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Original Research

Antimicrobial and Antioxidant Potential of *Streptomyces* sp. RAMPP-065 isolated from Kudremukh soil, Karnataka, India

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Abstract

Actinomycetes are among the industrially and therapeutically relevant microorganisms and are known to produce useful products such as antibiotics, enzymes, vitamins etc. Among actinomycetes, genus *Streptomyces* is known to produce a great array of products. In the present study, we have recovered a *Streptomyces* species RAMPP-065 from Western ghats soil of Kudremukh, Karnataka, India and determined its antimicrobial and antioxidant activity. The isolate was recovered on Starch casein agar and identified as *Streptomyces* species on the basis of cultural, microscopic, staining and biochemical characteristics. Fermentation was carried out in Starch casein broth for 7 days and filtered. The culture filtrate was extracted with ethyl acetate and the solvent was evaporated to get the extract. Antimicrobial activity of extract was tested against 8 bacteria and 2 fungi by agar well diffusion method. Gram positive bacteria were more sensitive to extract than Gram negative bacteria. Among fungi, susceptibility to extract was higher in *Candida albicans* than *Cryptococcus neoformans*. The extract showed a dose dependent scavenging of DPPH free radical as revealed by bleaching of DPPH radical color with increase in concentration of extract. In ferric reducing assay, the absorbance was found to increase with increase in extract concentration. Total phenolic content of extract, as estimated by Folin-Ciocalteau method, was 59mg Gallic acid equivalents/gram. The scavenging and reducing activity of extract were lesser when compared to reference compounds. The soils of Western ghats are rich sources for microorganisms with potent biological activities. To the best of our knowledge, this is the first report on bioactivity of *Streptomyces* species from Kudremukh soil. Further studies are to be carried out to characterize the *Streptomyces* isolate and the active principles present in the extract.

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INTRODUCTION

Among the well characterized industrially and therapeutically relevant microbes, actinomycetes remain the major source of novel useful products such as antibiotics, enzymes, vitamins etc.Streptomycetes are Gram positive aerobic members of the order Actinomycetales within the class Actinobacteria and have a G+C content of 69-78 mol%. They produce extensive branching aerial and substrate mycelia. Aerial mycelia (sporophore) are produced as culture ages and later develop into chains of spores by the formation of cross-walls. The morphological features of aerial mycelium are regarded as more significant for characterization than the substrate mycelium and include mode of branching, configuration of spore chains and surface of spores.The genus *Streptomyces* was proposed by Waksman and Henrici and classified in the family Streptomycetaceae on the basis of morphology and subsequently cell wall chemotype.*Streptomyces* species differ from other actinomycetes by their cell wall type which is characterized as Type I (LL-diaminopimelic acid and glycine present, but characteristic sugars absent). The *Streptomyces* is

well known among actinomycetes and is noticed mainly because of the ability to produce and secrete a large variety of bioactive secondary metabolites, mainly antibiotics. They produce over 2/3rd of the antibiotics which are clinically useful. Although a number of antibiotics have been isolated from *Streptomyces*, these represent only a small fraction of bioactive compounds produced so far. Therefore, screening soils for isolation of new *Streptomyces* species producing bioactive metabolites, particularly from unexplored areas, is still a valuable venture (Anderson and Wellington, 2001; Biswas*et al*., 2012).

Western Ghats are one of the thirty four biodiversity hotspots in the world. They have been grandly known for their flora and fauna. A few studies have been conducted on the biological activities of actinomycetes recovered from soils of Western Ghats of Karnataka such as Agumbe and Kodachadri (Kekuda *et al*., 2010a; Kekuda *et al*., 2010b; Kekuda *et al*., 2011a; Shobha and Onkarappa, 2011; Gautham *et al*., 2011; Gautham *et al*., 2012). Kudremukh, also called Kudremukha, is a mountain range in Chikkamagalure district, Karnataka, India. The name literally means 'horse face' (in the local language Kannada) and refers to a particular picturesque view of a side of the mountain that resembles the same. It is one of the largest declared wildlife protected area of a tropical wet evergreen type of forest in the Western Ghats. Screening for actinomycetes from Kudremukh area of Western Ghats of Karnataka has not been investigated much. In the present study, we report bioactivities namely antimicrobial and antioxidant potential of a *Streptomyces* species designated RAMPP-065 recovered from a soil sample of Kudremukh.

MATERIALS AND METHODS

Isolation of the Organism

The *Streptomyces* sp. RAMPP-065 was isolated from a soil sample collected from Kudremukh region of Western Ghats of Karnataka, India. The top layer of soil was removed and the soil was collected, in a sterile plastic pouch, from a depth of 10cm and dried at 40°C. The soil sample was serially diluted in 0.85% saline (up to 10^{-5}), plated on Starch casein agar (SCA) medium and incubated at 30 °C for about a week. The isolate obtained was subculture done SCA slants and stored at -20°C.

Identification of the Organism

The isolate RAMPP-065 was characterized to genus level by cultural (size, texture, color of substrate and aerial mycelium, diffusible pigment production), microscopic (spore arrangement in Cover slip method), staining (Gram's and Acid fast staining) and biochemical (starch hydrolysis, gelatin liquefaction, casein hydrolysis, sugar fermentation, catalase test, urease test, nitrate reduction, L-tyrosine degradation) characteristics (Holt *et al*., 1994; Aneja, 1996; Cappuccino and Sherman, 1999; Taddei*et al*., 2006).

Extraction of Bioactive Crude Metabolites

Well grown culture of the isolate was used for bulk cultivation by surface fermentation to get the bioactive metabolite. The isolate was grown in 250ml conical flasks containing 100ml of sterile Starch casein broth. The flasks were incubated at 30°C for 7 days aerobically. After incubation, the contents of the flasks were filtered through four fold muslin cloth followed by Whatman No. 1 filter paper and centrifuged. The clear supernatant was extracted thrice with equal volume of ethyl acetate in separation funnel and the solvent layers were pooled. The solvent was evaporated *in vacuo* to yield dark brown colored extract. The extract was lyophilized in freeze drier and used to screen bioactivities(Riyanti *et al*., 2009; Kekuda *et al*., 2010b).

Antibacterial Activity of Solvent Extract

The solvent extract was subjected for antibacterial activity by Agar well diffusion method against four Gram positive bacteria namely *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus pyogenes* and four Gram negative bacteria namely *Escherichia coli*, *Vibrio cholerae*, *Pseudomonas aeruginosa* & *Klebsiellapneumoniae*. The test bacteria were grown in sterile Nutrient broth (peptone 5g, beef extract 3g, sodium chloride 5g, distilled water 1000ml, pH 7.0) overnight and used for inoculation. The broth cultures of bacteria were inoculated uniformly on sterile Nutrient agar (peptone 5g, beef extract 3g, sodium chloride 5g, distilled water 1000ml, agar- 20g, pH 7.0) plates using sterile cotton swab. Wells of 6mm diameter were bored in inoculated plates using sterile cork borer. Solvent extract (10mg/ml of sterile distilled water) and standard antibiotic (Streptomycin, 1mg/ml of sterile distilled water) were transferred to respectively labeled wells using sterile droppers. The plates were left for 30 minutes and then incubated at 37°C for 24 hours. The zone of inhibition formed around the well was measured. The experiment was carried thrice and average reading was noted (Kekuda *et al.,* 2012).

Antifungal Activity of Solvent Extract

Antifungal activity of solvent extract was tested against two human pathogenic fungi namely *Candida albicans* and *Cryptococcus neoformans* by Agar well diffusion method. The test fungi were grown in sterile Sabouraud's dextrose broth (peptone 10g, dextrose 40g, distilled water 1000ml, pH 5.6). The broth cultures of fungi were inoculated uniformly on sterile Sabouraud's dextrose agar (peptone 10g, dextrose 40g, Agar 20g, distilled water 1000ml, pH 5.6) plates using sterile cotton swab. Wells of 6mm diameter were bored in inoculated plates using sterile cork borer. Solvent extract (10mg/ml of sterile distilled water) and standard antibiotic (Fluconazole, 1mg/ml of sterile distilled water) were transferred to

respectively labeled wells using sterile droppers. The plates were left for 30 minutes and then incubated at 37^{o}C for 48 hours. The zone of inhibition formed around the well was measured. The experiment was carried thrice and average reading was noted (Kekuda *et al.,* 2012).

Antioxidant activity of Solvent Extract

DPPH Free Radical Scavenging Assay

The radical scavenging ability of solvent extractand Ascorbic acid (standard) was tested on the basis of the radical scavenging effect on the DPPH free radical. Different concentrations of extract and standard (10 to 400 µg/ml) were prepared in methanol. In clean and labeled test tubes, 2ml of DPPH solution (0.002% in methanol) was mixed with 2ml of different concentrations of extract and standard separately. The tubes were incubated at room temperature in dark for 30 minutes and the optical density was measured at 517nm using UV-Vis Spectrophotometer. The absorbance of the DPPH control was also noted. The scavenging activity of the extract was calculated using the formula: Scavenging activity (%) = [(A–B)/ A] x 100, where A is absorbance of DPPH and B is absorbance of DPPH and extract/standard combination (Kekuda *et al*., 2011b).

Ferric Reducing Assay

Different concentrations of solventextract and Tannic acid (standard), namely 10 to 400µg/ml, in 1ml of methanol were mixed in separate tubes with 2.5ml of phosphate buffer (200mM, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The tubes were placed in water bath for 20 minutes at 50^{o}C, cooled rapidly and mixed with 2.5ml of 10% trichloroacetic acid and 0.5ml of 0.1% Ferric chloride. The amount of iron (II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700nm after 10minutes. The increase in absorbance of the reaction mixtures indicates increased reducing power (Kekuda *et al*., 2011b).

Total Phenolic Content of Solvent Extract

Folin-Ciocalteu (FC) method was used to determine total phenolic content of the extract. The reaction mixture consisted of a dilute concentration of extract (0.5ml) mixed with 0.5ml of F-C reagent (1:1 diluted) and 4ml of sodium carbonate (1M) in clean test tube. The tubes were allowed to stand for 15 minutes and the absorbance was measured at 765nm in Spectrophotometer. A standard curve was prepared by using increasing concentrations of Gallic acid in methanol. The total phenolic content was expressed in terms of mg Gallic acid equivalents (GAE) per gram of extract (Kekuda *et al*., 2011b).

Statistical Analysis

All data were expressed as mean±SD of the number of experiments (n=3). Past software version 1.92 was used.

RESULTS AND DISCUSSION

Isolation and Characterization of *Streptomyces* sp. RAMPP-065

The isolate RAMPP-065 was recovered on SCA agar by soil serial dilution plating method. Theisolate was identified as a species of *Streptomyces* on the basis of cultural and microscopic (spore chain morphology). The substrate mycelium was well developed, whitish in color and branched. The colony was powdery and impregnated inside the medium. The aerial mycelium was observed after 4 days of incubation and was light grey in colour. The isolate showed the presence of light brown diffusible pigment. The spore arrangement was observed in Cover slip method and was found to be spira (Figure 1).The staining and biochemical characteristics of *Streptomyces* sp. RAMPP-065 is depicted in Table 1. The isolate was Gram positive and nonacid fast. Hydrolysis of starch, gelatin and casein was observed. Only acid was produced from the fermentation of glucose. Nitrate reduction and degradation of tyrosine was observed. The isolate elaborated catalase and urease.

Table 1: Characteristics of *Streptomyces* sp. RAMPP-065.

Parameter	RAMPP-065
Gram's staining	Gram positive
Acid-fast staining	Non acid-fast
Starch hydrolysis	+
Casein hydrolysis	+
Gelatin liquefaction	+
Sugar fermentation	+ (acid only)
Nitrate reduction	+
Catalase test	+
Urease test	+
L-tyrosine degradation	+

'+' positive; '-' negative

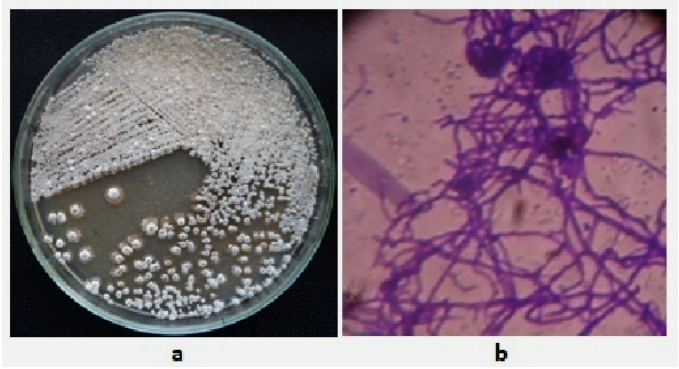

Figure 1: a) Culture of *Streptomyces* sp.RAMPP-065 b) Spore arrangement.

Antibacterial Activity of Solvent Extract

The efficacy of ethyl acetate extract to inhibit the growth of Gram positive and Gram negative bacteria was determined by agar well diffusion method. The antibacterial activity of the extract was higher against Gram positive bacteria when compared with Gram negative bacteria. Among bacteria, high susceptibility was observed in case of *B. cereus* followed by others. Gram negative bacteria namely *K. pneumoniae*, *V. cholerae* and *P. aeruginosa* were inhibited to lesser extent. Inhibition caused by extract was lesser when compared with antibiotic. In case of standard antibiotic also, inhibition of Gram positive bacteria was higher (Table 2).

Table 2: Antibacterial activity of solvent extract and standard.

Test bacteria	Zone of Inhibition in mm	
	Extract	Streptomycin
E. coli	16±0.41	33±0.54
S. pyogenes	18±0.88	39±0.36
K. pneumoniae	14±0.03	35±0.21
B. cereus	23±0.56	38±0.50
V. cholerae	14±0.44	35±0.50
B. subtilis	16±0.41	39±0.56
S. aureus	18±0.58	39±0.96
P. aeruginosa	14±0.99	36±1.00

Antifungal Activity of Solvent Extract

It was observed that susceptibility to extract was higher in *C. albicans* than *C. neoformans*. Inhibition caused by standard antifungal was marked when compared to extract (Table 3).

Table 3: Antifungal activity of solvent extract and standard.

Test fungi	Zone of inhibition in mm	
	Extract	Flucanozole
C. albicans	16±0.58	26±0.61
C. neoformans	14±0.46	24±0.69

Antioxidant Activity of Solvent Extract

DPPH Radical Scavenging Activity

The scavenging activity of different concentrations of solvent extract and Ascorbic acid (standard) was tested by DPPH free radical scavenging assay. Both extract and standard exhibited dose dependent scavenging of DPPH* (free radical) by converting into DPPHH. The scavenging activity of ascorbic acid was greater than that of solvent extract (Figure 2).

Ferric Reducing Activity

The result of reducing power of different concentrations of solvent extractand tannic acid is presented in (Figure 3). In this study, the absorbance at 700nm was found to increase with the increase in concentration of extract and standard which is suggestive of reducing power.

Total phenol content of extract

Folin-Ciocalteu method was employed to determine total phenolic content of the extract. The phenolic content in the extract was expressed as mg GAE/g of extract and it was found to be 59±0.88 mg GAE/g extract.

Actinomycetes are exploited as a source of secondary metabolites particularly antibiotics. Out of the approximately 10,000 known antibiotics, 45-55% is produced by Streptomycetes. Numerous antimicrobial substances from actinomycetes have been isolated and characterized including aminoglycosides, anthracyclines, glycopeptides, beta-lactams, macrolides, nucleosides, peptides, polyenes, polyester, polyketides, actionomycins and tetracyclines. Most of the antibiotics are extracellular metabolites and have been used as herbicides, anticancer agents, drugs, immunoregulators and antiparasitic drugs (Kekuda *et al*., 2010c).Among the various genera of actinomycetes, the genus *Streptomyces* is represented by the largest number of species and varieties, which differ greatly in their morphology, physiology and biochemical activities. Also, majority of the antibiotic producing actinomycetes falls into this genera, which led to the growing economic importance for this group of organisms. This resulted in screening, isolation and description of numerous new species. Various characteristics such as morphological (structure of substrate mycelium, the nature and formation of aerial mycelium, the structure and branching of pseudohyphae containing spores, and the spore surface), cultural (growth on media, color of aerial and substrate mycelia, formation of soluble pigments etc) and biochemical (utilization of carbon sources, proteolytic properties, usage of nitrogen compounds, presence of oxidases and reductases) characters are used for the classification of *Streptomyces* sp. among others (Taddei *et al*., 2006). In our study, we have characterized the isolate as a species of the genus *Streptomyces* based on its typical morphological and microscopic characters.

Secondary metabolites exerted a marked impact on the control of infectious diseases, and the development of pharmaceutical industry. Bacteria in nature have been exposed to a wide range of antibiotics. To survive, bacteria developed antibiotic resistance mechanisms. This resistance increasingly limits the effectiveness of current antimicrobial drugs. The problem is not just antibiotic resistance but also multidrug resistance. In 2004, more than 70% of

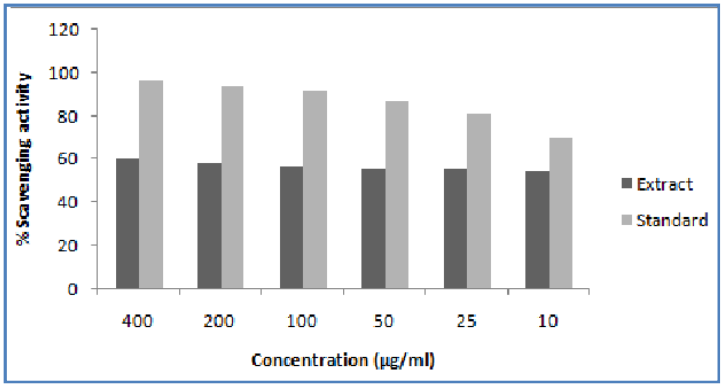

Figure 2: DPPH radical scavenging activity of solvent extract and standard.

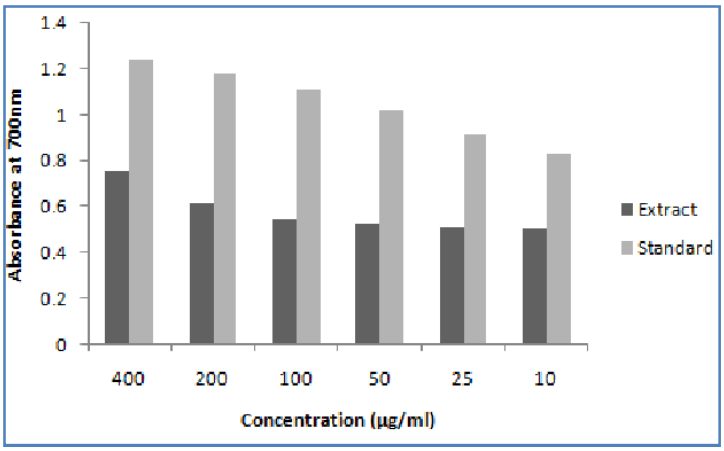

Figure 3: Ferric reducing activity of solvent extract and standard.

pathogenic bacteria were estimated to be resistant to at least one of the currently available antibiotics. The so-called 'superbugs' are emerging at a rapid rate. *S. aureus*, which is resistant to methicillin, is responsible for many cases of infections each year. New antibiotics that are active against resistant bacteria are required (Demain and Sanchez, 2009). A few studies have been conducted on efficacy of actinomycetes of Western Ghats of Karnataka against bacteria and fungi. Shobha *et al*. (2011) screened and found the broad spectrum efficacy of six isolates of Streptomycetes against fungi *C. albicans* and *C. neoformans*. Gautham *et al*. (2011) screened antimicrobial activity of a *Streptomyces* species GOS-1 isolated from soil of Agumbe, Karnataka. GOS-1 was found to possess a broad spectrum antibiotic activity, inhibiting 25 of the 28 test organisms comprising 16 bacteria, 05 yeast and 07 filamentous fungi. The antibiotic was effective against both human and plant pathogens. Gautham *et al*. (2012) studied antimicrobial activity of *Streptomyces* species isolated from Western ghat soils of Agumbe, Karnataka.The antibiotic spectrum of the isolates revealed activity to varied extents, the metabolites had antibacterial and antifungal activity. In our study also, the solvent extract showed a marked inhibition of Gram positive and Gram negative bacteria and two human pathogenic fungi *C. albicans* and *C. neoformans*.

DPPH is relatively stable nitrogen centered free radical that easily accepts an electron or hydrogen radical. DPPH radicals react with suitable reducing agents as a result of which the electrons become paired off forming the corresponding hydrazine. The solution therefore loses colourstoichometrically depending on the number of electrons taken up (Blois, 1958). In a previous study, Kekuda *et al*. (2010a) showed a dose dependent scavenging of DPPH free radical by butanol extract of two *Streptomyces* species isolated from Agumbe, Karnataka. In this study, the scavenging activity of extract was found to be dose dependent i.e., higher the concentration, more was the scavenging activity. Though the DPPH radical scavenging abilities of the extract was less than that of ascorbic acid, the study showed that the extract has the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung *et al*., 2002). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al*., 1995). However, the antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued

hydrogen abstraction, and radical scavenging (Diplock, 1997). Kekuda *et al*. (2010a) showed a marked increase in absorbance with increase in concentration of butanol extract of two *Streptomyces* species isolated from Agumbe, Karnataka in Ferric reducing assay. In this study, the solvent extract has shown dose dependent reducing activity as revealed by increase in absorbance with increase in concentration of extract.

CONCLUSION

The study revealed antimicrobial and antioxidant potential of *Streptomyces* species tested. Screening soils of Western ghat of Karnataka should be carried out to isolate and characterize potential actinomycetes capable of producing bioactive metabolites. To the best of our knowledge, this is the first report on bioactivity of *Streptomyces* species from Kudremukh soil. Further studies are needed to characterize the isolate, purify bioactive metabolites and determine antimicrobial and antioxidant efficacy.

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