterobacter* sp., *Salmonella* Para Typhi A, C and D from Medical Diagnostic Laboratory of Sajjadiyeh, Kerman, Iran and *Pseudomonas syringae* pv *syringae* and *Xanthomonas* sp. from Research Laboratory of Dept. of Plant Pathology, University of Kerman, Iran.

Isolation of Actinomycetes from Soil: Soil samples were collected from grasslands, orchards and vegetable fields in different localities of Kerman, Sistan and Baloochestan and Hormozgan, south and south east Provinces of Iran. Several samples randomly were selected from mentioned localities using an open-end soil borer (20 cm in depth, 2.5 cm in diameter) as described by Lee and Hwang[22] and Miyadoh[2]. Soil samples were taken from a depth of 10-20 cm below the soil surface. The soil of the top region (10 cm from the surface) was excluded. Samples were air-dried at room temperature for 7-10 days and then passed through a 0.8 mm mesh sieve and were preserved in polyethylene bags at room temperature before use. Samples (10 g) of air-dried soil were mixed with sterile distilled water (100 ml). The mixtures were shaken vigorously for 1 h and then allowed to settle for 1 h. Portions (1 ml) of soil suspensions (diluted 10^{-1}) were transferred to 9 ml of sterile distilled water and subsequently diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Inocula consisted of adding aliquots of 10^{-3} to 10^{-6} soil dilutions to autoclaved CGA (1 ml^{-25} ml CGA) at 50°C before pouring the plates and solidification. Three replicates were considered for each dilution. Plates were incubated at 30°C for up to 20 days. From day 7 on, Actinomycetes colonies were isolated on CGA, incubated at 28°C for one week and stored refrigerated as pure cultures before use. For screening studies 1300 pure Actinomycetes isolates were collected.

Screening procedures: Each Actinomycete isolate was lawn cultured on CGA medium and incubated at 29°C for 5- 7 days. From well grown cultures, 6 mm Agar disks were prepared as described by Boyd[23] using sterile cork borers and transferred to fresh lawn cultures of *E. coli*, *P. rettgeri*, *P. syringae* pv *syringae* and *S. epidermidis*. These bacteria were used in the preliminary screening surveys since they were the most sensitive. Actinomycete isolates with the broadest spectrum of activity were selected and tested as described against all of the bacteria mentioned previously. Tested media with pathogenic bacteria were incubated at 37°C and those of saprophytic bacteria incubated at 29°C for 24 h. The activity was recorded by measuring the diameter of inhibition zones (DIZ) in mm as described by Boyd[23].

Submerged Cultures and Antibacterial Crude Preparation: *Streptomyces* isolate No. 419, having the widest spectrum of activity was cultured in CG medium on rotary shakers with 130 RPM. After 10 days the cultures were harvested, to exclude spores and mycelia it was filtered through two layers of cheese cloth and then Watman filter paper # 1. The clarified sap was then dried to dark crude under reduced air at 50°C and kept refrigerated before use.

Preparation of Antibacterial Paper Disks for Bioassays: Sterile paper disks (No. 740-E, Disks for the Assay of Penicillin and Other Antibacterial Substances, Schleicher and Schuell BioScience, Inc.) were used to prepare

antibacterial paper-disks for Bioassays as described by Acar and Goldstein[24]. Concentration of 3 mg ml^{-1} of crude was prepared in dimethyl sulfoxide (DMSO): methanol (1:1, v/v) (DM solvent) and by a micro syringe 0.01 ml aliquots (0.03 mg) were adsorbed to each of paper disks and dried under reduced air at room temperature overnight. These disks were used in our and other laboratories for elucidation of the antibacterial spectrum of activity. Controls included incorporation of paper disks with CG medium and DM solvent only.

Evaluation by other Laboratories: Antibacterial spectrum of activity of *Streptomyces* isolate No. 419 paper-disks was evaluated by our and other laboratories including: Medical Diagnostic and Research Laboratory of Kashani Hospital, Kerman, Iran; Institute of Pasteur, Tehran, Iran; Pathology Research Laboratory of College of Medicine, Univ. of Medical Sciences, Kerman, Iran; Medical Diagnostic Laboratory of Sajjadiyeh, Kerman, Iran and Research Laboratory of Dept. of Plant Pathology, Univ. of Kerman, Iran. They tested the paper disks on the mentioned bacteria according to the procedure described by Boyd[23]. They performed the tests in triplicates and average of DIZs was recorded.

RESULTS AND DISCUSSION

Isolation of actinomycetes: From many soil samples collected from different localities of Kerman, Sistan and Baloochestan and Hormozgan, south and south east Provinces of Iran, 1,300 pure Actinomycete isolates were collected from 1992 through 1995. The most active isolates of *Streptomyces* were identified at genus level according to Kudo[16].

Yield of crude extract: Approximately 1.5- 2 g of crude was recovered from 1 L of submerged cultures. Concentration of 3 mg ml^{-1} of crude was prepared in dimethyl sulfoxide (DMSO): methanol (1:1, v/v) (DM solvent) and by a micro syringe 0.01 ml aliquots (0.03 mg) were adsorbed to each of paper disks and dried under reduced air at room temperature overnight. These disks were used in our and other laboratories for elucidation of the antibacterial spectrum of activity. Controls included incorporation of paper disks with CG medium and DM solvent only, however no antibacterial activity noticed in controls.

Antibacterial bioassays: The bioassay results of *Streptomyces* isolate No. 419 at 0.03 mg paper-$disk^{-1}$ against tested bacteria is indicated in Table 1. Because of wide range of spectrum of activity, the active principle named as Broadspectrim. *Shigella flexneri* and *Staphylococcus albus* were resistant and other tested G+ and G- bacteria were susceptible to it.

Table 1: Antibacterial results of crude extract of Broadspectrim at 0.03 mg paper-$disk^{-1}$ measured by Diameter of inhibition zones (DIZ, mm).

Bacteria	DIZ
Bacillus anthracis	18
Bacillus subtilis	22
Citrobacter diversus	30
Citrobacter freundii	24
Corynebacterium diphtheriae	28
Enterobacter sp.	20
Escherichia coli	30
Klebsiella pneumoniae	27
Micrococcus luteum	22
Proteus vulgaris	22
Proteus rettgeri	26
Proteus mirabilis	22
Proteus morganii	29
Pseudomonas fluorescens	14
Pseudomonas aeruginosa	23
Pseudomonas syringae pv *syringae*	22
Salmonella Para Typhi A	17
Salmonella Para Typhi B	21
Salmonella Para Typhi C	16
Salmonella Para Typhi D	17
Salmonella typhi	26
Sarcinia sp.	22
Serratia marcescens	24
Shigella dysentery	23
Shigella flexneri	R
Shigella sonnei	17
Staphylococcus aureus	28
Staphylococcus albus	R
Staphylococcus epidermidis	28
Streptococcus pneumoniae	16
Vibrio cholera Eltor (INABA)	20
Xanthomonas sp.	18

R: Resistant

Actinomycetes produce more than half of the world's antimicrobials and are consequently becoming valuable tools in searching for new principles. The emergence and dissemination of antibacterial resistance is well documented as a serious problem worldwide[25- 27]. Smith *et al.*[28] express that "The emergence of bacterial resistance threatens to return us to the era before the development of antibiotics". The perspective of rapid emergence of drug resistance among bacterial pathogens shows that the potencies of prevalent antibiotics are decreasing steadily, leading to reduced useful-period of drugs. This situation implies the need for new and safe antimicrobials for replacement with invalidated antimicrobials or use in antibiotic rotation programs[29- 31].

Broad antibacterial-spectrum of Broadspectrim has made it a proper candidate for further studies. Isolation and structural elucidation of Broadspectrim and determination of its biological properties are under investigation by Research Laboratory of Daru Pakhsh Pharmaceuticals Co., Tehran, Iran.

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