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Actinobacteria from soil as potential free radical scavengers

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ABSTRACT

Aims: Actinobacteria, the Gram positive bacteria are known to produce about 10,000 bioactive secondary metabolites. Among them, 80% of the metabolites are produced by *Streptomyces* like antibiotics, anticancer, antimicrobial, antioxidant, enzymes, etc. But most of the *Streptomyces* species are still to be explored for the different bioactivities. Hence, the present work is designed to evaluate the antioxidant activities of the secondary metabolites produced by the strains of *Streptomyces* designated as OS-6 and TES-25 on the free radicals generated in the different *in vitro* assays.
Methodology and results: The antioxidant activity of ethyl acetate and chloroform extracts of OS-6 and TES-25 was analyzed by performing different antioxidant assays *viz.* molybdate ion reduction assay, DPPH radical scavenging assay, ABTS radical cation decolourization assay, reducing power assay, deoxyribose degradation assay, lipid peroxidation assay, superoxide anion scavenging assay and DNA nicking assay. The total phenolic content was also determined. The findings showed that maximum hydrogen or electron donating and hydroxyl radical scavenging activities were exhibited by OS-6 ethyl acetate extract in all the assays.
Conclusion, significance and impact of study: The potent antioxidant activity may be due to the presence of different polyphenolic compounds as depicted from the total phenolic content of the extracts. Further study includes the isolation of compounds responsible for the antioxidant activity.

Keywords: Actinobacteria, antioxidant, secondary metabolites, free radicals

INTRODUCTION

The important biological process in all living beings is oxidation. During the process of oxidation reactions, free radicals are formed. These free radicals lead to damage of lipids, proteins, enzymes and nucleic acids which leads to aging and a large number of degenerative diseases (Duan *et al.*, 2006). Antioxidants have the ability to scavenge free radicals and thus aid in the prevention of degenerative diseases (Sheikh *et al.*, 2009). They have the potential of either suppressing or delaying the chain reaction formed by free radicals (Halliwell *et al.*, 1992). The previous research showed that actinobacteria possesses antioxidant potential (Isik *et al.*, 2006).

Actinobacteria are gram positive bacteria. They are included in the phylum Actinobacteria and order actinomycetales that is constituted of approximately 80 genera having saprotrophic mode of nutrition and have origin in terrestrial soil. They are capable of degrading complex biopolymers like lignocelluloses, hemicelluloses, pectin, keratin and chitin (Vijayakumar *et al.*, 2007). They have filamentous or branching growth pattern that results in extensive colony or mycelium. Approximately 100 genera of actinobacteria reside in soil (Lo *et al.*, 2002). Their number and type differs according to different soil conditions like pH, temperature, moisture and nutrients present in soil. Over 22,500 compounds have been isolated from micro-organisms of which 45% are extracted from actinobacteria, 38% from fungi and 17% from unicellular bacteria (Demain and Sa'nchez, 2009). Each strain of actinobacteria has the capability to form 10-20 metabolites (Sosio *et al.*, 2000; Bentley *et al.*, 2002). Among actinobacteria, *Streptomyces* is known to produce 80% of the natural products till today (Bull and Stach, 2007). Waksman and Henrici proposed the genus *Streptomyces*. It is included in the family Streptomyceae. The difference between *Streptomyces* and other genus of actinobacteria is in the structure of cell wall. The cell wall of *Streptomyces* is of Type I (LL-diamino pimelic acid and glycine present but absence of sugars). They have high G+C content in their DNA. About 10,000 bioactive compounds are synthesized by actinobacteria of which 7600 are obtained from *Streptomyces* which shows that it is the largest group that produces bioactive microbial metabolites. They are the group of micro-organisms that are most economic and half of the bioactive secondary metabolites like antibiotics, anticancer drugs and enzymes are produced by them (Amrita *et al.*, 2012). These

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metabolites are highly significant as they have antimicrobial property on a large number of microbes which are pathogenic (Krishnakumari *et al.*, 2006; Ceylan *et al.*, 2008; Ghadin *et al.*, 2008). These are highly valuable for industrial, pharmaceutical and therapeutic uses (Tokiwa and Buenaventurada, 2004). A few numbers of *Streptomyces* have been studied for their antioxidant potential but most of the secondary metabolites from them are yet to be isolated and screened. Keeping in mind the innumerable uses and advantages of actinobacteria which includes genus *Streptomyces*, the present study is concentrated on the antioxidant capability of two strains of *Streptomyces*.

MATERIALS AND METHODS

Streptomyces strains

Streptomyces species, OS-6 and TES-25, exhibiting antioxidant activity, were procured from Department of Microbiology, Guru Nanak Dev University, Amritsar and maintained on Starch Casein Nitrate Agar (SCNA) slants, and in the form of spore suspensions and mycelia fragments at -70 °C in 20% v/v glycerol in an ultra-low temperature freezer.

DNA isolation, amplification and sequencing of 16S rDNA

DNA isolation was done according to the method recommended by Marmur (1961). The 16S rDNA gene was amplified using primers 27F (5ˈ-AGAGTTTGATCCTGGCTCAG-3ˈ) and 1492R (5ˈ-AGAAAGGAGGTGATCCAGGC-3ˈ). The PCR product got sequenced from Institute of Microbial Technology (IMTECH), Chandigarh (India). The obtained 16S rDNA sequences of the strains were compared for similarity with the sequences of reference species of bacteria contained in GenBank, (which is available at http://www.ncbinlm) using BLAST.

Extraction of metabolites

The bioactive metabolites were obtained from cultures of both the strains. The strains were inoculated in 250 mL conical flasks containing 50 mL of sterile starch casein nitrate broth. The flasks were then incubated for 5 days at 28 °C in shaker at 180 rpm. Centrifugation of the contents of the flasks was carried out at 10,000 rpm for 15 min at 4 °C. Equal volume of ethyl acetate and chloroform in separation funnel was used for two times for the extraction of the clear supernatant obtained and the solvent layers were collected. The rotary evaporator was used for the evaporation of solvents in order to yield the extract. Lypholysis of the extracts was done in freeze drier and used to test the bioactivities. The extracts obtained were OS-6 chloroform extract, OS-6 ethyl acetate extract, TES-25 chloroform extract and TES-25 ethyl acetate extract abbreviated as OCE, OEAE, TCE and TEAE respectively.

Estimation of Total Phenolic content (TPC)

The total phenolic content in the extracts was calculated by the use of Folin ciocalteau method according to Yu *et al.* (2002). To 0.1 mL of extract, 1 mL of distilled water was added. It was followed by the addition of 0.5 mL of Folin Ciocalteau Reagent (1:1) and 1.5 mL of 20% Na_2Co_3. This mixture was kept for incubation at room temperature for 2 h. Final volume was made 10 mL by addition of water and the absorbance was read at 765 nm. The calibration curve of gallic acid which was used as a standard was plotted and the amount of TPC was estimated as mg/g (Gallic Acid Equivalents) using the calibration curve. The curve for gallic acid was plotted as absorbance versus concentration and the equation obtained was $y = 0.0022x + 0.0152$ (R^2= 0.9502), where, y = absorbance; x = different concentrations of gallic acid.

Antioxidative assays

The antioxidants terminate the free radical chain reaction either by donating a hydrogen atom (or electron) or by scavenging the hydroxyl radicals that are extremely reactive oxygen species possessing the capability to modify almost every molecule in the living cells. In the present study, the free radical scavenging potential of the ethyl acetate and chloroform extracts of OS-6 and TES-25 was analyzed by performing different *in vitro* assays. The hydrogen or electron donating capability of extracts was analyzed by molybdate ion reduction assay, DPPH radical scavenging assay, ABTS radical cation decolourization assay and reducing power assay while the hydroxyl radical scavenging assays performed were deoxyribose degradation assay, lipid peroxidation assay, superoxide anion scavenging assay and DNA nicking assay.

Molybdate ion reduction assay

The molybdate ion reducing ability of the extracts was calculated according to the method proposed by Prieto *et al.* (1999). A 0.3 mL (100 µg/mL) of sample solution was mixed with 3 mL of reagent solution which was comprised of 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. This mixture was kept for incubation for 90 min at 95 °C and followed by cooling at room temperature. The absorbance was read at 695 nm. The standard curve was plotted with 20-200 µg/mL concentrations of ascorbic acid. The standard showed the regression equation as, $y = 0.004x + 0.067$ ($R^2 = 0.990$); Here, y = absorbance obtained at 695 nm and x = concentration of ascorbic acid used. The reducing capability was calculated from the standard curve and expressed as mg Ascorbic Acid Equivalents (AAE) / 100 mg dry weight of extracts.

DPPH radical scavenging assay

The hydrogen donating capacity of extracts was measured by performing DPPH (2, 2’ diphenyl-1- picryl hydrazyl) assay according to the method given by Blois

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(1958). This assay includes the mixing of 0.3 mL of extract solution with 3 mL of 0.1 mM methanolic DPPH solution. The absorbance was measured at 517 nm. The decrease in absorbance was observed with increase in concentration of extract solution. The radical scavenging potential was calculated from the equation *(1-AS/AC)* × *100*; *AC* = Absorbance of Control, *AS* = Absorbance of Sample solution.

ABTS radical cation decolourization assay

This assay was done according to the method given by Re *et al.* (1999) for measuring the hydrogen donating capacity of extracts. $ABTS^{+\cdot}$ is soluble in both organic and aqueous media, stable in a wide range of pH and exhibit outstanding spectral qualities. Hence, it can be used to evaluate the antioxidant ability of both hydrophilic and lipophilic compounds. The mixing of two reagents 7 mM of ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and 140 mM of potassium persulfate generates $ABTS^{+\cdot}$ cation radicals. In the reaction mixture, Potassium Persulfate was diluted to 2.45 mM. The reaction mixture was incubated for 12-16 h under dark conditions at 30 °C. After 16 h of incubation, ethanol or PBS (pH = 7.4) was added to dilute the reaction mixture in order to get the absorbance of 0.700 ± 0.020 at 734 nm. The scavenging potential of $ABTS^{+\cdot}$ was measured on mixing 1.9 mL of $ABTS^{+\cdot}$ solution with 0.1 mL of extract solution. The absorbance of reaction mixture was read for 0 to 6 min at 734 nm. The percentage inhibition of extracts was calculated with the help of formula: Inhibition (%) = *(1-AS/AC)* × *100*; *AC* = Absorbance of Control, *AS* = Absorbance of Sample solution.

Reducing Power assay

The method was proposed by Oyaizu (1986) and employed for measuring the reducing potential. 1 mL of extracts having different concentrations was added to 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was kept for incubation at 50 °C for 20 min. It was followed by the addition of 2.5 mL of 10% TCA and centrifugation was done at 3000 rpm for 10 min. 2.5 mL of supernatant was diluted with 2.5 mL of distilled water. 0.5 mL of $FeCl_3$ (0.1%) was added to the reaction mixture. Absorbance of end product known as prussion blue coloured complex was read at 700 nm. The absorbance was increased with increased concentration of the extract which was correlated with the increased reducing capacity of extracts. The standard rutin was used for the comparison of results. The percentage reduction was calculated with the formula *[1-(1-As/AC)]* × *100*; *AC* = Absorbance of standard at maximum concentration tested and *AS* = Absorbance of sample.

Deoxyribose degradation assay

Hydroxyl radical scavenging potential in non-site and site-specific manner of extracts was demonstrated by the method proposed by Halliwell *et al.* (1987) and Arouma *et al*. (1987). EDTA was added to the Haber Weiss reaction mixture constituting 100 µL EDTA (1 mM), 20 µL $FeCl_3$ (10 mM), 100 µL H_2O_2 (10 mM) and 100 µL ascorbic acid (1 mM) and extract (1 mL) in 320 µL phosphate buffer (50 mM) of pH 7.4 in non-site specific assay. 100 µL phosphate buffer (50 mM) was used in place of EDTA solution in site specific assay. Ascorbic acid (1 mM) reduced Fe^{3+} to Fe^{2+} ions. The mixture was incubated for 1 h at 37 °C. TBA in 25 mM NaOH (1 mL, 0.5%) and TCA (1 mL, 10% w/v aqueous solution) were mixed with 1 mL of above mixture. Heating of mixture was done on water bath at 80 °C for 90 min. The absorbance of pink chromogen produced was read at 532 nm. The percentage inhibition was calculated as: % Hydroxyl radical scavenging capacity = *(1- AS/AC)* × *100*; *AC* = Absorbance of Control, *AS* = Absorbance of Sample solution.

Lipid peroxidation assay

The method proposed by Halliwell and Gutteridge (1989) with slight modifications was followed, to estimate the amount of malondialdehyde (MDA). The protective ability of extracts was demonstrated by mixing 1 mL of different concentrations of extracts with 0.15 M KCl (0.5 mL) and 10% egg yolk (0.5 mL). For initiation of peroxidation, 100 µL of 10 mM ferric chloride was added. It was incubated at 37 °C for 30 min. The process of lipid peroxidation was checked by the formation of thiobarbituric acid reactive substances (TBARS). TBARS were determined by adding 2 mL of ice-cold HCl (0.25 N) constituting 15% trichloroacetic acid (TCA), 0.5% TBA, and 50 µL of 0.5% butylated hydroxytoluene (BHT) to the reaction mixture. The reaction mixture was heated at 100 °C for 60 min. Cooling and centrifugation of samples were done and absorbance of supernatants obtained was read at 532 nm. The percentage inhibition was calculated as: % Hydroxyl radical scavenging capacity = *(1- AS/AC)* × *100*; *AC* = Absorbance of Control, *AS* = Absorbance of Sample solution.

Superoxide anion scavenging assay

This assay was proposed by Nishikimi *et al.* (1972) and used for measuring the superoxide anion scavenging potential. The process of generation of superoxide anions was non-enzymatic in a PMS-NADH system constituted of phenazine methosulphate and reduced nicotinamide adenine dinucleotide. Blue coloured formazan dye was formed by reduction of nitro blue tetrazolium. In this method, 1 mL of extract was mixed with 156 µM NADH (1 mL), 60 µM NBT (1 mL) and 468 µM phenazine methosulphate (1 mL) in phosphate buffer (pH = 8.3). PMS was added for initiation of reaction mixture followed by its incubation at 25 °C for 10 min. The absorbance was read at 560 nm and the percentage inhibition was calculated using the formula *(1- AS/AC)* × *100*; *AC* = Absorbance of Control, *AS* = Absorbance of Sample solution.

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DNA nicking assay

This assay was performed according to the method given by Lee *et al.* (2002). It evaluated the potential of extracts to protect super coiled pBR 322 from destroying effects of hydroxyl radicals produced by Fenton's reagent The reaction mixture constituted of 0.3 µL of plasmid DNA, 10 µL Fenton's reagent (30 mM H_2O_2, 50 µM ascorbic acid, and 80 µM $FeCl_3$) to which different concentrations of extracts were added (100 µg/mL, 200 µg/mL, 500 µg/mL and 1000 µg/mL) and addition of distilled water to make the final volume to 20 µL. Equal volume of distilled water was added in place of Fenton's reagent in negative control. It was followed by incubation at 37 °C for 30 min. 2.5 µL of bromophenol blue, tracking dye was added in loading of each sample. The analysis of DNA was done on 1% agarose gel (0.5 g of agarose was dissolved in 50 mL of 1x TBE buffer having pH of 8-8.3) followed by staining with ethidium bromide (3 µL). The positive control used was rutin. Densitometric analysis was done to examine the DNA damage quantitatively with the help of AlphaEase software. The calculation of percentage of different forms of DNA i.e. supercoiled (Form I), open circular (Form II) and double stranded nicked and linear (Form III) was performed.

Statistical analysis

All the experiments were performed in triplicates. The results were interpreted as inhibition (%) ± SE. Regression studies were carried out along with analysis of multiple comparisons by using one-way analysis of variance (ANOVA). Statistical significance was considered at $p \leq 0.05$. The calculation of IC_{50} value i.e. the concentration of extract in µg/mL used for scavenging 50% free radicals was determined from regression equation.

RESULTS AND DISCUSSION

Microorganisms are a source of large number of bioactive compounds. These compounds possess highly specific biological potential which are a major source of interest for their extraction and isolation. *Streptomyces* is the largest genus of actinobacteria which is highly significant in the manufacturing of antibacterials, antifungals, anti tumor agents and immunosuppressants. 75% of the commercially and medically useful antibiotics are the products of *Streptomyces* (Miyadoh, 1993). A large number of compounds isolated from *Streptomyces* exhibited antioxidant potential *viz.* isoflavonoids (Komiyama *et al.*, 1989), diphenazithionin (Hosoya *et al.*, 1996), dihydroherbimycin A (Chang and Kim, 2007), polysaccharide (He *et al.*, 2008) and protocatechualdehyde (Kim *et al.*, 2008). The present study involves the evaluation of antioxidant potential of strains of *Streptomyces* species designated as OS-6 and TES-25.

On the basis of comparison of sequences using BLAST analysis the strains exhibiting antioxidant activity were classified as *Streptomyces* spp. The almost complete 16S rDNA sequence (1499 bp) of strain OS-6 was aligned with all available *Streptomyces* sequences in Eztaxon database and it showed the maximum (100%) similarity with *Streptomyces hydrogenans* NBRC 13475 (T) (AB184868). The 16S rDNA gene sequence (GenBank, accession of TES-25 strain (1475 bp) was most similar (99.72% similarity) to *Streptomyces tanashiensis* LMG 20274 (T) and *Streptomyces nashillensis* NBRC 13064 (T).

Total Phenolic content**:** Phenolics are responsible for scavenging reactive oxygen species. It is due to their redox properties, electron donating properties and singlet oxygen quenching properties (Nagulendran *et al.*, 2007). The maximum amount of total phenolic content was exhibited by OEAE i.e. 84.3 mg/GAE which was followed by TEAE (82.18 mg/GAE), OCE (60.06 mg/GAE) and TCE (48.24 mg/GAE) (Table 1).

Table 1: Total Phenol Content of extracts in terms of mg Gallic Acid Equivalents/100 mg dry weight of extract.

S. No	Extract	mg GAE /100 mg ± S.E.
1	OCE	60.06 ± 1.183
2	OEAE	84.3 ± 0.397
3	TCE	48.24 ± 1.786
4	TEAE	82.18 ± 0.948

Hydrogen or electron donating assays

In molybdate ion reduction assay, extracts reduce Mo (VI) to Mo (V) that resulted in the formation of green coloured phosphate/ Mo(V) complex at acidic pH that determines the process of donation of electrons (Prieto *et al.*, 1999; Dorman *et al.*, 2003). The standard curve for ascorbic acid obtained was $y = 0.0044x+0.0947$ ($R^2= 0.9806$) and the results were determined in terms of AA equivalents in mg/100 mg dry weight of extract. Table 2 shows that maximum molybdate ion reduction potential was exhibited by OEAE (83.7) followed by TEAE (74), OCE (59.3) and TCE (53.09) at the same concentration tested.

DPPH radical scavenging assay confirms the antioxidant activity in a short time. *In vivo* ROS (reactive oxygen species) are categorized as oxide radical, hydrogen peroxide and hypochlorous acid. In the presence of certain transition metals, hydrogen peroxide and superoxide undergo interaction to form highly reactive oxidizing species called the hydroxyl radical. Hydrogen donating potential of antioxidants is responsible for their effects on DPPH radical. DPPH is a stable free radical undergo reaction with stable free DPPH radical of purple that is able to accept an electron. The antioxidants colour and lead to the formation of a yellow coloured 1,1-diphenyl-2-picryl hydrazine (Deora *et al.*, 2009). The reducing potential of DPPH radicals was analyzed by reduction in absorbance at 517 nm by the scavenging activity of antioxidants. Figure 1A shows the DPPH scavenging potential of different extracts. The maximum radical scavenging potential was exhibited by OEAE i.e.

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Table 2: Molybdate ion reduction ability (in terms of mg ascorbic acid equivalents/ 100 mg dry weight of extract).

S. No.	Extract	Molybdate ion reduction ability (in mg ascorbic acid equivalents/100 mg dry weight of extract) ± S.E.
1	OCE	59.3 ± 2.423
2	OEAE	83.7 ± 0.91
3	TCE	53.09 ± 1.517
4	TEAE	74 ± 0.607

82.57% at 1000 µg/mL concentration. The other extracts showed scavenging potential in the order TEAE (79.07%)> OCE (76.97%)> TCE (72.71%) at the maximum concentration tested. The potential of extracts to reduce DPPH was increased after 5 min and the steady state was reached after 12-13 min. The standard ascorbic acid was used to compare the IC_{50} values of extracts (Table 3). The OEAE showed least IC_{50} value 2.63 i.e. followed by TEAE (46.81 µg/mL)> OCE (51.88 µg/mL)> TCE (89.03 µg/mL). Similar result was also observed in the ethyl acetate extract (1 mg/mL) of actinobacteria mycelia of *Streptomyces* stain Eri 12 which was isolated from the rhizosphere region of *Rhizoma curcumae* from the Ya'an city of Sichaun province in the Southwest China (Zhong *et al.*, 2011). Compound 5-(2,4-dimethylbenzyl)pyrrolidin-2-one (DMBPO) (10 µg/mL) exhibited 59.32% DPPH radical scavenging activity which was isolated from marine actinobacteria *Streptomyces* sp. VITSVK5 (Saurav and Kannabiran, 2012). Marine *Streptomyces* sp. VITTK3 also exhibited DPPH radical scavenging potential of 96% (5 mg/mL) (Thenmozhi *et al.*, 2010). Phenolic compounds JBIR-94 and JBIR-125 exhibited DPPH radical scavenging potential isolated from *Streptomyces* sp. having IC_{50} values of 11.4 and 35.1 µM respectively (Kawahara *et al.*, 2012). *Streptomyces* sp. LK-3 exhibited DPPH radical scavenging potential of 76% (100 µg/mL). The extracts showed dose dependent response.

ABTS radical cation decolourization assay is one of the methods for testing the antioxidant potential (Re *et al.*, 1999). The oxidation of ABTS with potassium persulphate results in the formation of radical monocation of 2,2'-azinobis (3-ethylbenzathiazoline-6-sulfonic acid) ($ABTS^{+\cdot}$) and it gets reduced by the hydrogen donating activity of antioxidants. $ABTS^{+\cdot}$ scavenging potential is presented in Figure 1B. The maximum $ABTS^{+\cdot}$ scavenging potential was observed in OEAE (87.43%) at 1000 µg/mL followed by TEAE (84.29%), OCE (74.21%), TCE (69.08%) at the same concentration tested. The IC_{50} values of extracts were observed in the order OEAE (49.89 µg/mL)> TEAE (121.15 µg/mL)> OCE (352.48 µg/mL)> TCE (354.24 µg/mL). The results of extracts were compared with gallic acid used as a standard.

Antioxidants play a vital role in reducing the Fe^{3+}/complex to the ferrous form. The amount of ferrous/Fe^{2+} is measured by the Perl's Prussian blue complex formed at 700 nm (Chung *et al.*, 2002). The reducing capability of a compound plays an important role in determining its antioxidant potential (Meir *et al.*,1995). The ability of extracts to reduce Fe(III) to Fe(II) was analyzed by reducing power assay. It measures the electron donating ability of extracts. The reducing power capacity was measured in comparison of absorbance of different extracts with respect to rutin. Reducing potential of the extracts increased with increasing concentration (Figure 1C) i.e. the extracts showed linear correlation of absorbance with increase in concentration. Similar results were obtained where increase in absorbance was

Table 3: IC_{50} values of extracts in different *in vitro* assays.

Assays	Extracts/standard	IC_{50} (µg/mL)
DPPH Assay	Ascorbic acid	7.69
	OCE	51.88
	OEAE	2.63
	TCE	89.03
	TEAE	46.61
ABTS assay	Ascorbic acid	19.10
	OCE	352.48
	OEAE	49.89
	TCE	354.24
	TEAE	121.51
Reducing power	Rutin	202.35
Assay	OCE	254.16
	OEAE	141.17
	TCE	290.03
	TEAE	152.47
Non site specific	Gallic acid	1.45
deoxyribose	OCE	239.84
degradation assay	OEAE	4.40
	TCE	357.80
	TEAE	16.11
Site specific	Gallic acid	57.97
deoxyribose	OCE	198.34
degradation assay	OEAE	184.93
	TCE	252.14
	TEAE	186.79
Lipid peroxidation	Rutin	71.52
Assay	OCE	212.93
	OEAE	21.32
	TCE	259.82
	TEAE	75.18
Superoxide anion	Gallic acid	22.19
scavenging assay	OCE	217.02
	OEAE	26.84
	TCE	314.19
	TEAE	27.66

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observed with increase in concentration of butanol extracts of two species of *Streptomyces* isolated from Agumbe, Karnataka (Kekuda *et al.*, 2010). OEAE exhibited maximum reducing ability of 84.51% at 1000 µg/mL in terms of the standard rutin whereas TEAE showed 80.66% percent inhibition which was followed by OCE (75.24%). TCE exhibited least reducing ability of 69.32% at the same concentration tested.

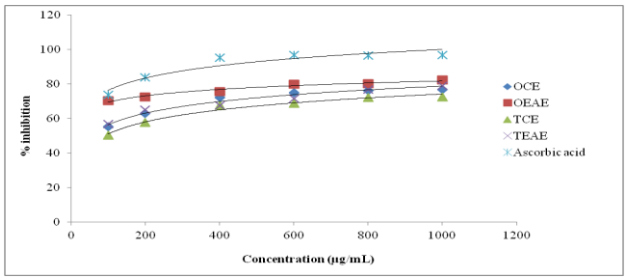

Figure 1A: Antioxidant potential of extracts in DPPH radical scavenging assay**.**

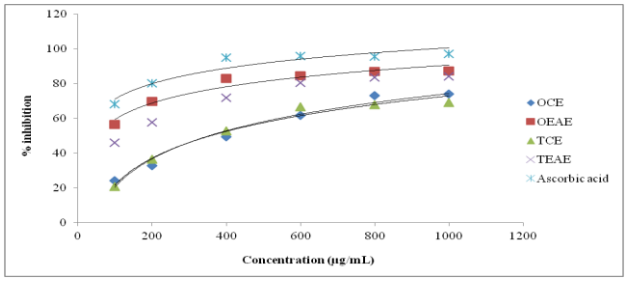

Figure 1B: Antioxidant potential of extracts in ABTS cation decolurization assay.

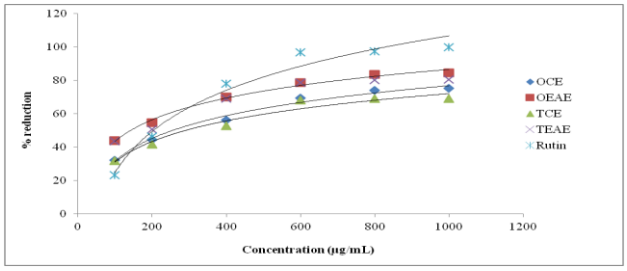

Figure 1C: Antioxidant potential of extracts in reducing power assay.

Hydroxyl radical scavenging assays

In deoxyribose degradation assay, deoxyribose is used as a detector molecule. Deoxyribose is an important component of DNA is used to reveal the damage caused by EDTA (Gutteridge, 1987). The extracts exhibited significant activity in the presence of EDTA by scavenging the ·OH radicals in the free solution and provide protection against the deterioration of deoxyribose. It was further noticed that the extracts were also capable in the chelation of Fe(III) in the absence of EDTA which results in its unavailability to deoxyribose and hence inhibit the formation of ·OH radicals at a particular site. The hydroxyl radical scavenging potential of extracts in non site specific and site specific manner are shown in Figure 1D and 1E respectively. From the results, it is clear that extracts showed scavenging potential in both non site and site specific assays in a dose dependent manner. OEAE showed the maximum hydroxyl radical potential (82.12%) at 1000 µg/mL. TEAE and OCE also exhibited the radical scavenging potential of 75.43% and 69.48% respectively. The least inhibitory potential was observed in TCE (67.46%) at the same concentration tested in non site specific assay. In site specific assay, OEAE was the most effective extract which exhibited 74.33% hydroxyl radical scavenging capacity. OEAE was followed by TEAE and OCE where percentage inhibition observed was 70.25% and 61.32% respectively. The least activity was exhibited by TCE (60.64%). The standard gallic acid was used for comparison of results. The IC_{50} values of different extracts are shown in Table 3. OEAE exhibited minimum IC_{50} value 184.93 µg/mL and 4.40 µg/mL in both site specific and non site specific assays. The extracts showed prominent effects in non site specific assay on comparison with site specific assay which signifies that they are better ·OH radical scavengers as compared to chelating agents.

In lipid peroxidation assay, lipids having many number of C-C double bonds undergo oxidative deterioration. The process of lipid peroxidation is initiated by ferrous sulphate by the formation of ·OH radicals or ferryl perferryl complex in the Fenton's reaction. The reaction can be stopped by a number of ways like non formation of ferryl-perferryl complex, scavenging the ·OH radical or the superoxide radical, changing the Fe^{3+}/Fe^{2+} radical or by the process of chelation of iron. In the present study, lipid peroxidation initiated by ferrous sulphate in egg yolk homogenate is opposed by the extracts (Nagulendran *et al*., 2007). All the extracts exhibited lipid peroxidation *a*bility in a dose dependent fashion as shown in Figure 1F. OEAE exhibited remarkable inhibition of 82.95% at 1000 µg/mL. The potent antioxidant activity was also observed in TEAE (80.80%) and OCE (75.11%). TCE showed least percentage inhibition of 72.30% at the same concentration evaluated.

In superoxide anion radical scavenging assay, cellular reactions linked with various enzyme system such as lipoxygenases, peroxidase, NADPH oxidase and xanthine oxidase lead to the generation of superoxide radicals (Miller *et al.*, 1993). Superoxide anion cause direct or indirect damage by the formation of hydrogen peroxide, ·OH, peroxy nitrate or singlet oxygen species during the process of aging and pathological events. Inhibition of blue NBT occurs with the help of antioxidants. The antioxidants lead to the decrease of absorbance at 560 nm which shows the utilization of $O^{2\cdot-}$ in the reaction mixture. The results of superoxide anion radical scavenging are shown in Figure 1G. The maximum superoxide scavenging potential was observed in OEAE (86.56%) at 1000 µg/mL. The effective scavenging activities were also exhibited by TEAE (77.80%) and OCE

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(73.09%). TCE showed least percentage inhibition of 69.29%. It was determined that the extracts showed percentage inhibition in a dose dependent manner. The least IC_{50} value was observed in OEAE (26.84 µg/mL) while TCE showed maximum IC_{50} value (314.19 µg/mL). The standard gallic acid was used for the comparison of results.

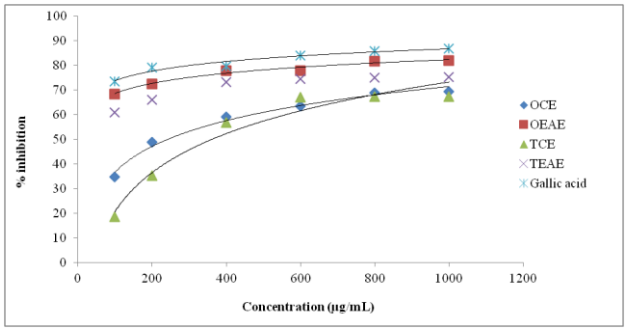

Figure 1D: Antioxidant potential of extracts in non site specific deoxyribose degradation assay.

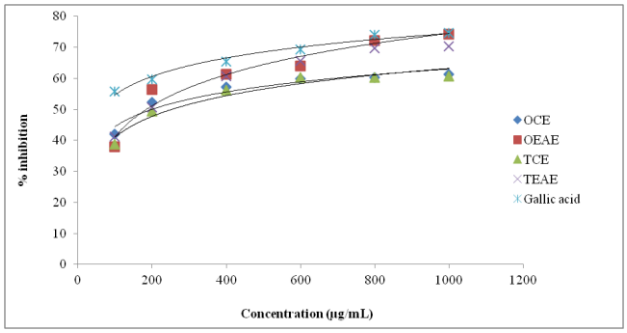

Figure 1E: Antioxidant potential of extracts in site specific deoxyribose degradation assay.

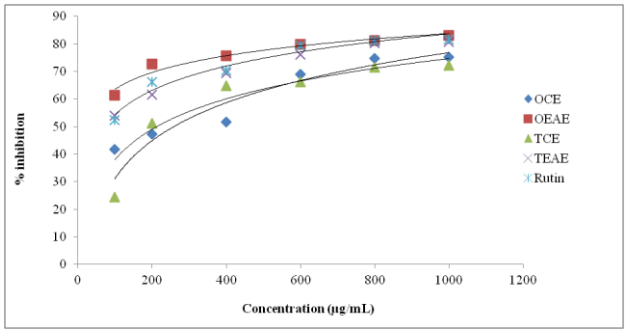

Figure 1F: Antioxidant potential of extracts in lipid peroxidation assay.

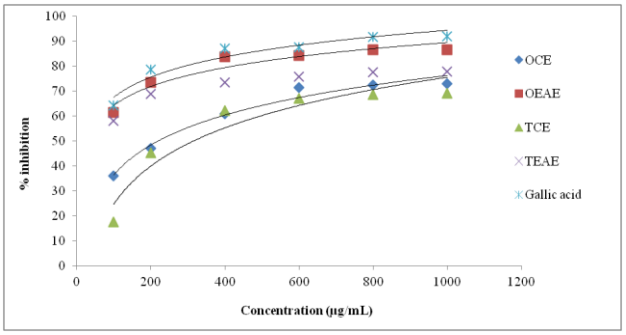

Figure 1G: Antioxidant potential of extracts in superoxide anion scavenging assay.

In DNA nicking assay, increase in the single stranded and double stranded nicked (Form II) and linear forms of DNA (Form III) has been observed on dissolving the plasmid DNA (pBR322) in the Fenton's reaction mixture by the formation of hydroxyl radicals. When extracts are added to pBR322 DNA comprising Fenton's reaction mixture, the formation of Form II (single stranded nicked DNA) and Form III (double stranded nicked and linear DNA) is minimized and Form I (supercoiled) DNA integrity is maintained (Russo *et al.*, 2000). The DNA damage protection potential of different extracts is shown in Figure 2A, 2B, 2C and 2D. The densitometric analysis indicated the protection of DNA from the hydroxyl radicals generated by Fenton's reagent. It is clear from Tables 4-7 and Figure 3A, 3B, 3C and 3D that the amount of supercoiled DNA, in the presence of extract and FR, was found to be 68.4% (at 1000 µg/mL), 75.1% (at 200 µg/mL), 69.6% (at 1000 µg/mL) and 64.7% (at 100 µg/mL) in case of OCE, OEAE, TCE and TEAE respectively.

Antioxidants help in combating the effect of free radicals by different mechanisms such as hydrogen or electron donating ability, free radical scavenging capability, modulation of gene expression, etc. The arrangement of functional groups about the nuclear structure is responsible for providing antioxidant activity to the phenolics. The present study is focused on the antioxidant potential of ethyl acetate and chloroform extracts of the bioactive metabolites of two strains of *Streptomyces* sp. designated as OS-6 and TES-25. OEAE showed maximum total phenolic content as compared to other extracts. The hydrogen or electron donating capacity of extracts was determined using molybdate reduction capacity, DPPH radical scavenging, ABTS radical cation scavenging and reducing power assay. Hydroxyl radical scavenging ability was determined using deoxyribose degradation assay, lipid peroxidation assay and DNA nicking assay whereas $O_2^{\cdot-}$ scavenging ability was estimated using superoxide anion scavenging assay. In majority of the antioxidant assays, OEAE showed maximum percent inhibition with least IC_{50} values in comparison to other extracts. The results were found to be statistically significant at $p \leq 0.05$ as depicted by one way ANOVA and Tukey's HSD post hoc test in all the *in vitro* models.

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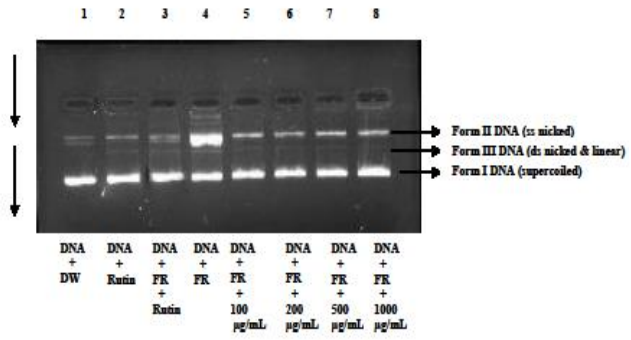

Figure 2A: DNA nicking assay for OS-6 chloroform extract.

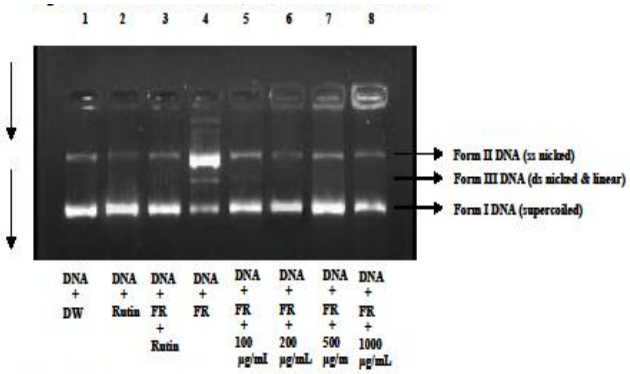

Figure 2B: DNA nicking assay for OS-6 ethyl acetate extract.

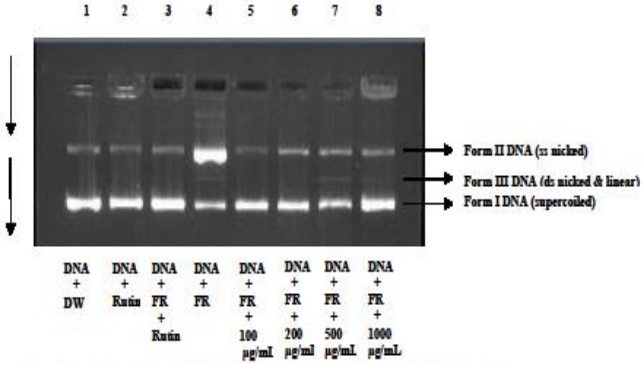

Figure 2C: DNA nicking assay for TES-25 chloroform extract.

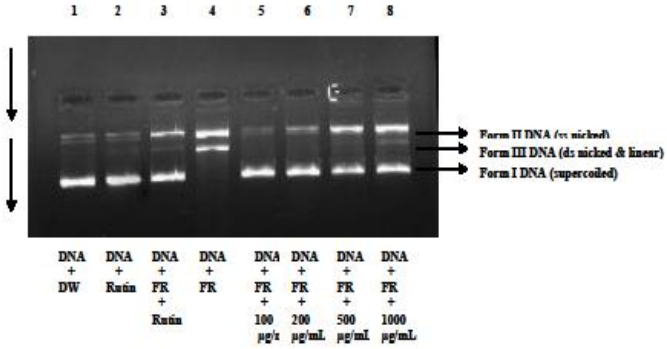

Figure 2D: DNA nicking assay for TES-25 ethyl acetate extract.

Table 4: Densitometric analysis of different forms of DNA after treatment with different concentrations of OCE.

		Control	Rutin	FR	FR + Rutin	100 µg/mL	200 µg/mL	500 µg/mL	1000 µg/mL
Form DNA	I	73.6	72.8	68.1	37.4	59.7	65.9	61.3	68.4
Form DNA	II	17.9	18.0	23.3	52.6	28.5	25.9	30.4	25.2
Form DNA	III	8.5	9.1	8.6	10.0	11.8	8.2	8.2	6.4

Table 5: Densitometric analysis of different forms of DNA after treatment with different concentrations of OEAE.

		Control	Rutin	FR	FR + Rutin	100 µg/mL	200 µg/mL	500 µg/mL	1000 µg/mL
Form DNA	I	74.3	78.4	74.0	25.3	70.1	75.1	74.8	70.7
Form DNA	II	19.3	15.3	19.5	68.0	23.1	18.3	19.1	21.9
Form DNA	III	6.5	6.3	6.6	6.7	6.9	6.6	6.1	7.5

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Table 6: Densitometric analysis of different forms of DNA after treatment with different concentrations of TCE.

		Control	Rutin	FR	FR + Rutin	100 µg/mL	200 µg/mL	500 µg/mL	1000 µg/mL
Form DNA	I	70.0	70.5	71.0	29.0	69.0	63.1	59.0	69.6
Form DNA	II	23.8	22.5	22.7	66.1	24.2	30.0	32.7	24.0
Form DNA	III	6.2	6.9	6.3	4.9	6.8	6.9	8.3	6.4

Table 7: Densitometric analysis of different forms of DNA after treatment with different concentrations of TEAE.

		Control	Rutin	FR	FR + Rutin	100 µg/mL	200 µg/mL	500 µg/mL	1000 µg/mL
Form DNA	I	70.1	68.4	56.3	5.4	64.7	63.9	48.8	47.0
Form DNA	II	23.2	23.7	36.7	55.8	27	27.9	43.2	45.1
Form DNA	III	6.7	7.9	7.0	38.7	8.3	8.1	8.0	7.9

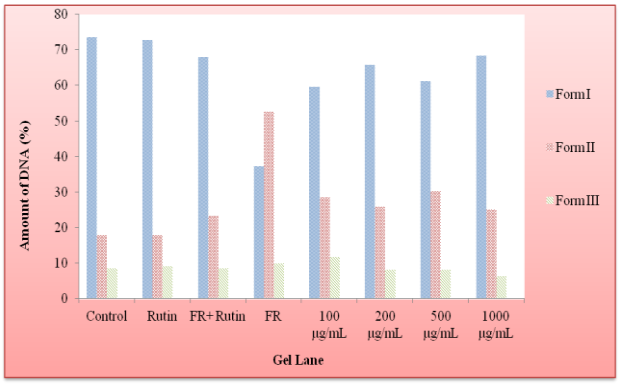

Figure 3A: Densitometric analysis of protective effects of OCE in the presence of hydroxyl radicals generated in DNA nicking assay.

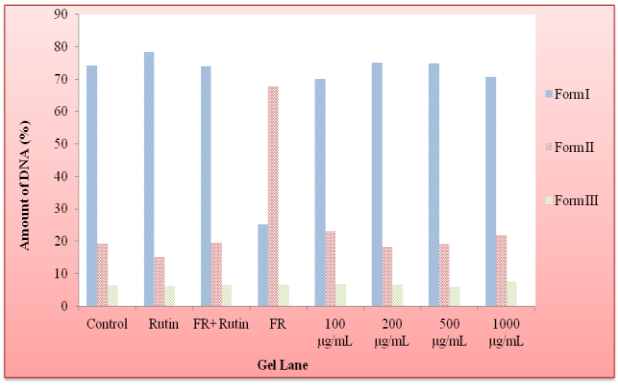

Figure 3B: Densitometric analysis of protective effects of OEAE in the presence of hydroxyl radicals generated in DNA nicking assay.

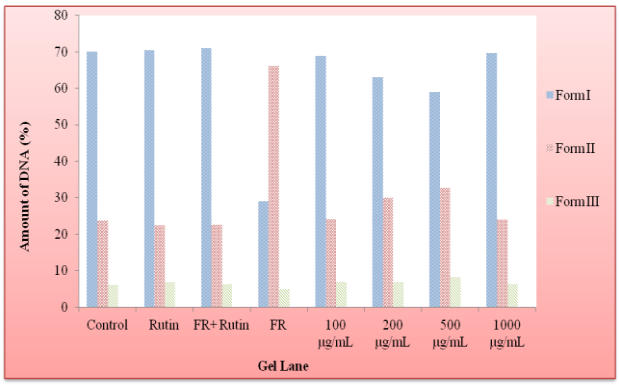

Figure 3C: Densitometric analysis of protective effects of TCE in the presence of hydroxyl radicals generated in DNA nicking assay.

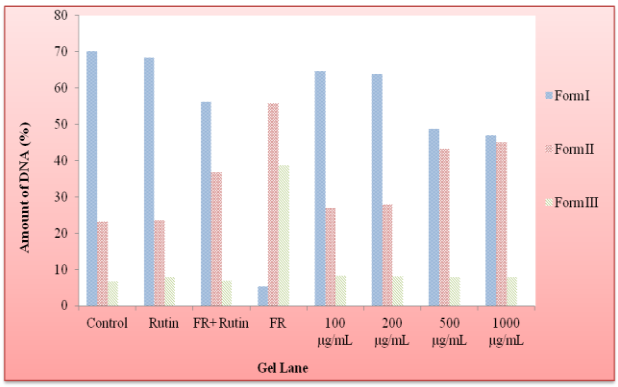

Figure 3D: Densitometric analysis of protective effects of TEAE in the presence of hydroxyl radicals generated in DNA nicking assay.

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CONCLUSION

From the present work, it is concluded that the ethyl acetate and chloroform extracts of strains of *Streptomyces* species designated as OS-6 and TES-25 exhibited effective chain breaking antioxidant potential by transforming the effect of free radicals produced in various *in vitro* assays by the process of hydrogen or electron donation or their subsequent scavenging. The antioxidative potential showed correlation with the total phenolic content of the extracts. The maximum activity was observed in the OS-6 ethyl acetate extract in all the assays. The observed activities may be the result of bioactive compounds present in the solvent extract. The present study is still in its initial stages for the discovery of compounds that are responsible for antioxidant activity. Further study includes the purification and identification of such compounds.

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