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Isolation and Purification of Two Metabolites (KGG32-A & KGG32-B) from a Soil Bacterium, *Streptomyces* sp., KGG32

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ABSTRACT

This research is concerned with the isolation, purification and partial characterization of two metabolites from culture filtrate of *Streptomyces* sp. KGG32, by solvent extraction, silica gel chromatography, UV and FT-IR spectroscopic analyses. This strain is a highly stable antimicrobial metabolite producer against different microorganisms such as *S. aureus*, *B. cereus*, *B. subtilis*, *Kocuria rhizophila*, *E. coli*, *Enterococcus faecalis* and *C. albicans* under *in-vitro* growth conditions. In order to obtain crude extract, ten liters of the fermentation broth was extracted with different solvents and concentrated until dryness. The crude extract was subjected to silica gel column chromatography and the fractions showing the highest antimicrobial activity were pooled and determined by TLC for purity. Extraction of fermentation broth, various separation and purification steps led to isolation of two pure active compounds having a retention factor of 0.22 (methanol: water, 3:1, v/v) and 0.64 (ethanol: water, 3:1, v/v), respectively. MIC values of the isolated compounds were found between 5.209–25.0 µg/mL. © 2011 Friends Science Publishers

Key Words: Antimicrobial activity; Antibiotics; Fermentation; Secondary metabolites; *Streptomyces*; Soil

INTRODUCTION

The resistance of numerous pathogenic bacteria and fungi to commonly used antibiotics is an urgent focus of research and new antifungal and antibacterial compounds are necessary to combat these pathogens. To prevent exponential emergence of microorganisms becoming resistant to the clinically available antibiotics already marketed, the periodic replacement of existing antibiotics is necessary. Filamentous soil bacteria belonging to the genus *Streptomyces* are aerobic Gram-positive branching bacilli, the majority of which are soil saprophytes (Atalan, 1993; Ben Fguira *et al.,* 2005; Ripa *et al.,* 2010). Much effort has long been focused upon the genus *Streptomyces*, which is the most abundant and a recoverable actinomycete group in soil. Extensive screening of this taxon has led to the discovery of many novel strains that produce useful secondary metabolites (Otoguro *et al.,* 2001; Ramakrishnan *et al.,* 2009). A variety of bioactivities is associated with secondary metabolites produced by *Streptomyces*, including antibacterial, antifungal, antiviral, antitumoral, and enzyme inhibitory compounds (El-Mehalawy *et al.,* 2005; Sonya & Galal, 2005; Wu *et al.,* 2007; Àarthi *et al.,* 2009; Venkatachalam *et al.,* 2009). Tens of thousands of such compounds have been isolated and characterized, many of which have been developed into drugs for treatment of wide range of diseases in human, veterinary and agriculture. Antibiotics have played a major role in the increase in life expectancy in the last 50 years and reduced morbidity and mortality due to infectious diseases. The search for new, safer, broad-spectrum antibiotics with novel mechanisms of action has been progressing slowly. The development of new antimicrobial agents is an urgent medical need.

The aim of this study is isolation and purification of two secondary metabolites from a soil actinobacteria, designated *Streptomyces* sp. KGG32. In addition, partial characterization and antimicrobial activity of these metabolites are presented.

MATERIALS AND METHODS

Microorganisms and growth conditions: The *Streptomyces* sp. KGG32 was isolated previously (Oskay, 2006; Oskay *et al.,* 2010) from the soil sample collected from Geçitköy Pond, North Cyprus. This strain was isolated by soil dilution plate technique using glycerol-asparagine agar (ISP5) supplemented with nystatin (50 µg/mL), cycloheximide (50 µg/mL) and nalidixic acid (10 µg/mL) in order to minimize undesirable fungal and Gram-negative bacteria contamination, respectively. The culture was maintained on yeast extract–malt extract agar (ISP2) slants (Atalan, 1993). Spore suspensions of *Streptomyces* sp. KGG32 in 15% glycerol deposited at –20°C in culture collection of Biology Department, Celal Bayar University, Manisa, Turkey.

The following test organisms were used for the

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determination of minimum inhibitory concentration (MIC) of the antibiotics: *S. aureus* ATCC 6538P, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* CCM 99, *Kocuria rhizophila* ATCC 9341, *E. coli* ATCC 39628, *Klebsiella pneumoniae* ATCC 10031, *Enterococcus faecalis* ATCC 29212, methicillin-resistant *S. aureus* ATCC 95047, *Salmonella typhimurium* CCM 5445 and *C. albicans* ATCC 10231 (Table I). Cultures of test bacteria were grown in Mueller-Hinton Broth (Oxoid) at 37°C for 24 h and stored in nutrient agar slants at 4°C. Yeast was cultured on yeast extract malt extract broth at 30°C for 48 h and it maintained on the potato dextrose agar (PDA, Oxoid). The bacteria were obtained from the Department of Biology, Ege University (İzmir/Turkey).

Fermentation conditions: A loopful of pure culture of the strain grown on ISP2 was transferred aseptically into the 40 mL seed medium [(sucrose 10.0 g, meat extract 3.0 g, yeast extract 1.0 g, bacteriologic peptone 5.0 g, $CaCO_3$ 1.0 g, K_2HPO_4 1.0 g, KH_2PO_4 1.0 g, trace element solution 1.0 mL and distilled water 1000 mL] in 250 mL Erlenmeyer flasks and incubated at 30°C for 48 h on a rotary shaker (180 rpm). The fermentation medium was inoculated with 5% (v/v) of a preculture after 48 h growth into a 500 mL Erlenmeyer flask containing 200 mL of the seed medium and incubated at 30°C under the standard condition of aeration and agitation (180 rpm). Each of the resulting culture broths (approximately 200 mL) obtained following the growth of KGG32 was separated from the mycelium by centrifugation at 10.000 rpm for 10 min. The supernatant was used for the isolation of active compounds.

Extraction, isolation and purification of active compounds: The isolation and purification procedure for active compounds is shown schematically in Fig. 1. In order to obtaining cell free supernatant, culture broths of *Streptomyces* sp. KGG32 were filtered through Whatman paper (no: 4) and centrifuged at 10.000 rpm for 10 min. The extraction (at room temperature, 140 rpm, 1 h) of the metabolites was carried out by the same volume of methanol based on best solubility and maximum antimicrobial activities. Organic layers of the fermentation broths were concentrated under reduced pressure. In addition, water layer of culture broths were concentrated by using vacuum at 70°C and combined with methanolic extracts to obtain the crude extract. The crude extract dissolved in a small volume of methanol (15 mL) was chromatographied on silica gel G60 (70-230 mesh, Merck) column (20 mm × 400 mm, Isolab) eluted with methanol and hexane (3:1) solvent system and 12 fractions were collected. Each fraction was bioassayed using *S. aureus* and *E. coli* and the active fractions were determined. The active fractions V and X were combined and further purified on a Sephadex LH-20 (particle size 25-100 µ, Sigma-Aldrich). Forty seven organic fractions were collected and tested for activity. Afterwards, methanolic active fractions VII and IX; ethanolic active fractions XXV and XXVII were combined, concentrated and labeled as KGG32-A and KGG32-B, respectively. KGG32-A and KGG32-B were checked for purity by using TLC using the solvent system methanol: chloroform: distilled water (10:2:1) and ethanol: ammonia: distilled water (8:1:1), respectively and visualized using UV light or the anisaldehyde–sulfuric-acid (1:1, prepared in acetic acid) color reaction. The R_f factor for each band was measured.

Spectroscopic measurements: UV absorption maxima of KGG32-A (in methanol) and KGG32-B (in ethanol) were measured with a UV/Visible spectrophotometer (UV-1601, Shimadzu). Infrared spectrum was taken on a PerkinElmer FT-IR System spectrophotometer BX series in KBr tablets.

Antimicrobial spectrum: The microtiter broth dilution technique was performed by using the CLSI standards (CLSI, 2003 & 2006). Briefly, a sterile 96 round-bottom well plate was labeled. A volume of 100 µL of antimicrobial compound solution was pipetted into the first row of the plate. To all other wells 50 µL of double strength Mueller Hinton broth or Potatoes Dextrose broth was added. Serial dilutions were performed using a micropipette (A1-A10). Tips were discarded after use such that each well had 50 µL of the test material in serially descending concentrations. Then, 50 µL of broth containing bacterial suspension (5×10^6 cfu/mL) or yeast (5×10^5 cfu/mL) was added to each well. Each column of wells contained a single antimicrobial agent in progressive dilutions and was inoculated with a single microorganism. Each plate had a set of both a growth (A11) and sterility control (A12). To compare the antibacterial and antifungal activities, neomycin, gentamycin (for bacteria) and nystatin (for fungi) were used as standard antibiotics. Plates were sealed with clean film to ensure that microorganisms did not become dehydrated. The plates were prepared in triplicate and placed in an incubator set at 37°C for 18–24 h and at 30°C for 48 h, respectively for bacteria and *C. albicans*. After incubation, added 10 µL of 0.2%, 3-5 Triphenyl tetrazolium chloride (TTC) solution to each well of microtitre plate. The plates containing TTC were incubated one h at 37°C for reaction. The color change was then assessed visually. Any color changes from purple to pink, which showed the growth of organism. MIC concentration does not exhibit reduction of TTC into formazan so the MIC was defined as that the lowest inhibitory concentration of the antimicrobial agent contained in the microtiter well in which the absence of visual color change (colorless) first observed. The average of nine values was calculated and that was the MIC for the test material and bacterial strain. Results were statistically analyzed by Minitab 13.20 (Minitab Inc., 2000) program and data were presented as the mean ± standard deviation.

RESULTS AND DISCUSSION

Culture broth of *Streptomyces* sp. KGG32 was used as a starting material for the isolation of antibiotics. The cultures grown in sucrose-supplemented medium at 30°C were used to isolate antimicrobial compounds from the

Table I: Physicochemical properties of the antibiotic KGG32-A and KGG32-B

Property	KGG32-A	KGG32-B
Appearance	Yellow	White
Solubility	Slightly soluble in *n*-butanol, ethyl alcohol, Petroleum ether, methyl chloride and Benzene), Soluble in water and methanol	Soluble in water and ethyl alcohol
Functional groups		
1) Ninhydrin test	Positive (free amino group present)	Positive (free amino group present)
2) Sulphuric acid test	Positive (unsaturation present)	Positive (unsaturation absent)
3) Ferric chloride test	Negative (Di-ketons or phenolic OH absent)	Negative (phenolic OH absent)
4) Anisaldehyde	Negative	Negative
UV λ max (nm)	275, 286.5 (Methanol)	396 (ethyl alcohol)
IR υ_{max} (KBr) (cm^{-1})	3439-2924-2352-1641-1633-1404-1121-1054	3456-3417-2918-2358-1636-1479-1381-1118-760-615
TLC R_f	(a)[a] 0.22 (b) 0.40	(c) 0.64 (d) 0.78
Antimicrobial spectrum	Sa[b], Bs, Bc, Kr, Ec, Kp, Ef, Ca, MRSA, Styp	Sa, Bs, Ec, Ef, Ca, Kr

[a] Solvent system, a) methanol: water (3:1 v/v), b) methanol: chloroform: water (10:2:1 v/v), c) ethanol: water (3:1 v/v), d) ethanol: ammonia: water (8:1:1 v/v)

[b] Microorganisms: Sa; *Staphylococcus aureus* ATCC 6538P, Bs; *Bacillus subtilis* ATCC 6633, Bc; *Bacillus cereus* CCM 99, Kr; *Kocuria rhizophila* ATCC 9341, Ec; *Escherichia coli* ATCC 39628, Kp; *Klebsiella pneumoniae* ATCC 10031, Ef; *Enterococcus faecalis* ATCC 29212, MRSA; methicillin-resistant *Staphylococcus aureus* ATCC 95047, Styp; *Salmonella typhimurium* CCM 5445, Ca; *Candida albicans* ATCC 10231

Table II: MIC values (µg/mL) of the isolated compounds KGG32-A, KGG32-B and standard antibiotics against various microorganisms

	Compounds		Standard antibiotics		
TM[a]	KGG32-A	KGG32-B	NEO[d]	GE	NYS
Sa	6.25±2.344[b] (4.688–9.375)[c]	12.50±4.69 (9.375–18.75)	27.08±9.88 (18.75–37.50)	3.907±1.172 (2.344–4.688)	ND[e]
Bc	7.813±2.344 (4.688–9.375)	13.54±4.94 (9.375–18.75)	7.292±2.471 (4.688–9.375)	5.73±2.067 (4.688–9.375)	ND
Kr	5.209±1.562 (4.688–9.375)	11.46±4.13 (9.375–18.75)	3.386±1.235 (2.344–4.688)	2.865±1.034 (2.344–4.688)	ND
Ec	8.333±2.067 (4.688–9.375)	16.67±4.13 (9.375–18.75)	5.73±2.067 (4.688–9.375)	6.25±2.344 (4.688–9.375)	ND
Bs	7.27±2.497 (4.688–9.375)	14.58±4.94 (9.375–18.75)	7.813±2.344 (4.688–9.375)	5.209±1.562 (4.688–9.375)	ND
Ef	6.771±2.47 (4.688–9.375)	15.63±4.69 (9.375–18.75)	6.25±2.344 (4.688–9.375)	4.427±0.781 (2.344– 4.688)	ND
Ca	20.83±6.25 (18.75–37.50)	25.0±9.38 (18.75–37.50)	ND	ND	0.488±0.293 (0.293–1.172)

[a] TM; Test microorganisms: Sa; *Staphylococcus aureus* ATCC 6538P, Bc; *Bacillus cereus* CCM 99, Kr; *Kocuria rhizophila* ATCC 9341, Ec; *Escherichia coli* ATCC 39628, Bs; *Bacillus subtilis* ATCC 6633, Ef; *Enterococcus faecalis* ATCC 29212, Ca; *Candida albicans* ATCC 10231

[b] Data presented as the mean value of nine determinations ± standard deviation

[c] Minimum and maximum values are shown in parentheses

[d] Standard antibiotics: NEO; Neomycin, GE; Gentamycin, NYS; Nystatin

[e] ND: Not determined

filtrate. Antibiotics were isolated from the culture medium by extraction and column chromatography on silica gel G60 and Sephadex LH20. According to obtained data, these antibiotics are partly soluble in solvents with a wide range of polarities, such as *n*-butanol, ethanol, methyl chloride, benzene and petroleum ether. The preliminary experiments showed that among the 47 fractions collected, fractions VII, IX, XXV and XXVII showed good activity and other fractions did not exhibit any activity against tested microorganisms. Two pure antibiotics were isolated from a fermentation of *Streptomyces* sp. KGG32 and named as KGG32-A and KGG32-B.

UV and Infrared spectrometry (IR) are being routinely used for the analytical estimation of various antibiotics. In the present investigation, UV scans of KGG32-A dissolved in methanol were performed. Absorbance maxima were obtained at 275 and 286.5 nm, indicating the existence of a carbon–carbon double bond. UV scans of KGG32-B in absolute alcohol were also performed. Major absorbance maxima were obtained at 396 nm. The FT-IR spectrum of the component KGG32-A exhibits absorption at 3439.77 cm^{-1}, which indicates hydroxyl groups, peaks at 1641.95 cm^{-1} (C=N groups) and at 1633.56 cm^{-1} (C=C groups) indicate a aromatic double bonds (Fig. 2). In addition, absorption at 2924.36 cm^{-1} shows the presence of -C-H- groups, peaks at 1404.19 cm^{-1} and 1054.54 cm^{-1} indicate -C-O- groups. The band at 1121.67 cm^{-1} is due to aliphatic -C-N- groups. The FT-IR spectrum of the component KGG32-B shows the presence of -OH functions (wide peak at 3456.58 cm^{-1}). Absorption at 3417.36 cm^{-1}, which is indicative of the -N-H- group, at 2918.76 cm^{-1} indicates C-H, at 1479.72 cm^{-1} indicates $–C-H_2–$, at 1381.81 cm^{-1} indicates $–CH_3$, at 1118.88 cm^{-1} indicates C-N, and at 1636.36 cm^{-1} indicating a $–C(NH_2)=O$ groups (Fig. 3). In correlation with data from UV and IR absorption maxima indicated that the isolated antibiotics, KGG32-A and KGG32-B were showed glycopeptide and peptide characteristic, respectively. Other physicochemical properties of KGG32-A and KGG32-B are summarized in Table I.

The antimicrobial activity of the antibiotics from *Streptomyces* sp. KGG32 was evaluated by *in vitro* growth inhibition assay on microtiter plates. Both the compound KGG32-A and KGG32-B showed remarkable antimicrobial activity against the tested microorganisms (Table II). In case of some tested microorganisms, KGG32-A was more potent

Fig. 1: Isolation and purification procedure of KGG32-A and KGG32-B from fermentation broths

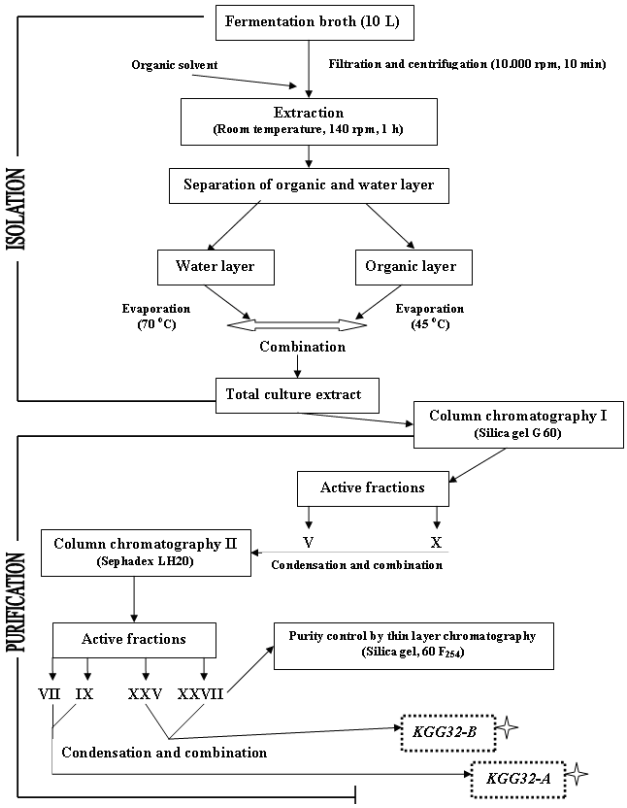


Fig. 2: The FTIR spectrum of the component KGG32-A (KBr)

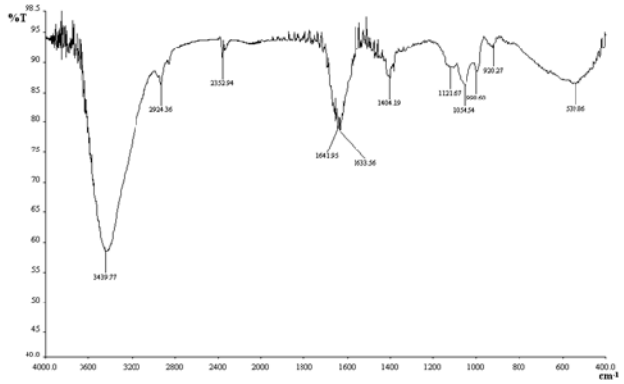
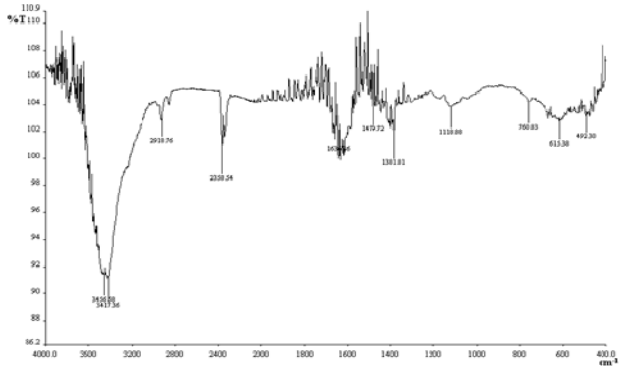


Fig. 3: The FTIR spectrum of the component KGG32-B (KBr)



than KGG32-B. At lower concentrations, both antibiotics showed a remarkable antibacterial activity against the tested bacteria in comparison with the standard neomycin and gentamycin. In the present investigation it was also found that both the compound showed comparatively better antibacterial activity against the Gram-positive bacteria than the Gram-negative bacteria.

The MIC is not a constant for a given antibiotics, because it is affected by the nature of the test microorganism used. The MIC values of the KGG32-A in increasing order against *Kocuria rhizophila*, *S. aureus*, *Enterococcus faecalis*, *B. subtilis*, *B. cereus*, *E. coli* and *C. albicans* were 5.209, 6.25, 6.771, 7.27, 7.813, 8.333 and 20.83 µg/mL, respectively. For KGG32-B, the best MIC was 11.46 µg/mL against *K. rhizophila* shown in Table II. The MIC values of KGG32-A and KGG32-B against *C. albicans* were 20.83 and 25.0 µg/mL, respectively which indicated, that isolated antibiotics were less active in comparison with the antifungal antibiotic nystatin (MIC=0.488 µg/mL). Guo *et al.* (2009) reported that a novel cyclic hexadepsipeptide antibiotic (NW-G01), from the fermentation broth of *Streptomyces alboflavus* exhibited strong antibacterial activity against *B. subtilis*, *B. cereus*, *S. aureus*, MRSA and their MIC values were 3.90, 3.90, 7.81 and 7.81 µg/mL, respectively. Ripa *et al.* (2010) noticed that ethyl acetate extract of *Streptomyces rajshahiensis* (RUPA08-PR), which showed antibacterial activity against a number of both Gram positive and Gram-negative bacteria but did not have antifungal activity under *in vitro* conditions. MIC values of the isolated compound were found between 32 and 128 mg/mL. Oligomycins A and C, the macrolide antibiotics from *Streptomyces diastaticus* showed a strong activity against selected plant pathogenic fungi but no activity against bacteria (Yang *et al.,* 2010). *Streptomyces aburaviensis* (Kut-8), reported to produce antibiotics against Gram-positive and Gram-negative bacteria such as *S. aureus*, *B. cereus*, *B. subtilis*, *E. coli*, *Enterobacter aerogenes*, *P. vulgaris*, and *Salmonella typhosa* (Thumar *et al.,* 2010). Similarly, ethyl acetate extract of *Streptomyces* strain RM42 was reported to active against *E. coli*, *S. typhi*, *S. aureus*, *C. albicans* and *B. subtilis* (Remya & Vijayakumar, 2008).

Over 9,000 biologically active molecules have been isolated from actinomycetes of which over 60 have been useful in medicine, agriculture and/or research. About 80% of these are made by members of the genus *Streptomyces* (Demain, 2009). The genus *Streptomyces* is represented in nature by the largest number of species and varieties, producing the majority of known antibiotics among the family *Actinomycetaceae*. Several species of *Streptomyces* from different soil and water samples were isolated and they found a virtually unlimited source of natural secondary

metabolites. Many kinds of them are used as pharmaceutical and agrochemical products and they have a wide variety of chemical structures, including tetracyclines, macrolides, quinocyclines and meroparamycin (Itoh *et al.,* 1986; Ubukata *et al.,* 1995; Trejo-Estrada *et al.,* 1998; Bouizgarne *et al.,* 2006; El-Naggar *et al.,* 2006; Charoensopharat *et al.,* 2008; Konya *et al.,* 2008; Ramakrishnan *et al.,* 2009). Recent comprehensive studies have shown that beneficial actinobacteria can be readily isolated from sediment (Àarthi *et al.,* 2009; Manivasagan *et al.,* 2009), soil (Atalan *et al.,* 2000; Venkatachalam *et al.,* 2009; Oskay *et al.,* 2010; Ripa *et al.,* 2010) and rhizosphere samples (Ramakrishnan *et al.,* 2009; Ting *et al.,* 2009) and these include a variety of well-established antibiotic producers such as *Streptomyces* sp., *Actinomadura* sp. and *Streptoverticillium* sp. In addition, some of the actinobacteria such as *Actinobispora, Microbispora, Saccharomonospora, Glycomyces* and *Nocardiopsis* were also found to be antibiotic producers (Manivasagan *et al.,* 2009; Ting *et al.,* 2009).

Despite the long list of currently available antibiotics and their applications, the increase in the resistance of human-pathogen populations to these compounds is of primary concern to the medical community and pharmaceutical industry and the problem is particularly severe in hospitals. The use of antibiotics inevitably selects for resistant microbes, so there is a continuing and cyclical need for new antibiotics. An antibiotic's useful lifetime begins to diminish before clinically significant resistance emerges, impelling the need for new drugs to combat the current generation of resistant pathogens. Therefore, new sources and strategies are required to find antimicrobial agents that combine a broad spectrum of activity with resistance to inactivation by bacterial enzymes. Recent reports show that species of *Streptomyces* remain an important source of antibiotics, with applications in medicine, veterinary medicine, and agriculture (El-Naggar *et al.,* 2006; Demain, 2009; Salamoni *et al.,* 2010). Ben-Fguira *et al.* (2005) isolated three antibiotics, named irumamycin (1a), X-14952B (1b) and 17-hydroxy-venturicidin from *Streptomyces* sp. strain US80, which active against Gram-positive, Gram-negative bacteria and fungi. Harindran *et al*. (1999) reported that the oxohexaene macrolide antifungal antibiotic, HA-1-92 showed promising antifungal activity against yeasts and filamentous fungi including human and plant pathogens. Park *et al.* (2008) determined another actinomycete peptide antibiotic, valinomycin isolated from *Streptomyces* sp. M10 and its potent antifungal activity against *Botrytis cinerea*. In another study, antibiotic TH818 that has a broad spectrum of antimicrobial activity was isolated from a culture broth of *Streptomyces fulvoviolaceus* 818 (Rachev *et al.,* 2003). However, in the present study, *Streptomyces* sp. KGG32 produced potent bioactive compounds, KGG32-A and KGG32-B was first time reported from the wild type of soil isolate. The present investigation also reveals the efficiency of the metabolites, produced by this strain against a variety of opportunistic pathogenic bacteria and fungi.

CONCLUSION

In the present study, it is clear that a soil bacterium of *Streptomyces* sp. KGG32 produced extracellular antibiotics (KGG32-A & KGG32-B), which are found very effective against pathogenic test microorganisms *in vitro*. Further investigations are needed in order to determine the structure of active compounds before it becomes a medical treatment. Efforts to establish the complete structure elucidation are in progress.

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