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In Vitro Antioxidant activity of Metabolite from *Streptomyces fradiae* strain GOS1

Gautham S A and Onkarappa R*
P.G. Department of Studies and Research in Microbiology, Sahyadri Science College (Autonomous), Kuvempu University, Shimoga -577203, Karnataka, India.

Abstract
Streptomyces species being the largest members of actinomycetes have been known for their metabolic capacities and form an august source of pharmacologically important compounds. The present study concentrated on *in vitro* evaluation of antioxidant property of n-butanol extract with antimicrobial potential from *Streptomyces fradiae* strain GOS1. The antioxidant activity was evaluated by total phenolic content, total reductive capability, DPPH assay, lipid peroxidation inhibition assay, nitric oxide scavenging activity, super oxide anion scavenging activity and metal chelating activity. The result showed moderate dose dependent antioxidant activity of the metabolite and is indicative of its role as a primary antioxidant.

*Corresponding author, Mailing address:
Onkarappa R*
E-Mail: *onkarappa.r@gmail.com, gautham.annappa@gmail.com

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INTRODUCTION:

Oxygen radicals and other reactive species are generated in biological systems either as by- products of oxygen reduction or by xenobiotic catabolism[1]. The reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot}$), hydroxyl radicals ($OH^{\cdot}$), nitric oxide (NO) and peroxy radicals ($ROO^{\cdot}$) are unstable and can attack key biomolecules such as lipids, proteins and nucleic acids[2]. The consequences of oxidation of these biomolecules have been linked to a variety of different human disorders, including atherosclerosis, cancer and disease of the nervous system [3]. Cells have a comprehensive array of antioxidant defense mechanisms to reduce free radical formation or limit their damaging effects [4].

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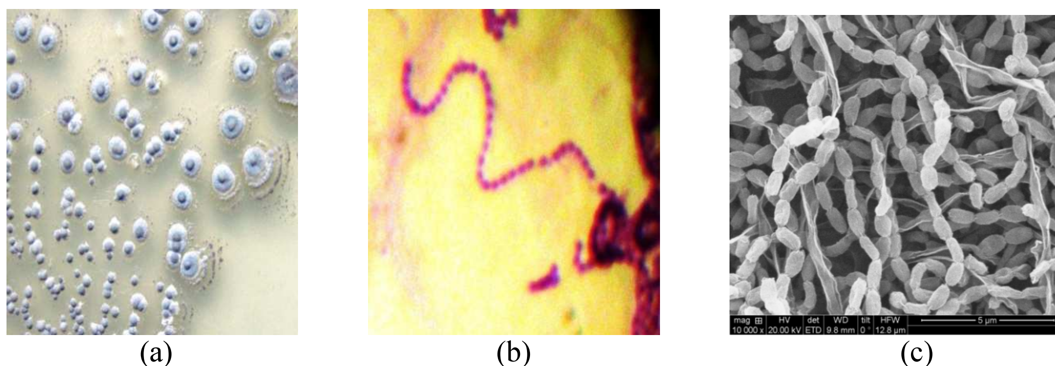
These mechanisms are not sufficient when the balance shifts to the side of free radical generation, thus body requires antioxidant supplements to reduce oxidative damages [5]. Antioxidants are molecules capable of preventing oxidative damage. Recent investigations suggest that antioxidant capacity of putative antioxidants can be attributed to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging in body cells alleviating lipid, protein oxidation and may reduce potential mutations. Therefore help prevent degenerative diseases and other pathologies [6,7].

The wide use of natural antioxidants as a replacement of conventional synthetic antioxidants in food and food supplements has been employed owing to the fact that natural products are considered to be a promising and safe source. Microbial natural products are notable not only for their potent therapeutic activities, but also for the fact that they frequently possess the desirable pharmacokinetic properties required for clinical development; many microbial natural products reach market without any chemical modifications, a testimony to the remarkable ability of microorganisms to produce drug like small molecules[8].

Among microorganisms the members of the order Actinomycetales have proved to be a particularly rich source of secondary metabolites with extensive industrial applications [9]. Traditionally actinomycetes have been the most prolific group in antibiotic production and have been the source of a good number of marketed antibiotics [10]. Mathematical models have suggested that the number of compounds still to be discovered from actinomycetes could well be above 10^5 - a tiny fraction of which has been unearthed so far [11].

The genus *Streptomyces* represents the largest group of actinomycetes and is the largest antibiotic-producing genus in the microbial world discovered so far. The number of antimicrobial compounds reported from the species of this genus per year increased almost exponentially [11]. Streptomycetes are used extensively in industry, due to their ability to generate a number of chemical compounds, including antibiotics, pigments, enzymes, enzyme inhibitors, antitumour agents, antifungal compounds, antiviral agents, pesticides etc[12, 13, 14]. The capacity of the members of this genus to produce commercially significant compounds, especially antibiotics, remains unsurpassed [15].

In pretext of the need of antioxidants and the rich and diverse secondary metabolic capacity of *Streptomyces*, the present study was carried out to assess the antioxidant potential of a bioactive fraction from *Streptomyces fradiae* strain GOS1 isolated from Western Ghats soils of Agumbe, Karnataka, India. *S. fradiae* strain GOS1 is a gram positive, non-acid fast, broad spectrum antibiotic producing, grey coloured isolate with an extended spiral spore chain arrangement and smooth spore surface arrangement (Figure 1)[16].



(a) (b) (c)

Figure 1: Colony and Microscopic characteristics of *Streptomyces fradiae* GOS1 [a- Colony on Starch casein agar; b- Spore arrangement (2000x), c- SEM of spore chain (10000x)]

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MATERIALS AND METHODS:

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).

Fermentation:

The isolate was grown in 250ml Erlenmeyer flasks containing 150ml of Starch Casein Nitrate (SCN) broth. The inoculated flasks were incubated at 30±2°C for 12 days. The growth was constantly observed for any possible contaminations and the broth was filtered through Whatmann Grade-01 filter paper[17].

Solubility and Metabolite Extraction:

The culture filterate was subjected to solubility tests in different solvents *viz.*, hexane, n-butanol, ethyl acetate, acetone, methanol and ethanol on polarity basis and LD50 values[18]. The solubility assessment was done by mixing broth and solvent (1:1v/v). The metabolite was found to be soluble in n-butanol. The extraction was carried out using sterile separating funnel to which equal volumes (1:1v/v) of broth and solvent were added and was vigorously agitated to ensure proper mixing of solvent and broth. The funnel was allowed to stand for 30 min. The organic fraction was separated, dried and used for antioxidant assays [19,20].

Antioxidant Activity:

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 assayed according to the protocol of Braca *et al.*[21]. 0.1ml of n-butanol extract (0-100µg/ml of methanol) was added to 3ml of 0.004% DPPH (in methanol) and the tubes were incubated in dark at room temperature for 30 min and the absorbance was measured at 517nm spectrophotometrically. Tertiary butyl hydroquinone (TBHQ) was used as standard. Absorbance of DPPH without extract/standard was also noted. The DPPH radical scavenging activity (%) was calculated using the formula,

Scavenging activity (%) = $[(A_o - A_e) / A_o] \times 100$

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

Total Reductive Capability:

1ml of different concentrations (0-100µg/ml) of n-butanol extract and the standard (Ascorbic acid) was mixed with 2.5ml of 0.2M phosphate buffer (pH 6.6) and 2.5ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. Reaction mixture was incubated at 50°C for 20 min, cooled to room temperature, 2.5ml of 10% trichloro acetic acid (TCA) was added and centrifuged (6500rpm at room temperature) for 10min. The upper layer of solution (2.5ml) was mixed with 2.5ml of distilled water and 0.5ml of 0.1% Ferric Chloride. Absorbance of the reaction mixture was measured at 700nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates the increase in reduction capability[22].

Metal Chelating Activity:

The chelating of ferrous ions by n-butanol extract and standard ethylene diamine tetra acetic acid (EDTA) was estimated according to the method of Dinis *et al.*[23]. The extract and standard (0-200µg/ml) were mixed with distilled water to make the volume to 3.0ml and 2mM 0.05ml of ferrous chloride ($FeCl_2$) solution was added. The reaction was initiated by the addition of 5mM ferrozine (0.2ml) and the mixture was vortexed and was incubated at room temperature for 10min. Absorbance of the solution was measured spectrophotometrically at 562nm. The control contained ferrous chloride and ferrozine complex formation molecules. The percentage of inhibition of Ferrozine-Fe^{2+} complex formation was calculated from the formula,

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.

Lipid Peroxidation Inhibition Assay:

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure lipid peroxidation formed using egg yolk homogenate as lipid rich media. 0.5ml of egg homogenate (10% in distilled water) and 0.1ml of extract and standard Butylated Hydroxyanisole (BHA) in the concentration range 0-125µg/ml were mixed in test tubes, the volume was made up to 1ml by adding distilled water. Finally 0.05ml of 0.07M ferrous sulphate ($FeSO_4$) was added to the mixture and incubated for 30min to induce the lipid peroxidation. Further 1.5ml of 20% acetic acid (pH 3.5), 1.5ml of 0.8% TBA and 0.05ml 20% TCA was added, the mixture was vortexed and was heated in a boiling water bath for 60min. After cooling 5ml of n-butanol was added and centrifuged at 3000rpm for 10min. The absorbance of the organic upper layer was measured at 532nm using a spectrophotometer[24]. The percentage inhibition of lipid peroxidation was calculated from the formula,

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 extract/standard.

Superoxide Radical Scavenging Activity:

The superoxide radical scavenging activity was assayed according to the method of Nishimiki *et al.*[25]. Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS-NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). All the solutions used in this experiment were prepared in phosphate buffer (pH 7.4). 1ml of NBT (156µM), 1ml of NADH (468µM) and 1ml of extract (000-125µg/ml) were mixed. The reaction was started by adding 1ml of PMS (60µM) and the mixture was incubated at 25°C for 5min followed by measurement of absorbance at 560nm spectrophotometrically. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. Ascorbic acid was used as standard. The percentage inhibition was calculated from the formula,

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 extract/standard.

Nitric Oxide Radical Scavenging Activity:

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2ml of 10mM sodium nitroprusside in 0.5ml of phosphate buffer saline (pH 7.4) was mixed with 0.5ml of extract and standard (curcumin) at various concentrations and the mixture was incubated at 25°C for 150min. From the incubated mixture 0.5ml was taken out and added into 1ml of sulfanilic acid reagent and incubated for 5min. Finally 1ml napthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30min before measuring absorbance at 540nm using spectrophotometer[26]. Nitric oxide radical scavenging activity was calculated using the formula,

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 extract/standard.

Total Phenolic Content:

The total phenolic content (TPC) of the extract was determined with the Folin-Ciocalteu reagent (FCR). 1.0ml of extract was mixed with 2.5ml FCR (diluted 1:10v/v) followed by 2ml of sodium carbonate (7.5% v/v) solution. The tubes were vortexed and allowed to stand for 90min at room temperature. Absorbance was measured against the blank at 750nm using a spectrophotometer. The TPC of the extract was expressed in terms of milligrams (mg) of gallic acid equivalents (GAE)/g of dry weight[27].

RESULTS:

DPPH Radical Scavenging Activity:

The scavenging of DPPH free radicals was observed by decrease in absorbance at 517nm induced by extract/standard. The results showed dose dependent scavenging activity. The percentage of inhibition of DPPH radicals by n-butanol extract and TBHQ at concentration of 100µg/ml was 33.33% and 68.4% respectively (Figure 2).

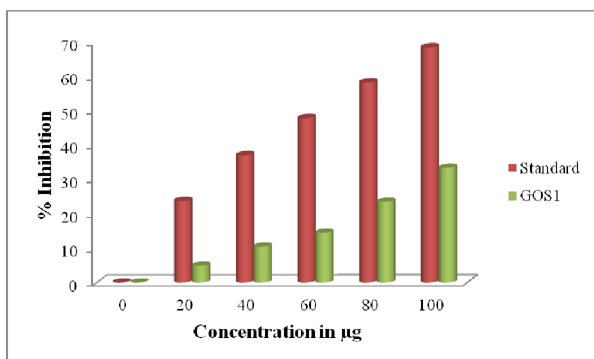

Figure 2: DPPH radical scavenging activity of n-butanol extract and TBHQ

Total Reductive Capability:

In total reductive assay, the increase in absorbance at 700nm with the increase in concentration showed the reducing capability of standard and the n-butanol extract. Ascorbic acid showed absorbance values of 1.15 and the n-butanol extract showed 0.51 absorbance units at concentrations of 200µg/ml respectively (Figure 3).

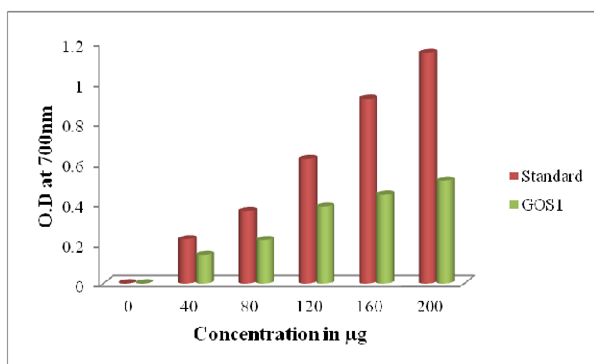

Figure 3: Total reductive capability of n-butanol extract and ascorbic acid

Metal Chelating Activity:

Metal chelating capacity of the extract was dose dependent and was evident by the decrease in absorbance at 562nm. EDTA showed higher chelating efficacy than extract. The percentage of metal chelating capacity at 200µg/ml of EDTA and n-butanol extract was found to be 70.48% and 39.54% respectively (Figure 4).

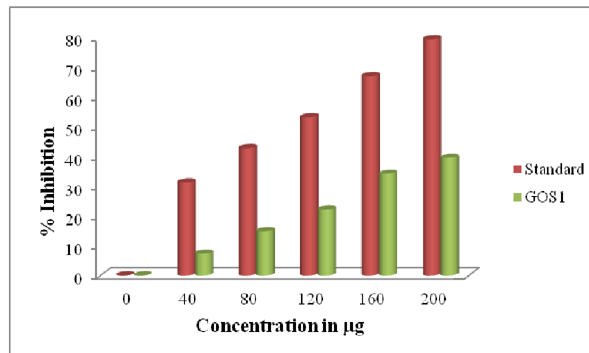

Figure 4: Metal chelating activity of n-butanol extract and EDTA

Lipid Peroxidation Inhibition Assay:

In lipid peroxidation inhibiton assay, n-butanol extract and BHA showed a dose dependent activity and the inhibition increased with the increase in concentration. The percentages of lipid peroxidation inhibition capacity of BHA and extract at 125µg/ml were found to be 90.93% and 68.13% respectively (Figure 5).

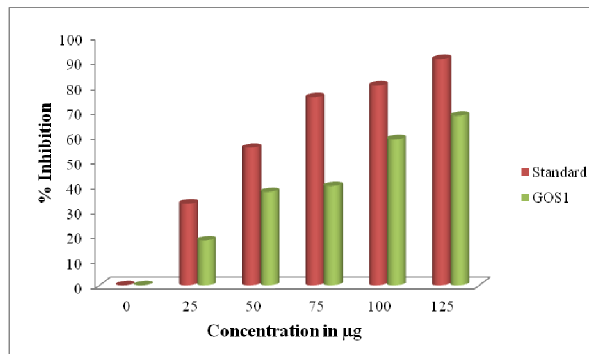

Figure 5: Lipid peroxidation inhibition by n-butanol extract and BHA

Superoxide Radical Scavenging Activity:

The percentage of superoxide scavenging activity by the n-butanol extract and the standard was dose dependent. The percentage radical scavenging activity of ascorbic acid and n-butanol extract at

125µg/ml was 69.25% and 48.28% respectively (Figure 6).

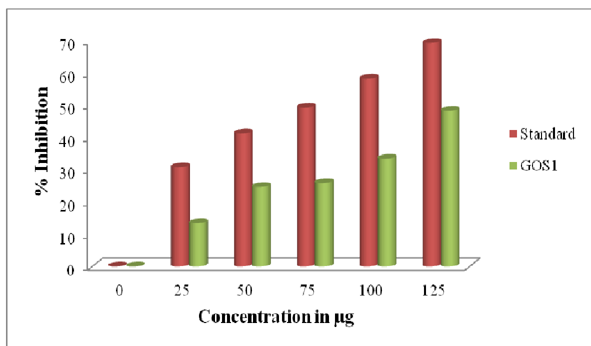

Figure 6: Superoxide radical scavenging activity of n-butanol extract and ascorbic acid

Nitric Oxide Radical Scavenging Activity:

In nitric oxide scavenging assay, the standard and the extract inhibited the nitric oxide radicals in a dose dependent manner. Curcumin and the n-butanol extract at 125µg/ml showed percentage inhibition of 79.25% and 50.98% respectively (Figure 7).

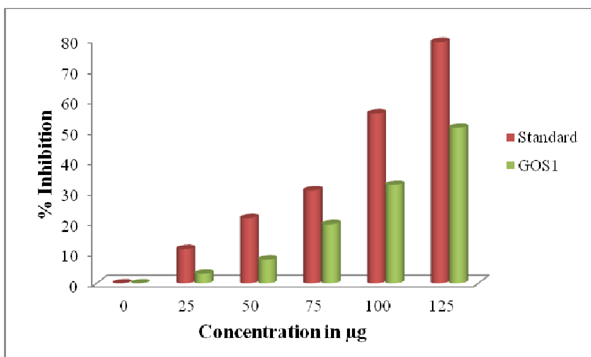

Figure 7: Nitric oxide radical scavenging activity of n-butanol extract and curcumin

Total Phenolic Content:

The total phenolic content, expressed as GAE, of n-butanol extract was found to be 1.23mg GAE/g of dry metabolite.

DISCUSSION

Owing to the complexity of the antioxidant materials and their mechanisms of action, it is obvious that no single testing method is capable of providing a comprehensive picture of the antioxidant profile of a studied sample and combination of different methods is necessary[28]. In the present study, the role of n-butanol extract of *Streptomyces* GOS1 as an antioxidant was assessed by seven different antioxidant models.

DPPH radical scavenging activity:

DPPH radical scavenging activity forms an easy, rapid and sensitive method for screening antioxidant molecules. The DPPH test provides information on the reactivity of the test compounds with a stable free radical. DPPH gives a strong absorption band at 517nm in visible region. When the odd electron becomes paired off in the presence of free radical scavenger the absorption reduces indicated by the change of color from deep violet to light yellow. The degree of reduction in absorbance is indicative of the radical scavenging potential of the test compound[29]. In the present study, the n-butanol extract showed moderate dose dependent activity. The extract though showed moderate activity in comparison to the standard TBHQ, the study was evident in determining the proton donating ability of the extract thus serving as free radical scavenger. The results obtained are in concordance with the results reported by Kekuda *et al.*[30], Zhong *et al.*[31,32] and Diraviyam *et al.*[33]. These previous results have also reported mild to moderate antioxidant activity of metabolites from *Streptomyces* sp.

Total Reductive Capability:

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. For the measurements of the reductive ability, the Fe^{3+} to Fe^{2+} transformation in the presence of n-butanol extract was investigated. In the present study the extract showed increase in activity with the increase in dose. The extract though showed moderate activity in comparison to the standard ascorbic acid. The results are concordant with earlier studies conducted by Kekuda *et al.*[30] and Thenmozhi and Kannabiran[34]. The reducing capacity of the compound may serve as significant indicator of its potential antioxidant activity[35].

Metal Chelating Effect:

Ferrous ion can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation

by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red coloured) formation is interrupted and as a result, the red coloured complex is decreased. Thus the rate of chelating effect of the coexisting chelator can be determined by measuring the rate of colour reduction[26]. The formation of ferrozine-Fe^{2+} complex is interrupted by the n-butanol extract in a dose dependent manner and similar results have been observed by Thenmozhi and Kannabiran[34]. Chelating agents that forms bonds with metal are effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion[26].

Lipid Peroxidation Inhibition Assay:

Lipid peroxidation is an accumulated effect of ROS, which leads to deterioration of biological systems. It may be initiated by reactive free radicals, which subtract an allylic hydrogen atom from a methylene group of polyunsaturated fatty acid side chains. This is accompanied by bond rearrangement that results in stabilization by diene conjugate formation. The lipid radical then takes up oxygen to form peroxy species[36]. Hydroxyl radicals are considered to be one of the rapid initiators of lipid peroxidation process, abstracting hydrogen atoms from polyunsaturated fatty acid which brings about peroxidation reactions of membrane lipids and also from each of the carbon atom of the sugar moiety of DNA causing oxidative damage to DNA. These effects have been implicated in mutagenesis, carcinogenesis and aging[37]. In our study, the n-butanol extract and standard was found to exhibit a concentration dependent lipid peroxidation inhibition. The extent of inhibition of lipid peroxidation by extract was lesser when compared to standard.

Superoxide Radical Scavenging Activity:

Superoxide radicals damage biomolecules directly or indirectly by forming H_2O_2, OH, peroxyl nitrate or singlet oxygen during aging and pathological events such as ischemic repurfusion injury. Superoxide has also been observed to directly initiate lipid peroxidation[38]. The superoxide anion radical scavenging assayed by PMS-NADH system reveled dose dependent scavenging activity of the n-butanol extract. In comparison to ascorbic acid, the n-butanol extract showed moderate activity.

Nitric Oxide Radical Scavenging Activity:

Nitric oxide is potent pleotrophic mediator of physiological process such as smooth muscle relaxant, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical which plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodialtion, antimicrobial and antitumor activities. Nitric oxide and superoxide radicals are involved in host defense, however over production of these radicals contributes to the pathogenesis of some inflammatory diseases. In pathological conditions nitric oxide radicals react with superoxide radicals and form potentially cytotoxic molecule peroxynitrite. Nitric oxide inhibitors have been shown to have beneficial effects on some aspect of inflammation and tissue damage seen in inflammatory diseases[39]. The n-butanol extract dose dependently inhibited the formation of nitric oxide radicals, suggesting its beneficial role in treating inflammatory conditions.

Total Phenolic Content:

Phenolics are the most wide spread secondary metabolite in plants as well as in microorganisms. This diverse group of compounds has received much attention as a potential natural antioxidant in terms of their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers[40]. The Folin-Ciocalteau method was employed to estimate the phenolic content of the n-butanol extract and revealed minimum quantity of phenolics.

The antioxidant and radical scavenging activity may partly be due to a wide variety of antioxidant constituents such as phenolics, ascorbate, flavonoids and carotenoids. The low quotient of the phenolic compounds in the extract might have attributed to the moderate antioxidant and scavenging activities observed in this study.

CONCLUSION:

The present study revealed the antioxidant property of the n-butanol extract of *Streptomyces fradiae* strain GOS1. Although the above represented results are moderate in comparison to the standard compounds, the results are indicative that the *Streptomyces* extract possess antioxidant properties and can serve as free radical inhibitors or scavengers acting possibly as primary antioxidants.

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