Journal of Young Pharmacists 5 (2013) 121–126

Contents lists available at ScienceDirect

Journal of Young Pharmacists

journal homepage: www.elsevier.com/locate/jyp

Original article

Molecular characterization and its antioxidant activity of a newly isolated *Streptomyces coelicoflavus* BC 01 from mangrove soil

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ARTICLE INFO

Article history:
Received 3 July 2013
Accepted 9 October 2013
Available online 28 December 2013

Keywords:
Actinomycetes
Antioxidants
Molecular characterization
Phylogenetic tree
Streptomyces

ABSTRACT

Objective: To isolate and identify the biologically active strain of *Streptomyces* species from mangrove soil of Visakhapatnam region.
Materials and methods: Actinomycetes are isolated by using starch casein agar media and four potential strains were selected to evaluate the antioxidant activity by using the standard methods DPPH, FRAP and total antioxidant capacity. Further, significant antioxidant activity strain characterized by morphological, physiological, biochemical and molecular characterization.
Results: 20 actinomycetes strains were isolated, among them four active isolates designated as BC 01, BC 02, BC 03 and BC 04 were studied for antioxidant activities. Of these four isolates, BC 01 showed a potent antioxidant activity when compared with other isolates. The morphological, biochemical and molecular characterization of the active isolate BC 01 belongs to the genus *Streptomyces* species. The phylogenetic tree was constructed and nucleotide blast in search indicated that the strain is 99.7% similarity with *Streptomyces coelicoflavus*.
Conclusion: The results of the present investigation proven that actinomycetes isolated from mangroves are potent source of antioxidants. The strain BC 01 exhibited a potential *in vitro* antioxidant activity; studies of actinomycetes from mangrove soil can be useful in discovery of novel species to get novel drugs.

1. Introduction

Mangrove ecosystem is an enormous source for isolating the actinomycetes that has the potential for producing the biologically active secondary metabolites. Mangroves are the untouched source for discovering the new actinomycetes. The surroundings of the mangrove environment are brackish and are enormously rich in organic matter due to its numerous microbial enzymatic and metabolic activities.[1] The mangrove ecosystem is nutritionally resourceful and soils are rich source of new species of *Streptomyces*, *Nocardiopsis* and various types of actinomycetes.[2] Actinomycetes are the major microbes in the soil micro-ecosystem[3] and are of special interest since they can produce a wide range of biologically active compounds.[4] They are noteworthy as antibiotic producers, comprising 80% of all known products and the *Streptomyces* species are especially regarded as source of antibiotics[5] and other important metabolites, which includes antifungal agents,[6] antioxidant,[7] antitumor agents,[8] antihelmintic agents,[9] herbicides[10] respectively.

The present research is focused towards the identification of natural antioxidant producing microorganisms, which serves as safe therapeutics. The melanin that was a natural antioxidant extensively produced from the majority of the *Streptomyces* species.[11] Other than melanin, natural antioxidants reported were Protocatechualdehyde from *Streptomyces lincolnensis* M-20[12] and Surugapyrone A from *Streptomyces coelicoflavus* USF-6280.[13]

Several studies were carried out to isolate new *Streptomyces* species from different habitats in order to produce new bioactive metabolites. The study on actinomycetes from mangroves with respect to antioxidant system is meager. Hence, the present study was focused on isolating different *Streptomyces* species from the mangrove soil in and around Visakhapatnam, Andhra Pradesh, India and to evaluate the antioxidant potential of the four isolates. The best isolate was further characterized by molecular studies to determine its taxon.

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0975-1483/$ – see front matter
http://dx.doi.org/10.1016/j.jyp.2013.10.002

2. Materials and methods

2.1. Sample collection

The soil sample was collected from 6 to 10 cm depth from mangrove regions in Visakhapatnam, Andhra Pradesh, India. Samples were stored under aseptic conditions until further use.

2.2. Isolation and characterization of actinomycetes

The media used for isolation of actinomycetes was starch casein agar media. The soil samples were serially diluted up to $10^{-5}$and 0.1 ml of the diluted sample were inoculated on the media by spread plate method. Rifampicin (5 μg/ml) and Nystatin (25 μg/ml) were added to the media to evade from contamination. The inoculated plates were incubated at 28 °C for 2 weeks. The actinomycetes colonies were identified by their chalky, firm and leathery texture.[14] These colonies were sub cultured and maintained at 4 °C for further use.

2.3. Extraction of bioactive compound

The pure culture of actinomycetes was cultivated by submerged fermentation on 100 ml of production medium, consisting glucose 1%, soya bean meal 1%, NaCl 1%, and $CaCO_3$ 0.1% and Incubated at 28 °C for 96 h at 180 rpm on a rotary shaker. After completion of fermentation, the broth was centrifuged and the supernatant was taken in a separating funnel mixed with ethyl acetate by vigorous shaking and kept without any disturbance for 1 h. The ethyl acetate phase that contains bioactive compound was separated under reduced vacuum. The obtained residue was used to evaluate antioxidant activity.

2.4. Assay of DPPH (1, 1, diphenyl-2-picryl hydrazyl) scavenging activity

The free radical scavenging activity was determined by the method of DPPH with some modifications.[15] Different concentration (5, 10, 15 & 20 μg/ml) of above residual extracts were dissolved in methanol and taken in test tubes separately. Ascorbic acid was used as a reference standard. DPPH 0.004% was freshly prepared in methanol. DPPH (2 ml) was added to each tube containing different concentrations of extracts (1 ml) and of standard solution (1 ml). It was shaken vigorously. They were then allowed to stand for 30 min for room temperature in dark place. The control was prepared without any extracts. Methanol was used for base line corrections and absorbance (OD) of sample was measured at 517 nm. The below formula was used to interpret the value of the sample.

$$\%\ \text{Radical Scavenging Activity} = [(\text{control O.D} - \text{sample O.D})/\text{control O.D}] \times 100.$$

2.5. Ferric reducing antioxidant power assay (FRAP)

FRAP assay was carried out according to the method of Benzie & Strain.[16] The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM $FeCl_3 \cdot 6H_2O$ in a ratio of 10:1:1, at 37 °C. 1.5 ml of the FRAP reagent was mixed with 0.5 ml of the sample and the absorbance was measured at 593 nm after 15 min. The results are expressed in μmoles/ml of ascorbic acid equivalents by calculating from calibration curve.

2.6. Determination of total antioxidant capacity

The total antioxidant capacity was evaluated by the phosphomolybdenum method.[17] 0.1 ml of sample was taken in methanol, combined with 1.9 ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated at 95 °C for 90 min. After the samples were cooled to room temperature, the absorbance was measured at 695 nm against a blank. Ascorbic acid equivalents were calculated using standard graph of ascorbic acid and values are expressed as ascorbic acid equivalents in μg/mL of extract.

2.7. Characterization of potent actinomycetes strain

The morphological, physiological, cultural and biochemical characteristics of the potent actinomycetes strain were studied. The morphology and the color of spore mass were identified by using optical microscope at 1000× magnification. The utilization of carbon sources and physiological characterization such as effect of temperature, pH, NaCl concentrations (2, 5, 7, 10, 12% w/v) were performed. For scanning electron microscope (SEM) the samples were fixed to SEM stubs using carbon tape followed by thin coating with platinum and examined with JEOL; JSM-6610LV scanning electron microscope.

2.8. Molecular characterization

The genomic DNA of strain BC 01 was isolated and amplified by PCR with forward primer 8-27f and reverse primer 1500r as described by Mayilraj.[18] The amplified DNA fragment was separated and purified by using a gel extraction kit and the purified PCR product was sequenced. The 16S rRNA gene sequence of strain BC 01 generated in this work (1379 bases) was aligned. A sequence similarity search was performed using GenBank BLASTN,[19] the sequences of closely related taxa were retrieved and aligned using the program CLUSTAL_X[20] and the alignment was corrected manually. A phylogenetic tree was constructed by using the neighbor-joining method[21] and distances between the sequences were calculated using Kimura's two-parameter model.[22] The topology of the phylogenetic tree was evaluated by the bootstrap resampling method[23] with 1000 replicates. *Kitasatospora sampliensis* MTCC 6546T (AY260167) was used as an out group. The guanine and cytosine (G + C) content was determined by thermal denaturation method.[24]

2.9. GC FAME analysis

The potent strain BC 01 was grown on tryptic soya broth (TSB) medium at 30 °C for 3 days for the identification of the cellular fatty acids. The fatty acids methyl esters (FAME) were obtained by saponification, methylation, extraction and washing of the freshly grown culture. The sample was analyzed by Hewlett Packard 5890A gas chromatography equipped with HP7673A auto sampler and a flame-ionization detector fitted with a 5% phenyl methyl silicon column (A25 m × 0.2 mm). The temperature of the oven was 170 °C–270 °C increasing at a rate of 5 °C per min and the injection-port temperature was 300 °C with hydrogen as carrier gas and nitrogen as make up gas. Fatty acid profiles were compared with profiles of the Sherlock Microbial Identification System (MIS) Software.[25]

2.10. Statistical analysis

All investigations were conducted in triplicate and the attained data was exposed to one way ANOVA by using Minitab ver 15 and the significance level was 0.05.

Table 1
DPPH radical scavenging activity of the four extracts.

% Inhibition				
Name of the extract	Concentration of the extract			
	5 μg/ml[a]	10 μg/ml[a]	15 μg/ml[a]	20 μg/ml[a]
BC 01	56.58 ± 12.07	61.13 ± 15.92	65.30 ± 18.20	68.91 ± 21.00
BC 02	52.41 ± 16.76	57.88 ± 22.47	62.46 ± 21.62	65.12 ± 23.00
BC 03	36.83 ± 4.84	41.15 ± 1.43	43.82 ± 2.66	46.41 ± 3.55
BC 04	37.91 ± 1.40	43.45 ± 2.60	47.00 ± 5.85	53.12 ± 11.95
Ascorbic acid	47.03 ± 15.90	51.24 ± 7.31	67.96 ± 25.69	70.64 ± 25.07
	$F = 1.61^{@}$	$F = 1.39^{@}$	$F = 1.23^{@}$	$F = 1.13^{@}$

[@]Not significant.
[a] Each value represents mean ± SD of three independent experiments.

Table 2
FRAP activity of the four extracts.

Ascorbic acid equivalents (μmoles/ml)				
Name of the extract	Concentration of the extracts			
	5 μg/ml[a]	10 μg/ml[a]	15 μg/ml[a]	20 μg/ml[a]
BC 01	64.00 ± 14.00	68.67 ± 11.02	72.00 ± 13.11	78.00 ± 15.10
BC 02	55.33 ± 13.32	60.00 ± 12.49	63.33 ± 11.02	68.67 ± 11.72
BC 03	59.33 ± 12.06	62.67 ± 14.19	68.00 ± 11.14	72.00 ± 12.00
BC 04	56.00 ± 8.00	62.67 ± 9.45	66.00 ± 9.17	72.00 ± 10.00
	$F = 0.32^{@}$	$F = 0.28^{@}$	$F = 0.32^{@}$	$F = 0.30^{@}$

[@]Not significant.
[a] Each value represents mean ± SD of three independent experiments.

3. Results

In the present study 20 actinomycetes strains were isolated from mangrove soil, Visakhapatnam, India. Among them four potent strains were designated as BC 01, BC 02, BC 03 and BC 04 were subjected to *in vitro* antioxidant activity. Compounds with high concentration exhibited highest rate of antioxidant capacity followed to the least.

DPPH is a stable free radical accepts protons from the antioxidant source and shows a decreasing absorption. In the present study, all the four extracts (BC 01–BC 04) were evaluated for their DPPH scavenging assay. It was witnessed that all the four extracts (BC 01–BC 04) showed an effective DPPH radical scavenging activity but BC 01 showed significant levels (68.91 ± 21.00) (Table 1) which were comparable with remaining three isolates. The DPPH activity was determined in percentage of inhibition.

FRAP a method used to detect the reducing potential of an antioxidant source. Tripyridyltriazine (ferric form) combines with antioxidant source results in the formation of a blue color complex (ferrous form) which was measured at 593 nm. The amount of iron reduced can be correlated with the amount of antioxidant capacity of isolates (Table 2). Activity was expressed in terms of ascorbic acid equivalents (μmoles/ml). Among all the four extracts the BC 01 showed highest activity (78.00 ± 15.10) of ascorbic acid equivalents.

Phosphomolebdenum method was used to evaluate the total antioxidant capacity (TAC) which involves the reduction of Mo (VI) to Mo (V) results in the formation of a green colored complex, which shows maximum absorbance at 695 nm. Among all the four extracts, TAC was found to be highest in BC 02 (99.33 ± 3.51 l) followed by BC 01 (93.33 ± 2.52). The total antioxidant capacity of all the four extracts at different concentrations used in this study shows the significant variance ($p < 0.05$), the results are expressed as the μg/ml of ascorbic acid equivalents and tabulated in Table 3.

Table 3
The total antioxidant capacity of the four extracts.

Ascorbic acid equivalents (μg/ml)				
Name of the extract	Concentration of the extract			
	5 μg/ml[a]	10 μg/ml[a]	15 μg/ml[a]	20 μg/ml[a]
BC 01	75.667 ± 3.512	82.333 ± 2.517	89.667 ± 2.082	93.33 ± 2.52
BC 02	63.333 ± 3.215	69.000 ± 1.732	77.000 ± 4.000	99.33 ± 3.51
BC 03	62.667 ± 3.512	72.333 ± 1.528	83.333 ± 7.371	97.67 ± 2.08
BC 04	67.000 ± 2.000	72.000 ± 4.000	76.667 ± 2.082	83.33 ± 2.52
	$F = 11.00^{*}$	$F = 14.63^{*}$	$F = 5.75^{*}$	$F = 21.10^{*}$

[*]5% level of significance ($p < 0.05$).
[a] Each value represents mean ± SD of three independent experiments.

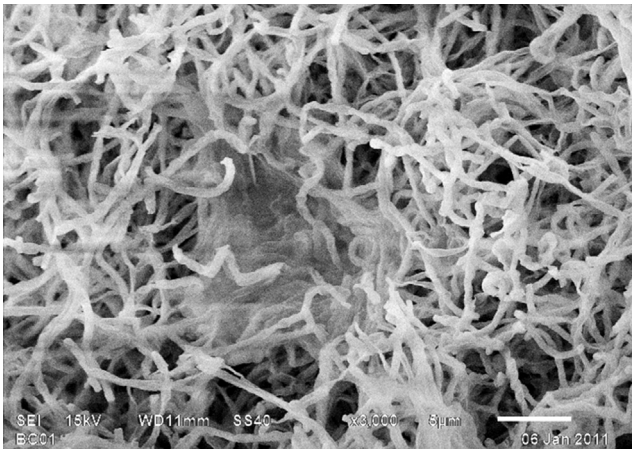

Fig. 1. Scanning electron microscope of the potent actinomycete strain BC 01.

Table 4
Morphological and biochemical characteristics of the potent actinomycetes strain BC 01.

Name of the test	Results
Substrate Mycelia on Starch casein agar	White
Aerial Mycelia on Starch casein agar	Gray
Pigmentation	Nil
Spore surface	Smooth
Spore bearing aerial hyphae	Flexous
Growth at temperature	25°–35 °C
Growth at pH	7.2–8.2
Indole production	+
Methyl red	+
Voges–Proskauer	+
Citrate utilization	+
Melanin formation	+
Gelatin hydrolysis	+
Tyrosine reaction	+
H_2S production	+
Casein hydrolysis	+
Starch hydrolysis	+
Nitrate reduction	+
Urease	–
Catalase	+
Gelatin	+
Carbon source utilization	
Glucose	+
Fructose	+
Galactose	+
Sucrose	–
Mannose	+
Mannitol	+
Sorbitol	+
Inositol	+
Xylose	–
Arabinose	–
Glycerol	+

+ – Positive, – – Negative.

Table 5
Fatty acid percentage profile of the potent actinomycetes strain BC 01.

Fatty acid	Percentage (%)
$C_{11:0}$	0.25
Iso-$C_{11:0}$	0.34
$C_{12:0}$	0.99
Iso-$C_{13:0}$	0.19
Iso-$C_{14:0}$	2.26
$C_{14:1}$Cis9/Trans9	1.04
$C_{14:0}$	1.13
Iso-$C_{15:0}$	9.74
Anteiso-$C_{15:0}$	17.80
$C_{15:0}$	1.65
Iso-$C_{16:1}$H	2.63
Iso-$C_{16:0}$	21.68
$C_{16:1}$Cis 9	2.07
$C_{16:0}$	8.84
$C_{16:0}$9 Methyl	4.39
Iso-$C_{17:0}$	5.44
Anteiso-$C_{17:0}$	11.58
Anteiso-$C_{17:1}$C	2.88
$C_{17:1}$Cis 9	0.65
Cyclo $C_{17:0}$	1.27
$C_{17:0}$	0.66
Iso-$C_{17:0}$2OH	0.24
$C_{18:0}$	0.81
$C_{19:1}$Trans 7	0.57

3.1. Morphological characteristic of potent isolate

The physiological and morphological observations revealed that the isolated strain had the characteristic features of the genus *Streptomyces*. The spore was smooth and non-motile; the rod shaped spore chain was borne on aerial mycelium. The aerial mycelia are abundant and developed without fragmentation (Fig. 1). The morphological, biochemical and physiological characteristics of the strain were tabulated in Table 4.

3.2. GC FAME analysis

Major cellular fatty acids Iso-$C_{16:0}$ (21.68%) and Anteiso-$C_{15:0}$ (17.80%) were identified by using GC FAME analysis (Table 5). Previous results showed that actinomycetes produce saturated and unsaturated fatty acids with branched chain lengths in the range of C_{10}–C_{18}.[26] Our results also showed that the fatty acids obtained are in the range.

3.3. Molecular characterization and phylogenetic analysis of the potent isolate BC 01

The purity of the genomic DNA was 1.795 and the genomic DNA G+C content of the strain BC 01 was 69.7 mol%. The 16S rRNA gene partial sequence was deposited in NCBI nucleotide database with Accession No JX126485 and the sequence was correlated with genus *Streptomyces* by using BLASTN. Sequence analysis revealed that the isolated strain showed highest (99.7%) 16S rRNA gene sequence identity with (a four nucleotide difference out of 1379 nt) with *S. coelicoflavus* NBRC 15399(T) of GenBank accession No. AB184650 and the analysis of the sequence confirmed that the strain belongs to the species *S. coelicoflavus*. A phylogenetic tree was reconstructed by using neighbor-joining method (Fig. 2) and the results stipulate that the strain BC 01 closely resembles with the genus *S. coelicoflavus* NBRC 15399T (AB184650).

By comparing the morphological and biochemical characteristics of the strain BC 01 with the standard literature of *S. coelicoflavus* ZG0656[27] and *Streptomyces aurantiacus*,[28] the strain BC 01 was confirmed to belong to *S. coelicoflavus*.

4. Discussion

The mangroves involve a massive quantity of novel actinomycetes, which are promising sources of bioactive metabolites. The mangrove environs are huge unexplored zone and are biochemically exceptional for generating a wide range of novel natural products comprises biologically active compounds. Now a days research is focused on the replacement of synthetic antioxidants with a natural one to preclude the side effects caused by synthetic antioxidants. A search is going for screening of a new source to discover natural ones.

DPPH, FRAP and Phosphomolybdenum are the proven methods to evaluate radical scavenging activity. In the present study four isolate extracts (BC 01–BC 04) were evaluated for their antioxidant activity. The results showed that four isolates were presenting substantial antioxidant activity, of these BC 01 exhibited potent

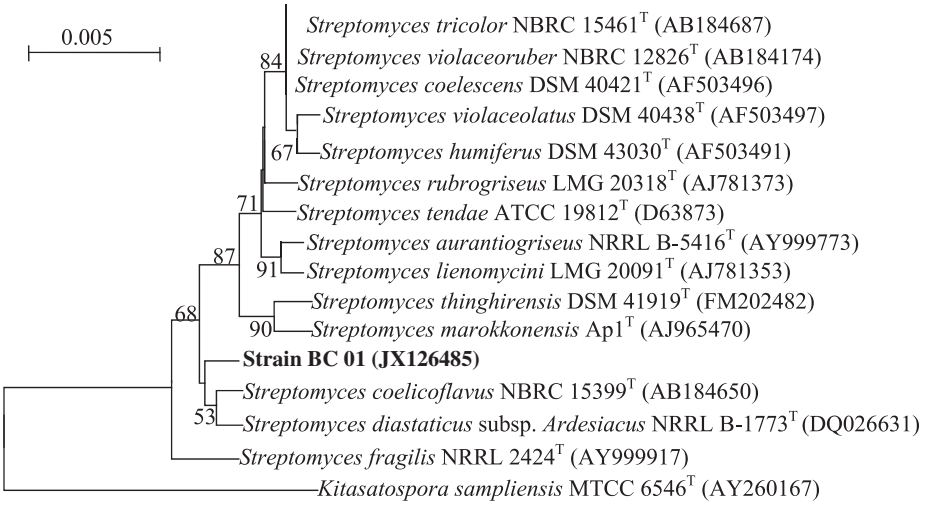

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA sequence (1379 bases), showing the phylogenetic relation between of strain BC 01 (JX126485) and the other type strains of closely related to *Streptomyces coelicoflavus* NBRC 15399T (AB184650). Bootstrap values (expressed as percentage of 1000 replications) of greater than 50% are shown at the branch points. Bar 0.005 substitutions per site and *Kitasatospora sampliensis* MTCC 6546T (AY260167) was used as out group.

activity. This is the first report of this kind from mangrove ecosystem of Visakhapatnam region. It shows that mangrove soil is also rich in bioactive compounds with high antioxidant activity. Comparable reports with rich source of *Streptomyces* species were reported in marine environment, a compound 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one extracted from marine *Streptomyces* VITSVK5 spp which exhibited potent antioxidant activity.[29] Similarly the *Streptomyces* VITTK3 species showed effective DPPH radical scavenging activity in both extracellular and intracellular metabolites[30] and also *Streptomyces* Eri12 isolated from rhizosphere of *Rhizoma curcumae* Longae was an aspirant from the nature source of antioxidants.[31]

Further studies were carried with BC 01 strain due to its highest antioxidant activity when compared with the other three isolates. The molecular characterization revealed that the strain belongs to the genus *Streptomyces* and for phylogenetic tree analysis exposes that the strain closely correlated with species *S. coelicoflavus*.

4.1. Description of *Streptomyces coelicoflavus*

The strain is gram-positive, aerobic and non-acid fast. Vegetative hypha is enormously branched, copious and fragmented. After 2 weeks of incubation aerial mycelia was gray to chalky white in starch casein agar media. Smooth and non-motile spores are observed. Glucose, fructose, galactose, glycerol, mannose, mannitol, and sorbitol, are used as carbon sources. Starch, gelatin and casein are hydrolyzed. IMVC tests are positive. The biochemical tests like nitrate reduction, H_2S production, catalase, tyrosine and melanin production were positive. The optimum growth of the isolate is 25°C–35 °C at pH 7.0–8.2. The strain was resistance against antibiotics streptomycin, kanamycin and tetracycline up to 100 μg/ml, but rifampicin is 50 μg/ml. In the cell wall the leading fatty acids found were Iso-$C_{16:0}$(21.68), Anteiso-$C_{15:0}$(17.80) and Anteiso-$C_{17:0}$(11.58). The guanine and cytosine content of the isolated DNA was found to be 69.7 mol%. The 16S rRNA gene partial sequence was deposited in NCBI nucleotide database with Accession No JX126485.

5. Conclusion

The present study was mainly focused on isolation and molecular characterization of potent actinomycetes from mangrove soil. Therefore, we conclude that microbes isolated from mangroves having potent bioactive secondary metabolites. Our results evidenced that the four extracts showed worthy antioxidant activity, which was produced from natural source. Among them, the isolate BC 01 revealed a potential *in vitro* antioxidant activity equally with the standard reference. The morphological, biochemical, physiological, molecular characterization confirmed that the strain belongs to the species *S. coelicoflavus*. Further, the bioactive compound is to be purified and the structural elucidation of the compound is to be done. The purified compound is to be analyzed for biological activities. The current attempt for isolation and characterization of actinomycetes from mangrove environs will be favorable for the identification of novel antibiotics.

Conflicts of interest

All authors have none to declare.

Acknowledgment

The author (K.V. Raghava Rao) gratefully acknowledges the University Grants Commission (UGC), New Delhi, India for providing financial assistance to study this work under Non-SAP scheme.

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