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Research Article

IN VITRO ANTIOXIDANT ACTIVITY OF ANTIBACTERIAL METABOLITES FROM *STREPTOMYCES SPECIES* KSRO-04

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ABSTRACT
Antioxidants have gained importance as pharmacological agents and have been consumed in various forms mainly as fruits, vegetables and dietary supplements. These antioxidants are majorly obtained from plant source or have been synthesized chemically. This study concentrated on the *in vitro* evaluation of antioxidant property of a water soluble antibacterial metabolite from *Streptomyces species* KSRO-04. The antioxidant activity was evaluated by Total phenolic content, total flavonoid content, Total antioxidant activity, DPPH assay, Lipid peroxidation inhibition assay, Nitric oxide scavenging activity, Super oxide anion scavenging activity, Metal ion chelating activity and total reductive capability. The metabolite was characterised using TLC and purity was assessed by HPLC. The UV absorption indicated absorption maximum of 360 nm suggesting a polyene nature of the metabolite. The metabolites showed a moderate antioxidant activity with good nitric oxide scavenging activity and also hinted that the anti oxidant activity is not solely due to the phenolic compounds. The metabolite from the *Streptomyces species* KSRO-04 is potent both as antimicrobial agent and an antioxidant agent which suggest its dual and beneficiary role as a pharmacological agent.
Keywords: Streptomyces, metabolite, anti-oxidant, antibacterial, HPLC.

INTRODUCTION

Oxidation is an essential biological process for energy production in many living organisms. In few instance excessive reactive oxygen species are produced *in vivo* during some oxidative reactions[1]. Reactive oxygen species (ROS), sometimes called active oxygen species, are various form of activated oxygen, which include free radicals such as superoxide ions (O_2) and hydroxyl radicals (OH), as well as non free radical species such as hydrogen peroxide (H_2O_2)[2,3]. In living organisms various ROSs are known to cause damage to lipids, proteins, enzymes and nucleic acids leading to cell and tissue injury implicated in the processes of aging as well as in wide range of degenerative diseases including inflammation, cancer, atherosclerosis, diabetes, liver injury, Alzheimer's disease, Parkinson's disease and coronary heart pathologies[4]. Recent investigation suggest that antioxidants can trap free radicals and neutralize potentially harmful reactive free radicals in body cells before they cause lipid, protein oxidation and may reduce potential mutations and therefore help prevent degenerative diseases and other pathologies[5]. Actinomycetes are prokaryotes with extremely various metabolic possibilities[6]. These are gram positive bacteria frequently filamentous and sporulating organisms with DNA rich in G + C from 57-75 %. Actinomycetes of about 100 genera exist in soil[7]. The filamentous actinomycetales produces over 10,000 bioactive compounds, 7,600 derived from Streptomycetes representing the largest group of bioactive microbial metabolites. The metabolites are of high significance as antimicrobial agents on wide range of pathogenic microorganisms[8-10]. The present study concentrated to evaluate the antioxidant activity of metabolite from *Streptomyces species* the largest antibiotic producing microorganisms. This would be a small step to know all possible potentials of an antimicrobial agent.

MATERIALS AND METHODS

This study was carried out in Department of Microbiology, Sahyadri Science College (A), Shimoga, Karnataka, India and Department of P.G. Studies and Research In Biochemistry, Kuvempu University, Davangere from June – July 2010.

Extraction of bioactive metabolite

The isolate was inoculated to Starch casein nitrate broth medium and incubated at 30 ± 2°C for 7-14 days on a rotary shaker. The broth was filtered using Whatmann filter paper number 1 and the culture filtrate was dried at 50°c. The dried residue was used for the analysis[11].

Chemicals and Reagents

Chemicals, such as ascorbic acid, Quercetin, gallic acid, 1, 1 Diphenyl-2-picrylhydrazyl (DPPH), NADH, Nitro Blue Tetrazolium (NBT), Catechin were procured from Sigma Chemical Co. (St Louis, MO, USA), Ferrozine, Ferrous Chloride; Folin-Ciocalteu reagent was from Spectrochem PVT. Ltd. All other chemicals unless and otherwise mentioned were obtained from Sisco Research Laboratories PVT. Ltd. (Mumbai, India).

Determination of total Phenolic content

The total phenolic content in the metabolite was determined with the Folin- Ciocalteu's reagent (FCR) according to the method of Slinkard and Singleton (1977)[12]. In brief, 1.0 ml of metabolite was mixed with 2.5 ml FCR (diluted 1:10 v/v) followed by 2 ml of Na_2CO_3 (7.5 % v/v) solution. The tubes were vortexed and allowed to stand for 90 minutes at room temperature. Absorbance of sample was measured against the blank at 750 nm using a spectrophotometer (Elico SL 159, India). The total phenolic content of the metabolite was expressed in terms of milligrams of gallic acid (mg GAE) per gram of dry weight.

Determination of total flavonoids content

Total flavonoids content of the metabolite was determined according to modified method of Zhishen *et al*., (1999)[13]. Briefly, 1 ml of test sample and 4 ml of water were added to a volumetric flask (10 ml volume), 0.3 ml of 5 % $NaNO_2$ was added to the reaction mixture, the mixture was incubated for 5 minutes at room temperature followed by addition of 0.3 ml of 10 % $AlCl_3.6H_2O$ and was again incubated for 6 minutes at room temperature, 2 ml of 1 M NaOH was added to the reaction mixture, immediately 2.4 ml of distilled water was added to make up the final volume to 10 ml. The absorbance of the reaction mixture was measured at 510 nm against a blank spectrophotometrically (Elico SL 159, India). Results were expressed as catechin equivalents (mg catechin/g dried metabolite).

DPPH radical scavenging activity

The free radical scavenging activity of the metabolite, based on the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined according to the method of Braca *et al*., (2001)[14]. The metabolite (0-100 µg/ml) and standard compound TBHQ (0-100 µg/ml) was added to distilled water to make the volume to 0.1 ml, 3 ml of 0.004 % DPPH (in methanol). The samples were dark incubated at room temperature for 30 minutes and the absorbance was measured at 517 nm spectrophotometrically (Elico SL 159, India). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The percent of DPPH radical scavenging activity was calculated as,

$$\text{Inhibition (\%)} = [(A_o - A_e) / A_o] \times 100$$

Where, A_o is the absorbance of the control and A_e is the absorbance of the metabolite/standard.

Metal chelating activity

The chelating of ferrous ions by the metabolite and standard (EDTA) was estimated by the method of Dinis *et al., (*1994)[15]. The metabolite and standard (0-200 µg/ml) were mixed with distilled water to make the volume to 3.0 ml, 2 mM $FeCl_2$ (0.05 ml) solution was further added. The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was vortexes and was incubated at room temperature for 10 minutes. Absorbance of the solution was measured spectrophotometrically (Elico SL 159, India) at 562 nm. The control contains $FeCl_2$ and ferrozine, complex formation molecules. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated from the formula,

$$\text{Inhibition (\%)} = [(A_o - A_e) / A_o] \times 100$$

Where, A_o is the absorbance of the control and A_e is the absorbance of the metabolite/standard.

Total Reductive Capability

The reductive ability was determined according to the method of Oyaizu (1986)[16]. Different concentrations (0-100 µg/ml) of the metabolite and the standard compound (Ascorbic acid) in distilled water was prepared, the volume was made up to 1.0 ml. This was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1 % potassium ferricyanide [$K_3Fe(CN)_6$]. Reaction mixture was incubated at 50°C for 20 minutes, 2.5 ml of 10 % trichloro acetic acid was added to the mixture, which was then centrifuged (6500 rpm at room temperature) for 10 minutes. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1 % $FeCl_3$. Absorbance of the reaction mixture was measured at 700 nm in a spectrophotometer (Elico SL 159, India). Increased absorbance of the reaction mixture indicates the increase in reduction capability.

Lipid peroxidation inhibition assay

A modified Thio barbituric acid reactive species (TBARS) assay Ohkowa, *et al.*, (1979)[17] was used to measure lipid peroxidation formed using egg yolk homogenate as lipid rich media[18]. 0.5 ml of egg homogenate (10 % in distilled water) and 0.1 ml of metabolite and standard (BHA) in the concentration range 100-500 µg/ml were mixed in test tube, the volume was made up to 1 ml by adding distilled water. Finally 0.05 ml ferrous sulphate (0.07 M) was added to the above mixture and incubated for 30 minutes to induce the lipid peroxidation. Further 1.5 ml of 20 % acetic acid (pH 3.5) and 1.5 ml of 0.8 % TBA and 0.05 ml 20 % TCA was added, the mixture was vortexes and was heated in a boiling water bath for 60 minutes. After cooling 5 ml of Butanol was added and centrifuged at 3000 rpm for 10 minutes. The absorbance of the organic upper layer was measured at 532 nm using a spectrophotometer (Elico SL 159, India). The percentage inhibition of lipid peroxidation was calculated from the formula,

$$\text{Inhibition (\%)} = [(A_o - A_e) / A_o] \times 100$$

Where, A_o is the absorbance of the control and A_e is the absorbance of the metabolite/standard.

Super oxide radical scavenging activity

Super oxide anion scavenging activity of the metabolite was measured according to the method of Nishimiki *et al*., (1972) with slight modification[19]. All the solutions used in this experiment were prepared in phosphate buffer (pH 7.4). 1 ml of NBT (156 µM), 1 ml of NADH (468 µM) and 1 ml of metabolite (Metabolite was added in the range of 000-125 µg/ml) were added. The reaction was started by adding 100 ml of PMS (60 µM) and the mixture was incubated at 25°C for 5 minutes followed by measurement of absorbance at 560 nm spectrophotometrically (Elico SL 159, India). Decreased absorbance of the reaction mixture indicated increased super oxide anion scavenging activity. The percentage inhibition was calculated from the formula,

$$\text{Inhibition (\%)} = [(A_o - A_e) / A_o] \times 100$$

Where, A_o is the absorbance of the control and A_e is the absorbance of the metabolite/standard.

Nitric Oxide scavenging

Nitric oxide radical scavenging activity was determined according to the method reported by Garrat (1964)[20]. 2 ml of 10 mM sodium nitro prusside in 0.5 ml of phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of metabolite at various concentrations and the mixture was incubated at 25°c for 150 minutes. From the incubated mixture 0.5 ml was taken out and added into 1 ml of Sulfanilic acid reagent and incubated for 5 minutes. Finally 1 ml napthyl ethyelene diamine dihydrochloride (0.1 % w/v) was mixed and incubated at room temperature for 30 minutes before measuring absorbance at 540 nm using spectrophotometer (Elico SL 159, India). Nitric oxide radical scavenging activity was calculated using the formula,

$$\text{Inhibition (\%)} = [(A_o - A_e) / A_o] \times 100$$

Where, A_o is the absorbance of the control and A_e is the absorbance of the metabolite/standard.

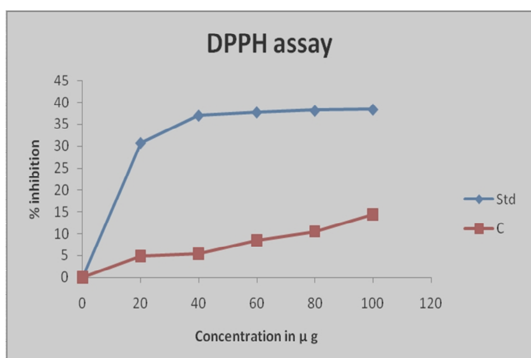

Figure 1: DPPH assay

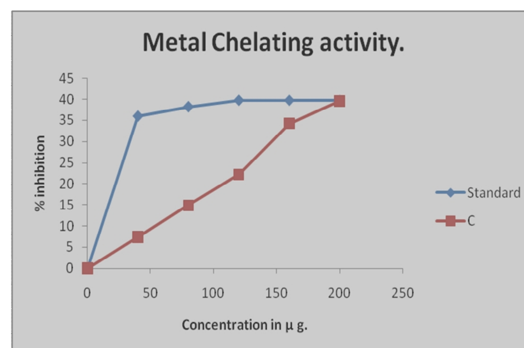

Figure 2: Metal chelating activity

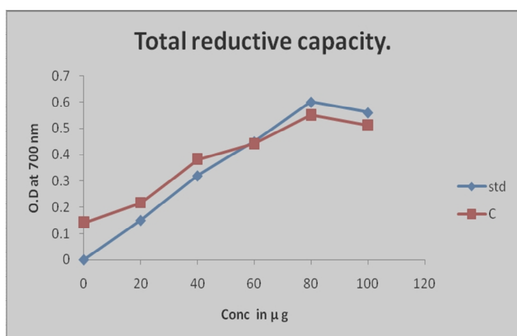

Figure 3: Total reductive capacity

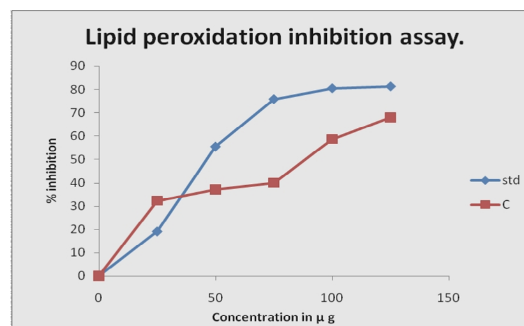

Figure 4: Lipid peroxidation inhibition assay

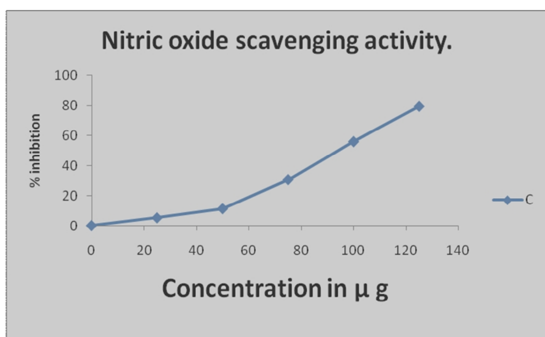

Figure 5: Super oxide radical scavenging activity

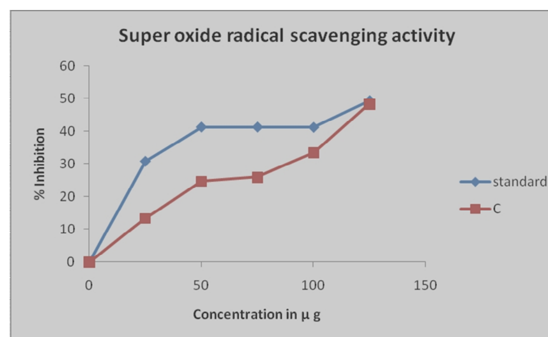

Figure 6: Nitric oxide scavenging activity

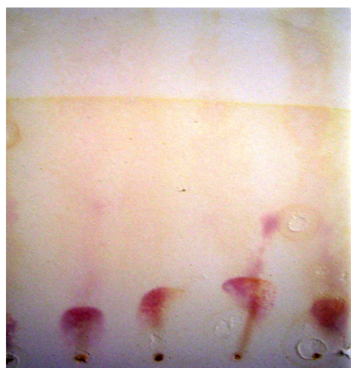

Figure 7: TLC

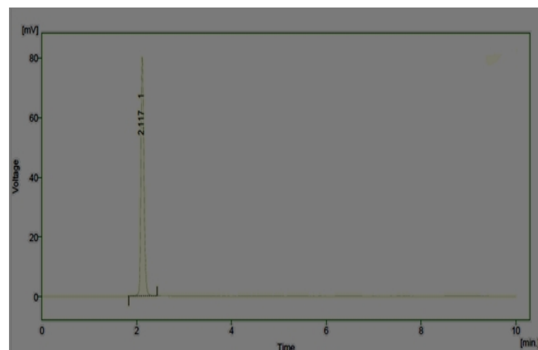

Figure 8: HPLC analysis

TLC

The water soluble metabolites containing bioactive components were concentrated and fractionated using Thin layer chromatography (TLC) on a 12 X 22 cm silica gel plate and developed with chloroform-ethanol-water (2:4:4) and n-butanol-acetic acid-water (4:1:2) solvent systems. Afterwards, the TLC plates were air dried. Detection was done by three ways. The TLC plates were exposed to iodine vapours, sprayed with vanillin and ninhydrin separately to develop the antibiotic, if any[21].

HPLC

The purity of the bioactive compound was tested using HPLC. The metabolite was dissolved in 1 ml of sterile distilled water. HPLC (Shimadzu) separation was performed using a C18-column (250 x 4.6 mm) at a flow rate of 1 ml/min and Pressure 225 kgf with 20 μl of sample injected. Water was used as mobile phase. The absorbance was monitored at 360 nm[21].

UV absorption spectra

The UV-Visible absorption spectra of the metabolite was determined with a (Elico SL 159, India) spectrophotometer at 300-400 nm to determine the $\lambda_{maximum}$ of the band.

Statistical analysis

All experiments were repeated three times. The data were recorded as mean ± deviation.

RESULTS AND DISCUSSION

Total phenolic content

Phenolics have been considered as the major antioxidant in terms of their ability to scavenge reactive oxygen species. The ability of phenol to act as an anti oxidant is due to their redox properties, electron donating properties and singlet oxygen quenching properties[22]. In the present study the total phenolic content was found to be 1.23 mg gallic acid equivalents/g of dry metabolite for gallic acid.

Total flavonoids content

It has been recognised that flavonoids show antioxidant activity and their effect on human health is considerable. The mechanism of action of flavonoids are through scavenging or chelating process[23,24]. In the present study the flavonoids content of metabolite was negligible.

DPPH radical scavenging activity

The DPPH radical scavenging model is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. ROS produced *in vivo* include super oxide radical, hydrogen peroxide and hypochlorous acid. Hydrogen peroxide and superoxide can interact in the presence of certain transition metal ions to yield a highly reactive oxidising species, the hydroxyl radical. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron. The antioxidants react with the stable free radical DPPH (deep violet colour) and convert it to 1, 1-diphenyl-2-picryl hydrazine with decolouration[25]. The reduction capability of DPPH radicals was determined by decrease in its absorbance at 517 nm induced by antioxidants. The percentage of inhibition of the standard and metabolite was observed in the order: BHA (38.4 ± 0.2 %), metabolite (14.39 ± 1.19 %) at a concentration of 125 µg/ml. The scavenging effect was in a dose dependent manner. These results provide a direct comparison of the antioxidant activity with BHA. The inhibition percentage is shown graphically in Figure 1.

Metal chelating activity

Ferrous ion commonly found in the food system is well known as an effective pro-oxidant. Ferrous ion can initiates lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxy radicals[2,26]. Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colour) formation is interrupted and as a result, the red colour of the complex is decreased. Thus the chelating effect of the coexisting chelator can be determined by measuring the rate of colour reduction[22] (Figure 2). In this assay, metabolite and standard compound both interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and are able to capture ferrous ion before ferrozine. The absorbance of Fe^{2+}-ferrozine complex was observed to be linearly decreased with the concentration of the metabolite (from 00 to 200 µg/ml). The percentage of metal chelating capacity at 200 µg/ml of metabolite and EDTA was found to be 39.55 ± 0.85 % and 39.76 ± 0.2 % respectively. The metal scavenging activity reveals that metabolite has a marked capacity to bind metal ions and was found to be very close to the standard compound EDTA, suggesting that its action as antioxidant may be attributed to its iron binding capacity.

Total reductive capability

The reductive capabilities of compounds are assessed by the extent of conversion of the Fe^{3+}/ ferricyanide complex to the Fe^{2+}/ ferrous form. The reducing powers of the compounds were observed at different concentrations, and results were compared with standard compound Ascorbic acid. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity of putative antioxidant has been attributed to various mechanisms, among which is prevention of chain initiation, binding of transition metal ion catalyst, and decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging[27]. In this study, the metabolite showed increased absorbance with increased concentration. Hence, it is capable to reduce metal ions complexes to their lower oxidation state or to take part in any electron transfer reaction. The reducing power of the metabolite and standard compound ascorbic acid exhibited the following order: Ascorbic acid (0.56 ± 0.02), metabolite (0.51 ± 0.02) absorbance units at 100 µg/ml (Figure 3).

Lipid peroxidation inhibition assay

Lipid peroxidation is the oxidative deterioration of lipids containing many number of carbon-carbon double bonds. Initiation of the lipid peroxidation by ferrous sulphate takes place either through feryl-perferryl complex or through $^{\cdot}OH$ radical by Fenton's reaction. The inhibition could be caused by the absence of ferryl – perferryl complex or by scavenging the $^{\cdot}OH$ radical or the super oxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the conversion of ferrous to ferric or by chelating the iron itself. The metabolite inhibited the lipid peroxidation induced by ferrous sulphate in egg yolk homogenate[22]. The percentages of lipid peroxidation inhibition capacity of 125 µg/ml doses of metabolite and BHA were found to be 67.88 ± 2.09 % and 81.16 ± 2.06 %

respectively. The inhibition percentage is shown graphically in Figure 4.

Super oxide anion radical scavenging activity

Super oxide radicals are produced by a number of cellular reactions associated with various enzyme system, such as lipoxygenases, peroxidise, NADPH oxidase and xanthine oxidase[1]. Super oxide anions damage directly or indirectly by forming H_2O_2, OH, peroxy nitrate or singlet oxygen during aging and pathological events such as ischemic reperfusion injury, super oxide has also been observed to directly initiate lipid peroxidation. In this method, $O_2^{\bullet -}$ derived from dissolved oxygen by PMS-NADH coupling reaction reduces the yellow dye (NBT) to produce the blue formazan, which is measured spectrophotometrically at 560 nm[33]. Antioxidants are able to inhibit the blue NBT formation. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of $O_2^{\bullet -}$ in the reaction mixture. The percentage of inhibition was in the order: Ascorbic acid (49.25 ± 0.2 %), metabolite (48.28 ± 2.01 %) at a concentration of 125 µg/ml. The inhibition percentage is shown graphically in Figure 5.

Nitric oxide scavenging activity

Nitric oxide (NO) is a potent pleotropic mediator of physiological process such as smooth muscle relaxant, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilatation, anti microbial and anti tumour activities[28]. Although nitric oxide are involved in host defence, over production of these two radicals contributes to the pathogenesis of some inflammatory diseases[29]. Moreover in the pathological conditions, nitric oxide reacts with superoxide anion and form potentially cytotoxic molecules. Nitric oxide inhibitors have been shown to have some beneficial effects on some aspects of inflammation and tissue damage seen in inflammatory diseases. The metabolite inhibited the nitric oxide in a dose dependent manner the percentage inhibition was 79.25 ± 1.65 % at a concentration of 125 µg/ml. The inhibition percentage is shown graphically in Figure 6.

TLC

The thin layer chromatogram of the metabolite was developed. On spraying ninhydrin Isolate S4 showed a purple band with R_f value 0.60. The chromatogram was as shown in Figure 7.

HPLC

HPLC analysis was performed to check purity of metabolite obtained. Solvent system was standardized with Water. The metabolite revealed a prominent peak as shown in the Figure 8. The results from all the above assays indicate that the metabolite has a moderate anti oxidant activity with good nitric oxide scavenging activity. From the results we can also conclude that the anti oxidant activity is not solely due to the phenolic compounds. Similar results of correlation on anti oxidant activity of plant extracts have been reported by Kahkonen (1999), Hopia and Heinonen (1999) and Khamsah (2006)[30-32]. Furthermore we can conclude that the metabolite from the *Strepromyces species* KSRO-04 is potent also as an antioxidant agent which suggests its dual and beneficiary role as a pharmacological agent. Further research has to be carried out to characterise the active principle. We would also like to conclude that if the active principle is both antibiotic and antioxidant such metabolites have to be used with additional care if formulated to drug based on the requirement or else will be a potential source for pathogenic microorganisms to develop resistance, a more severe and dangerous problem.

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