

## ***In vitro* Antioxidant Activity of a Metabolite 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 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 360nm 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 a potent antioxidant agent which suggests its beneficiary role as a pharmacological agent.

**Key words:** 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 and 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 produce over 10,000 bioactive compounds, 7,600 derived from Streptomyces representing the largest group of bioactive microbial metabolites. The metabolites are of high significance as antimicrobial agents on a 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.

### **MATERIALS AND METHODS**

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

**Extraction of Bioactive Metabolite:** The isolate (From western ghat soil samples) was inoculated onto Starch casein nitrate broth medium and incubated at  $30\pm 2^\circ\text{C}$  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  $50^\circ\text{C}$ . 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 [12]. 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.* [13]. 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.* [14] 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,

$$\text{Inhibition (\%)} = [(A_0 - A_c) / A_0] \times 100$$

where,

$A_0$  is the absorbance of the control and

$A_c$  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.* [15]. The percentage of inhibition of ferrozine- $\text{Fe}^{2+}$  complex formation was calculated from the formula,

$$\text{Inhibition (\%)} = [(A_0 - A_c) / A_0] \times 100$$

where,

$A_0$  is the absorbance of the control and

$A_c$  is the absorbance of the metabolite/standard.

**Total Reductive Capability:** The reductive ability was determined according to the method of Oyaizu [16]. 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 [17] was used to measure lipid peroxidation formed using egg yolk homogenate as lipid rich media [18]. The absorbance of the organic upper layer was measured at 532nm using a spectrophotometer (Elico SL 159, India). The percentage inhibition of lipid peroxidation was calculated from the formula,

$$\text{Inhibition (\%)} = [(A_0 - A_c) / A_0] \times 100$$

where,

$A_0$  is the absorbance of the control and

$A_c$  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.* [19] with slight modification. All the solutions used in this experiment were prepared in phosphate buffer (pH 7.4). 1 ml of NBT ( $156 \mu\text{M}$ ), 1ml of NADH ( $468 \mu\text{M}$ ) and 1 ml of metabolite (Metabolite was added in the range of  $000-125 \mu\text{g/ml}$ ) were added. The reaction was started by adding 100ml of PMS ( $60 \mu\text{M}$ ) and the mixture was incubated at  $25^\circ\text{C}$  for 5 min followed by measurement of absorbance at 560nm

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,

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

where,

$A_o$  is the absorbance of the control and

$A_c$  is the absorbance of the metabolite/standard.

**Nitric Oxide Scavenging:** Nitric oxide radical scavenging activity was determined according to the method reported by Garrat [20]. The absorbance was measured at 540 nm using spectrophotometer (Elico SL 159, India). Nitric oxide radical scavenging activity was calculated using the formula,

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

where,

$A_o$  is the absorbance of the control and

$A_c$  is the absorbance of the metabolite/standard.

**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  $\mu$ l of sample injected. Water was used as mobile phase. The absorbance was monitored at 360 nm [22].

**UV Absorption Spectra:** The UV-Visible absorption spectra of the metabolite was determined with a (Elico SL 159, India) spectrophotometer at 300-400nm to determine the  $\lambda_{\text{maximum}}$  of the band [23].

**Statistical Analysis:** All experiments were repeated three times. The data were recorded as mean  $\pm$  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 [24]. 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 mechanisms of action of flavonoids are through scavenging or chelating process [25, 26]. 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 [27]. 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 $\pm$ 0.2%), metabolite (14.39 $\pm$ 1.19 %) at a concentration of 125 $\mu$ g/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 was shown graphically in Fig. 1.

**Metal Chelating Activity:** Ferrous ion commonly found in the food system is well known as an effective pro-oxidant. Ferrous ion can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxy and alkoxy radicals [28, 29]. Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex

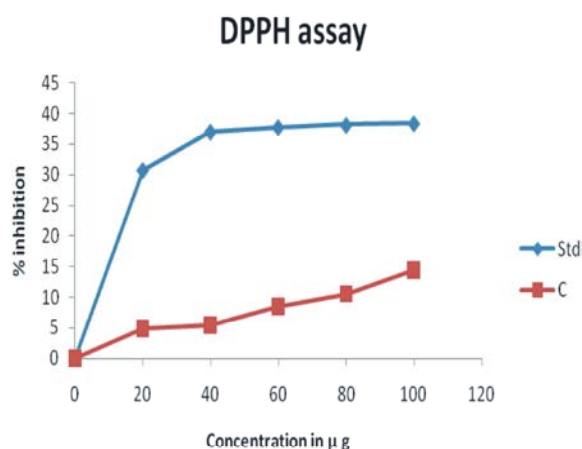


Fig. 1: DPPH assay of the metabolite

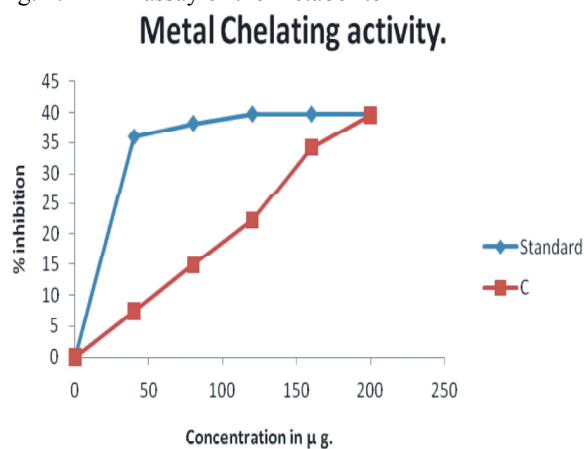


Fig. 2: Metal chelating activity of the metabolite

(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 [24] (Fig. 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  $\text{Fe}^{2+}$ -ferrozine complex was observed to be linearly decreased with the concentration of the metabolite (from 00 to 200µg/ml). The percentage of metal chelating capacity at 200 µg/ml of metabolite and EDTA was found to be  $39.55 \pm 0.85\%$  and  $39.76 \pm 0.2\%$  respectively.

The metal scavenging activity revealed 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  $\text{Fe}^{3+}$ / ferricyanide complex to the  $\text{Fe}^{2+}$ / 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 [30].

In this study, the metabolite showed increased absorbance with increased concentration. Hence, it was 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 ( $0.56 \pm 0.02$ ), metabolite ( $0.51 \pm 0.02$ ) absorbance units at 100µg/ml (Fig. 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 ferryl-perferryl complex or through  $\cdot\text{OH}$  radical by Fenton's reaction. The inhibition could be caused by the absence of ferryl – perferryl complex or by scavenging the  $\cdot\text{OH}$  radical or the super oxide radicals or by changing the  $\text{Fe}^{3+}/\text{Fe}^{2+}$  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 [24].

The percentages of lipid peroxidation inhibition capacity of 125 µg/ml doses of metabolite and BHA were found to be  $67.88 \pm 2.09\%$  and  $81.16 \pm 2.06\%$  respectively. The inhibition percentage was shown graphically in Fig. 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, peroxidase, NADPH oxidase and xanthine oxidase [31]. Super oxide anions damage directly or indirectly by forming  $\text{H}_2\text{O}_2$ ,  $\cdot\text{OH}$ , peroxy nitrate or singlet oxygen during aging and pathological events such as

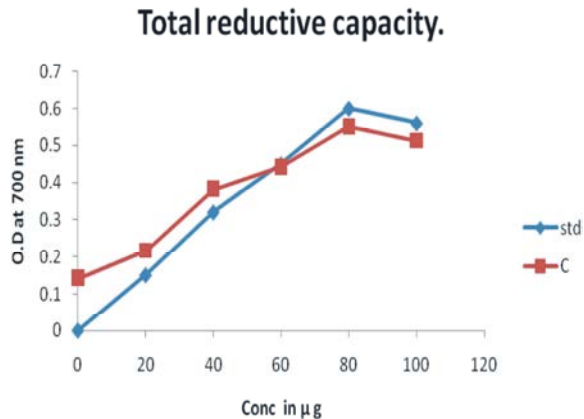


Fig. 3: Total reductive capacity of the metabolite

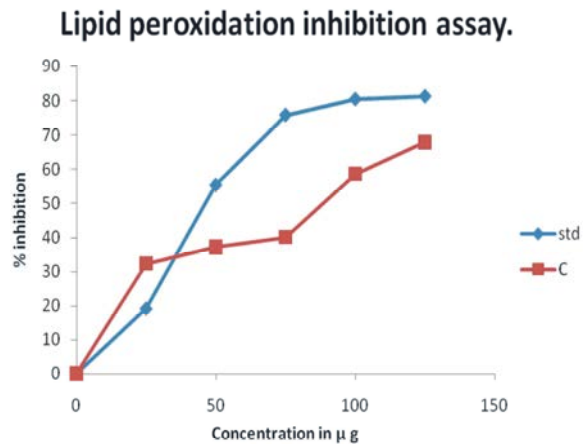


Fig. 4: Lipid peroxidation inhibition assay of the metabolite

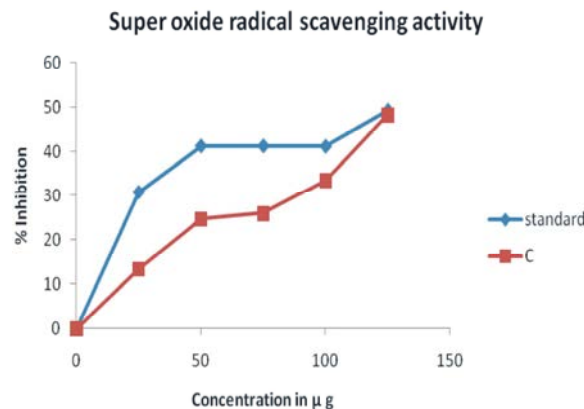


Fig. 5: Super oxide radical scavenging activity of the metabolite

ischemic reperfusion injury, super oxide has also been observed to directly initiate lipid peroxidation. In this method,  $\text{O}_2^{\bullet-}$  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. Antioxidants are able to inhibit the blue NBT formation. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of  $\text{O}_2^{\bullet-}$  in the reaction mixture.

**The Percentage of Inhibition Was in the Order:** Ascorbic acid ( $49.25 \pm 0.2\%$ ), metabolite ( $48.28 \pm 2.01\%$ ) at a concentration of  $125 \mu\text{g/ml}$ . The inhibition percentage was shown graphically in Fig. 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, vasodilation, antimicrobial and antitumour activities. Although nitric oxide is involved in host defence, over production of these two radicals contributes to the pathogenesis of some inflammatory diseases [32]. 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  $79.25 \pm 1.65\%$  at a concentration of  $125 \mu\text{g/ml}$ . The inhibition percentage was shown graphically in Fig. 6.

**TLC:** The thin layer chromatogram of the metabolite was developed. On spraying ninhydrin Isolate S4 showed a purple band with Rf value 0.60. The chromatogram was as shown in Fig. 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 Fig. 8.

The results from all the above assays indicated that the metabolite has a moderate anti oxidant activity with good nitric oxide scavenging activity. From the results we can also concluded 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 *et al.* [33] and Hopia and Heinonen [34].

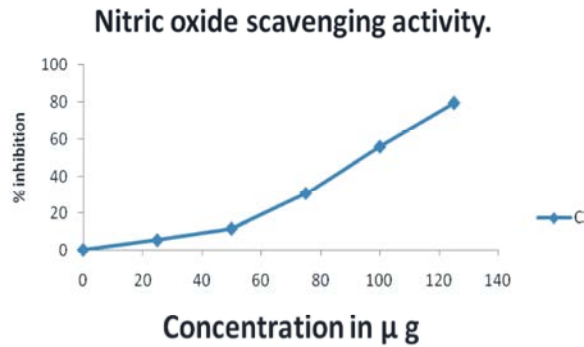


Fig. 6: Nitric oxide scavenging activity of the metabolite



Fig. 7: TLC of the bioactive metabolite

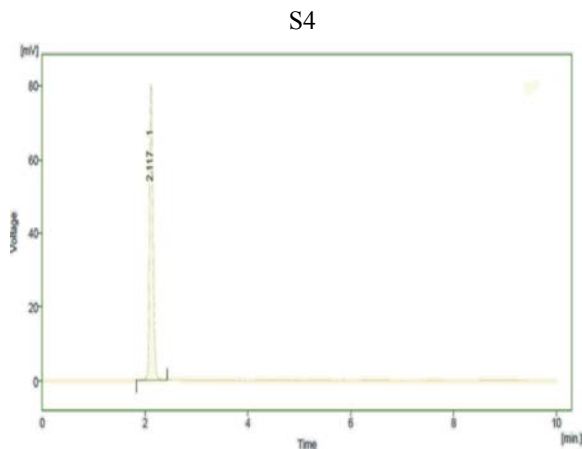


Fig. 8: HPLC analysis of the bioactive metabolite

It can be concluded that the metabolite from the *Streptomyces species* KSRO-04 is a potent antioxidant agent which suggests its beneficiary role as a pharmacological agent. Further research has to be carried out to characterize the active principle.

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