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Research Article

ANTIOXIDANT, ANTHELMINTIC AND ENZYME INHIBITORY POTENTIAL OF *STREPTOMYCES VARIABILIS* STRAIN PO-178 ISOLATED FROM WESTERN GHAT SOIL OF AGUMBE, KARNATAKA, INDIA

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ABSTRACT

The present study was carried out with an aim of determining antioxidant, anthelmintic and enzyme (amylase and pancreatic lipase) inhibitory activity of bioactive *Streptomyces variabilis* strain PO-178 isolated from a rhizosphere soil sample of Agumbe, Western Ghats of Karnataka, India. The strainPO-178 was grown in Starch casein nitrate broth for ten days at 30°C and the culture filtrate was extracted using butanol in separation funnel. Antioxidant potential of butanol extract was tested using four *in vitro* antioxidant models *viz*., DPPH radical scavenging, ABTS radical scavenging, metal chelating and Ferric reducing assay. The extract exhibited moderate antioxidant activity in all assays performed when compared with reference antioxidants. Anthelmintic activity of extract was screened using adult Indian earthworm. The extract caused paralysis and mortality of worms in a dose dependent manner. Enzyme inhibitory activity of butanol extract was determined against amylase and pancreatic lipase. The extract was found to exhibit dose dependent pancreatic lipase inhibitory and amylase inhibitory activity. Further studies on isolation and characterization of biologically active principles from butanol extract of strain PO-178 and their bioactivity determination are under progress.

Keywords: Western Ghats, Agumbe, *Streptomyces variabilis*, Antioxidant, Anthelmintic, Enzyme inhibitory activity

INTRODUCTION

Among various ecological habitats, soil is one of the richest reservoirs supporting a variety of organisms including microorganisms. Rhizosphere is the region of soil present in vicinity to plant roots and is influenced by secretions of plant roots called root exudates. This region is thought to be of great importance to plant health and soil fertility. The exudates from plant roots stimulate the growth of microbial populations in soil and thus, the microbial activity is greatest in rhizosphere region[1]. Actinomycetes forms a key part of soil microorganisms. The species of the genus *Streptomyces* are saprophytic and are commonly associated with soils. It has been found that about 90 % of soil actinomycetes are reported to be *Streptomyces* species. In soil, *Streptomyces* species significantly contribute to the turnover of complex biopolymers such as cellulose, pectin, lignin etc. They are noteworthy as they are the prolific producers to a range of bioactive metabolites including antibiotics[2-4]. Western Ghats of India covers an area of 1, 80, 000 km^2 and represent just under 6 % of the land area of India. The area represents > 30 % of all plant, fish, herpeto-fauna, birds and mammal species found in India which include some of the globally threatened species[5]. Agumbe, a region of Western Ghats of Shivamogga district, Karnataka, India is shown to be a rich reservoir for *Streptomyces* species having potent industrial and agricultural significance in terms of production of bioactive metabolites. *Streptomyces* species from Agumbe region of Western Ghats have shown to antimicrobial, antioxidant, enzyme inhibitory, insecticidal, cytotoxic, anthelmintic, analgesic, anti-inflammatory, CNS depressant and antipyretic activities[6-13]. *Streptomyces variabilis* strain PO-178 (Figure 1) of this study was isolated from a rhizosphere soil of Agumbe, Karnataka, India. The extract from this strain was shown to possess antimicrobial, insecticidal, cytotoxic, analgesic, anti-inflammatory, antipyretic and CNS depressant activity[13,14]. In continuation part of our previous study, the present study was conducted with an aim of determining antioxidant, anthelmintic and enzyme inhibitory activity of butanol extract of *S.variabilis* PO-178 isolated from rhizosphere soil of Agumbe, Karnataka, India.

MATERIALS AND METHODS

Fermentation and extraction of metabolite from strain PO-178

Sterile SCN broth containing Erlenmeyer flasks were inoculated with the spore suspension of well sporulated culture of PO-178. The flasks were incubated aerobically at 28°C for 10 days followed by filtering the broth through sterile Whatman No. 1 filter paper. The culture filtrate was centrifuged and the supernatant was subjected solvent extraction. Equal volume (1:1) of supernatant and butanol were taken in a separation funnel and agitated for about 30

minutes. Solvent layer was separated and the supernatant was again extracted with butanol. The solvent layers were pooled and evaporated to dryness at 40°C[9,15].

Antioxidant activity of butanol extract of isolate PO-178

DPPH free radical scavenging activity

2 ml of DPPH solution (0.002 % in methanol) was mixed with 2 ml of different concentrations (0-200 µg/ml) of butanol extract and reference standard (ascorbic acid) separately. The tubes were incubated in dark at room temperature for 30 minutes and the optical density was measured at 517 nm using UV-Vis spectrophotometer (Elico, SL-159). The absorbance of the DPPH control (2 ml DPPH + 2 ml methanol) was noted. The scavenging activity of the extract was calculated using the formula:

$$\text{Scavenging activity (\%)} = [(A - B) / A] \times 100$$

Where A is absorbance of DPPH control and B is absorbance of DPPH in the presence of extract/standard[6]. IC_{50} (Inhibitory Concentration) for extract was calculated, IC_{50} denotes the concentration of extract required to scavenge 50 % of DPPH free radicals.

ABTS radical scavenging assay

The ABTS radicals were generated by reacting 7 mM ABTS stock solution with 2.45 mM potassium per sulfate and the mixture was left at room temperature in the dark for 12–16 hours. The obtained radical solution was diluted with distilled water to an absorbance of 0.7 at 730 nm. 1 ml of different concentrations (0-200 µg/ml) of butanol extract and ascorbic acid (reference standard) was added to 4 ml of ABTS solution. The tubes were incubated for 30 minutes and absorbance was measured at 730 nm in a UV-Vis spectrophotometer (Elico, SL-159). The absorbance of the ABTS control (4 ml DPPH + 1 ml methanol) was noted. The scavenging activity was calculated using the formula:

$$\text{Scavenging activity (\%)} = [(A - B) / A] \times 100$$

Where A is the absorbance of the ABTS control and B is the absorbance of ABTS in the presence of extract/standard[16]. IC_{50} for extract was calculated. IC_{50} denotes the concentration of extract required to scavenge 50 % of ABTS free radicals.

Metal chelating assay

The chelating of ferrous ions by different concentrations of butanol extract and EDTA (reference standard) was determined by the method of Ebrahimzadeh *et al*[17]. Briefly, different concentrations of extract and standard (0-200 µg/ml) were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for ten minutes. After the mixture had reached equilibrium, the absorbance of the solution was then measured at 562 nm in a UV-Vis spectrophotometer (Elico, SL-159). The control contained $FeCl_2$ and ferrozine. The percentage of inhibition of ferrozine- Fe^{2+} complex formation was given by the formula:

$$\text{Percent inhibition} = [(A - B) / A] \times 100$$

Where A was the absorbance of the control, and B was the absorbance in the presence of the extract and standard. EC_{50} (effective concentration) was calculated for extract. EC_{50} denotes the concentration of extract required to cause 50 % chelating activity.

Ferric reducing activity

Different concentrations (0-200 µg/ml) of butanol extract and ascorbic acid (reference standard) in 1 ml of methanol were mixed separately with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (1 %). The tubes were incubated in water bath for 20 minutes at 50°C, cooled rapidly and mixed with 2.5 ml of trichloroacetic acid (10 %) and 0.5 ml of ferric chloride (0.1 %). The amount of iron (II)-ferricyanide complex formed was determined by measuring Perl's Prussian blue complex at 700 nm after 10 minutes. An increase in absorbance on increase in concentration indicates increased reducing power[6].

Anthelmintic activity of butanol extract of isolate PO-178

The anthelmintic effect of butanol extract was screened against adult Indian earthworms (*Pheretima posthuma*). The worms were washed with normal saline (0.85 %) to remove extraneous matter. In brief, 6 worms of equal size (6 cm long) were transferred into normal saline containing standard (1 %) and different concentrations of butanol extract (0-2 mg/ml of saline). The time taken for paralysis and death of worms was noted. Piperazine citrate was used as standard anthelmintic. Normal saline served as control[6].

Pancreatic lipase inhibitory activity of butanol extract of isolate PO-178

Lipase inhibitory activity of different concentrations of butanol extract (0-50 mg/ml) was tested by mixing 100 µl of each concentration of extract, 8 ml of oil emulsion and 1 ml of chicken pancreatic lipase followed by incubation of 60 minutes. The reaction was stopped by adding 1.5 ml of a mixture solution containing acetone and 95 % ethanol (1:1). The liberated fatty acids were determined by titrating the solution against 0.02 M NaOH (standardized by 0.01 M oxalic acid) using phenolphthalein as an indicator[9]. Percentage inhibition of lipase activity was calculated using the formula:

$$\text{Lipase inhibition} = [(A - B) / A] \times 100$$

Where A is lipase activity in the absence of extract, B is activity of lipase when incubated with the extract. IC_{50} for extract was calculated. IC_{50} denotes the concentration of extract required to cause 50 % inhibition of enzyme activity.

Amylase inhibitory activity of butanol extract of isolate PO-178

The inhibitory activity of butanol extract against amylase activity was tested against amylase (Diastase (Fungal) 3240, Lobachemie Laboratory reagents and fine chemicals, Mumbai, India) by following the method Karthik *et al*[18]. The enzyme (0.5 %) was prepared in phosphate buffer (pH 6.8). Briefly, 500 µl of different concentrations of extract (0-50 mg/ml) and 500 µl of 0.1M phosphate buffer (pH 6.8) containing amylase were incubated at 25°C for 10 minutes. After pre incubation, 500 µl of 1 % starch solution in 0.1 M phosphate buffer (pH 6.8) was added to each tube and further incubated at 25°C for 10 minutes. The reaction was stopped by addition of 1 ml of dinitro salicylic acid reagent. The same was performed for control where extract was replaced with buffer. The test tubes were placed in a boiling water bath for 10 minutes and cooled. To each tube, 10 ml of distilled water was added and the absorbance was measured at 540 nm. The percentage (%) inhibition was calculated using formula:

$$\text{Inhibition (\%)} = [A_{Control} - A_{Extract} / A_{Control}] \times 100$$

IC_{50} for extract was calculated which denotes the concentration of extract required to cause 50 % inhibition of enzyme activity.

RESULTS

DPPH free radical scavenging activity of butanol extract of isolate PO-178

Radical scavenging ability of different concentrations of butanol extract and ascorbic acid was evaluated using DPPH free radical assay. The extract exhibited antioxidant activity by scavenging DPPH* (free radical) and converting into DPPHH and the activity was found to be dose dependent (Figure 2). However, the scavenging potential of extract (IC_{50} 166.61 µg/ml) was found to be much lesser when compared with ascorbic acid (IC_{50} 3.7 µg/ml).

ABTS radical scavenging activity of butanol extract of isolate PO-178

Radical scavenging ability of different concentrations of butanol extract and ascorbic acid was also evaluated using ABTS radical assay. The extract exhibited dose dependent scavenging of ABTS radicals (Figure 3). However, the scavenging potential of extract (IC_{50} 225 µg/ml) was found to be much lesser when compared with reference standard (IC_{50} 3.74 µg/ml).

Metal chelating activity of butanol extract of isolate PO-178

Butanol extract of isolate PO-178 displayed a dose dependent metal chelating activity (Figure 4). The chelating potential of extract (EC_{50} 532.07 µg/ml) was found to be lesser when compared with the reference chelating agent (EC_{50} 13.60 µg/ml).

The reducing potential of butanol extract and ascorbic acid was determined by employing ferric reducing assay in which the reduction of Fe^{3+} to Fe^{2+} was investigated in the presence of different concentrations of extract and ascorbic acid. The absorbance at 700 nm was found to increase with the increase in concentration of extract indicating reducing potential of extract. The reducing potential of extract was lesser when compared with the reference standard (Figure 5).

Anthelmintic activity of butanol extract

The result of anthelmintic activity, in terms of time taken for paralysis and death of worms, of different concentrations of butanol extract is presented in Table 1. The extract exhibited dose dependent anthelmintic activity. Reference anthelmintic caused paralysis and death of worms in relatively shorter period of time when compared with butanol extract.

Pancreatic lipase inhibitory activity of butanol extract of isolate PO-178

Inhibitory activity of butanol extract was tested against chicken pancreatic lipase using olive oil as the substrate. The activity of lipase was affected dose dependently when incubated with butanol extract. An inhibition of > 50 % was observed at extract concentration 50 mg/ml (Figure 6). The IC_{50} value of extract was found to be 44.32 mg/ml.

Amylase inhibitory activity of butanol extract of isolate PO-178

Inhibitory activity of different concentrations of butanol extract was tested against amylase using starch as the substrate. The activity of amylase was affected dose dependently when incubated with the butanol extract. An inhibition of 45 % was observed at extract concentration 50 mg/ml (Figure 7). The IC_{50} value of extract was found to be 55.41 mg/ml.

DISCUSSION

DPPH assay is one of the widely used radical scavenging assays to determine the radical scavenging ability of compounds. DPPH is a stable, organic, nitrogen centered free radical with absorption maximum around 515-528 nm. On accepting an electron or hydrogen atom it becomes a stable diamagnetic molecule. The effect of antioxidants on scavenging DPPH radical is due to their hydrogen donating ability. The compounds (antioxidants) reduce the purple colored DPPH radical to a yellow colored compound diphenylpicrylhydrazine and the extent of color change depends on the hydrogen donating ability of the antioxidants[19-21]. In this study, the absorption of DPPH in the presence of various concentrations of butanol extract of PO-178 was measured at 517 nm. Although the scavenging activity of extract was lesser than that of ascorbic acid, it was evident that the extract showed hydrogen donating ability and the extract could serve as free radical scavengers, acting possibly as primary antioxidants[20]. The result is in justification with earlier studies which also reveal weaker scavenging activity of extracts[6,11,22,23]. Like DPPH assay, ABTS radical scavenging is another popular antioxidant assay which measures the radical scavenging nature of several types of compounds[16,24-27]. On interaction with ABTS, antioxidants either transfer electrons or hydrogen atoms to ABTS and thereby neutralizing the free radical character[27]. In the present study, the butanol extract of PO-178 showed lower scavenging potential when compared to reference standard. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted and eventually that the red color of the complex fades. In the present study, the formation of the Fe^{2+}-ferrozine complex was not completed in the presence of butanol extract, indicating that the extract chelates the iron and capture ferrous ion before ferrozine. The absorbance of Fe^{2+}-ferrozine complex decreased dose-dependently. Iron can stimulate lipid peroxidation by the Fenton reaction, and also accelerates peroxidation by decomposing lipid hydro peroxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. Metal chelating capacity is important since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents form bonds with a metal and are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion[28,29]. The data obtained in the present study reveal that the butanol extract demonstrate capacity for iron binding, suggesting that their action as peroxidation protector may be related to its iron binding capacity. Ferric reducing assay is performed to measure the reducing power of the compounds. In this assay, the reductants (antioxidants) would cause the reduction of Fe^{+3} to Fe^{+2} by donating an electron. The amount of Fe^{+2}complex formed can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability[20]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[30].

Table 1: Anthelmintic activity of butanol extract of isolate PO-178

Treatment	Concentration	Time (minutes)	
		Paralysis	Death
Butanol extract	0.25 mg/ml	0	0
	0.5 mg/ml	90	150
	1.0 mg/ml	65	126
	1.5 mg/ml	55	105
	2.0 mg/ml	35	58
Standard	1 %	16	24
DMSO	10 %	0	0

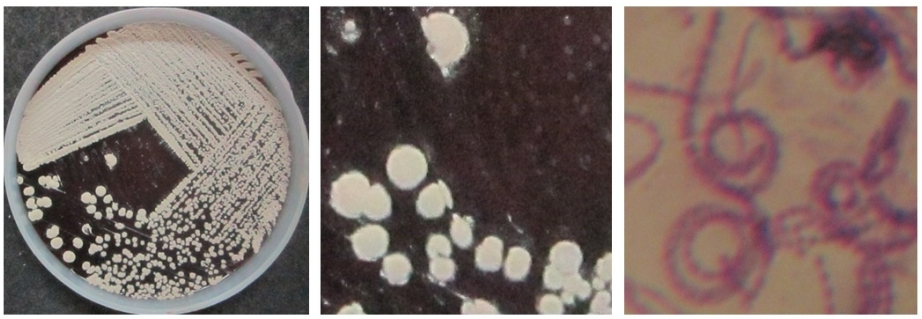

Figure 1: Culture and spore arrangement of PO-178[14]

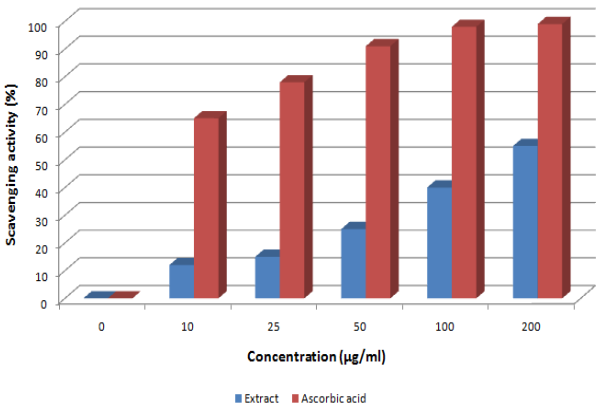

Figure 2: DPPH radical scavenging activity of butanol extract of isolate PO-178

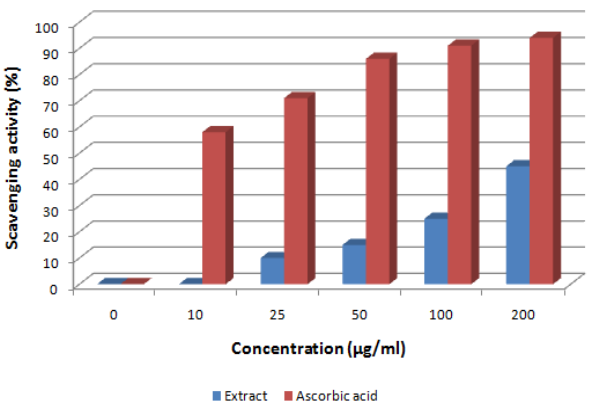

Figure 3: ABTS radical scavenging activity of butanol extract of isolate PO-178

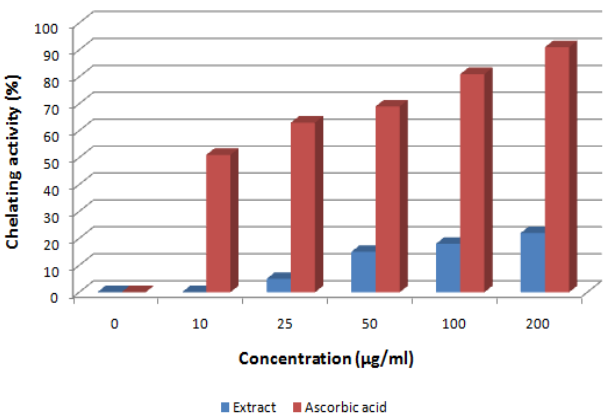

Figure 4: Metal chelating activity of butanol extract of isolate PO-178

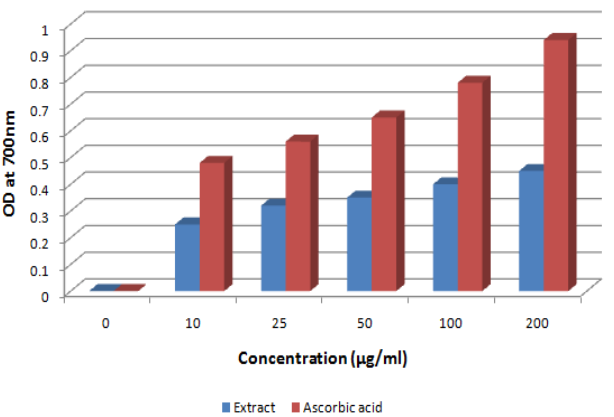

Figure 5: Ferric reducing activity of butanol extract of *S. variabilis* PO-178

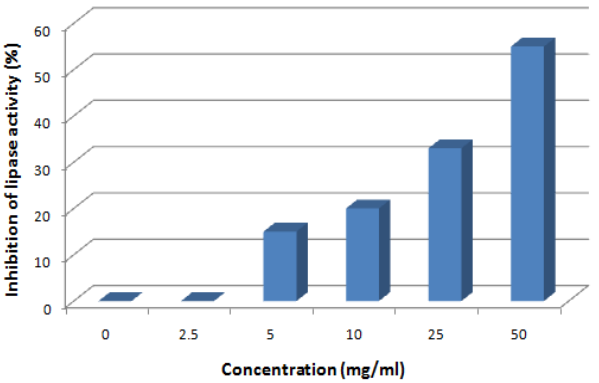

Figure 6: Pancreatic lipase inhibitory activity of butanol extract of isolate PO-178

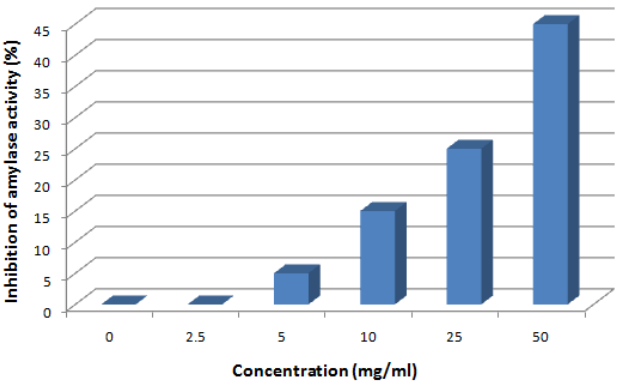

Figure 7: Amylase inhibitory activity of butanol extract of isolate PO-17

In the present study, we determined the Fe^{+3}/Fe^{+2} transformations in the presence of butanol extract of PO-178. In our study, the reducing power of the butanol extract increased with the increase of its concentration. However, the reducing potential of extract was lesser when compared with reference standard. However, it is evident that the extract possesses reductive potential and could serve as electron donors, terminating the radical chain reactions[20]. The result is in justification with the studies of Kekuda *et al*[6], Manasa *et al*[22] and Kekuda *et al*[23]. Helminthiasis is the major cause of morbidity worldwide in particular tropical countries and is often fatal in extreme conditions. Parasitic worms also infect livestock and crops, affecting food production resulting in low economy. Anthelmintic drugs that expel parasitic worms from the body have some major drawbacks such as resistance development in gastro-intestinal helminthes, high cost, adverse effects etc. This situation led to the discovery and development of anthelmintic agents from natural sources[31-34]. Actinomycetes possess marked anthelmintic activity in terms of inhibition of plant and animal parasites[6,35-38]. In this study, the anthelmintic activity of butanol extract of isolate PO-178 was determined using adult Indian earthworms due to their ready availability and anatomical and physiological resemblance to the human intestinal roundworm parasite[32]. The reference anthelmintic piperazine citrate showed higher activity than the extract. The predominant effect of piperazine citrate on the worm is to cause a flaccid paralysis that result in expulsion of the worm[31,32]. The extract of PO-178 not only demonstrated this property but also killed the worms; however, the time taken for this was much higher when compared with standard. In an earlier study, Kekuda *et al*[6] showed dose dependent mortality and death of earthworms by butanol extracts of two *Streptomyces* species isolated from western ghat soil of Agumbe, Karnataka, India. One of the most important strategies for treating obesity is the development of inhibitors of nutrient digestion and absorption. Pancreatic lipase inhibition is one of the most widely studied mechanisms used to determine the potential efficacy of natural products as anti obesity agents. Or list at, one of the two clinically approved drugs for obesity treatment, has been shown to act by inhibiting pancreatic lipase. Although it is one of the best-selling drugs worldwide, it is shown to causesome side effects such as oily stools, oily spotting and flatulence. This has prompted research for the identification of inhibitors that lack some of this side effects[39-42]. Naturally occurring compounds present an exciting opportunity for the discovery of newer anti-obesity agents. A dose dependent inhibition of chicken pancreatic lipase by butanol extract of PO-178 was observed in this study. Similar result was observed in our previous study where we have reported inhibitory activity against pancreatic lipase from chicken pancreas by butanol extract of a *Streptomyces* species[8,9]. Diabetes mellitus is a chronic endocrinal disorder caused by altered carbohydrate metabolism. It is characterized by an elevated blood glucose level. There are two main types of diabetes, type I and type II. The most prevalent form of diabetes is non-insulin dependent diabetes mellitus (type II) accounting for 90 % of cases throughout the world. The control of hyperglycemia is critical in the management of diabetes. Pancreatic α-amylase hydrolyzes the starch to oligosaccharides and maltose in small intestine. Membrane bound α-glucosidase hydrolyzes di- and oligosaccharides to glucose. Inhibition of these two enzymes decreases the rate of starch digestion leading to decreased post-prandial blood glucose levels especially in diabetic patients. Inhibition of carbohydrate hydrolyzing enzymes is one therapeutic approach to decrease the hyperglycemia, especially after a meal. One such drug is Acarbose which inhibits α-glucosidase enzymes in the brush border of the small intestines and pancreatic α-amylase. Other drugs that belong to this class are miglitol and voglibose. Acarbose reduces postprandial hyperglycemia and is used to treat type-2 diabetes. However, these drugs are shown to have gastrointestinal side effects such as abdominal pain, flatulence and diarrhea in the patients[18,43]. Actinomycetes have shown to be promising producers of amylase inhibitors[43-48]. In the present study, the butanol extract of PO-178 was found inhibitory to amylase and the effect was dose dependent.

CONCLUSION

In the present study, we found antioxidant, anthelmintic and enzyme inhibitory activity in the bioactive *Streptomyces variabilis* strain PO-178. The results of the present study highlighted that the soil of Agumbeis a reservoir for potent actinomycetes and hence further screening can be beneficial in isolation of bioactive actinomycetes which might be exploited in industries for production of novel metabolites. Further, purification of components responsible for

bioactivities from the butanol extract and their bioactivity determination are to be carried out.

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