

BIOLOGICAL ACTIVITIES OF *ALTERNARIA* SP. RL4 – A POTENT ENDOPHYTIC FUNGUS ASSOCIATED WITH *RAUVOLFIA SERPENTINA* L. BENTH.RANJAN GHOSH¹, SOMA BARMAN², PAVAN KUMAR JGS³, NARAYAN C MANDAL^{1*}¹Mycology and Plant Pathology Laboratory, Department of Botany, Visva-Bharati, Santiniketan – 731 235, West Bengal, India. ²Soil and Agrobio-engineering Laboratory, Department of Environmental Science, Tezpur University, Napaam, Tezpur – 784 028, Assam, India.³School of Bioscience and Technology, Vellore Institute of Technology, Chennai – 600 127, India.

Received: 24 May 2018, Revised and Accepted: 04 July 2018

ABSTRACT

Objectives: Endophytic fungi are considered as an important source of bioactive metabolites. The present study focused on the isolation of potent endophytic fungal strains from well-known medicinal plant *Rauvolfia serpentina* L. Benth. having biological activities.

Materials & Methods: Fungal endophytes were isolated from aerial parts of the plant and the potent strain was selected on the basis of antibacterial activities of cell-free supernatant (CFS). Ethyl acetate (EA) extraction of CFS was done and mode of action of EA fraction was checked against pathogenic bacteria. EA fraction was also analyzed by thin-layer chromatography (TLC). In addition, antioxidant activity was checked by 1,1-Diphenyl-2-picrylhydrazyl-free radical scavenging assay and anticancer activity was checked against breast cancer cell line MCF-7 by MTT assay.

Results: Among the different endophytic fungal isolates, CFS of *Alternaria* sp. RL4 produced prominent zones of inhibition against numbers of Gram-positive pathogenic bacteria including *Staphylococcus aureus* (12±0.5 mm) and *Listeria monocytogenes* (17±2.0 mm). Massive reductions in bacterial CFS were noticed upon treatment with EA fraction (2 mg/ml). It also showed strong cidal mode of action against Gram-positive organisms. TLC analysis revealed the production of two different compounds with antibacterial potentials. In addition, EA fraction of RL4 showed very good antioxidant property with an IC₅₀ value of 49.80±2.11 µg/ml. MTT assay also suggested the anticancerous properties of EA fraction of RL4.

Conclusion: *Alternaria* sp. RL4 could be a very good source of bioactive compounds for the development of new drugs.

Keywords: *Rauvolfia serpentina*, Endophytic fungi, Antimicrobial potential, Cidal mode of action, Antioxidant activity, Anticancerous activity.

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INTRODUCTION

Symbiotic relationship between plants and microorganisms is considered as one of the important factors for colonization of plants on the land. Among different types of microbial symbionts, endophytic fungi which live inside the plant tissues without producing any sign of existence play an important role in defense and plant growth promotion [1]. They take nutrition from the plant to survive in different biotic and abiotic stress conditions [2,3] and produce several bioactive compounds to protect the host plant. Endophytic fungi reported from various groups of plant are good sources of secondary metabolites and could serve as vectors for the introduction of genes that can confer pest resistance and for the expression of important traits [4,5]. Natural products from fungal endophytes have broad spectrum of biological activities, and these metabolites encompass a diverse range of structures including alkaloids, steroids, terpenoids, phenylpropanoids, quinines, peptides, and xanthenes [6]. Among the different bioactive secondary metabolites produced by microorganisms, 38% are of fungal origin [7]. Taxol, a potent anticancerous drug, produced by the endophytic fungus *Taxomyces andreae* was discovered from *Taxus brevifolia* [4].

Rauvolfia serpentina L. Benth. (family - Apocynaceae), commonly known as "Indian snakeroot" or "sarpagandha," is a species of flowering plant with useful medicinal values. It is native to the Indian subcontinent and East Asia (from India to Indonesia). *R. serpentina* contains a number of bioactive chemicals, including yohimbine, reserpine, ajmaline, deserpidine, rescinnamine, and serpentinine. *R. serpentina* is widely used medicinally both in modern medicinal system and also in ayurvedic, unani, and folk medicine. Nath *et al.* [8] have characterized *Colletotrichum gloeosporioides*, *Penicillium* sp., and *Aspergillus awamori* as fungal endophytes from *R. serpentina* with hypocholesterolemic,

antimicrobial, and antioxidant potential.

The types of endophytes within the same plant varies with different habitats, different parts of the plant as well as in different seasonal conditions [9]. In the present endeavor, we have tried to isolate and characterize endophytic fungi from *R. serpentina* collected from the lateritic soil of Santiniketan, West Bengal. The antimicrobial potential, antioxidant activity, and anticancerous property of the potent isolate were also studied during this period.

METHODS**Collection of plant materials**

Healthy and mature *R. serpentina* plants were randomly collected from the lateritic soil of Santiniketan, West Bengal, India. The plant materials were brought to the laboratory in sterilized polythene bags. Fresh plant materials were selected for the isolation of endophytic fungi.

Isolation and purification of endophytic fungi

Collected plant materials were washed in running tap water and stem as well as leaf samples were cut into small pieces. Leaves were cut into 3–4 mm in diameter and 0.5–1 cm in length without midrib and stem were cut into 0.5–1 cm in length. Plant materials were then surface sterilized with 4% sodium hypochlorite and finally washed for three times with sterilized distilled water. The sterilized stem and leaf samples were placed on potato dextrose agar (PDA) plates incorporated with streptomycin (100 µg/ml) to prevent bacterial contamination and incubated at 28°C for 3–4 days. After the emergence of fungal mycelia, they were purified by streaking and stored in PDA slants for further studies.

Characterization and identification of isolated fungi

The isolated fungal strains were characterized and identified by light microscopic studies [10]. A little amount of fungal mycelia was taken on a clean glass slide and stained with cotton blue. Excess stain was removed and mounted with a cover glass using lactophenol. The specimens were observed under light microscope (Olympus CH20i) and identified on the basis of their morphological characteristics.

Antimicrobial screening of isolated endophytes

To select the isolated endophytes with antimicrobial potentials, all of the isolated strains were grown in 100 ml of potato dextrose broth at 28°C for 7 days. The cell-free supernatants (CFS) were taken after filtration with Whatman No. 1 filter paper followed by centrifugation at 6000 rpm for 10 min and antimicrobial activities were checked by agar well diffusion method [11]. 50 µl CFS was added to the wells of agar plates. Zones of inhibition were observed and diameters were measured. Antimicrobial potentials of isolated endophytes were studied against a number of Gram-positive and Gram-negative pathogenic bacteria.

Extraction of culture filtrates of endophytic fungus

After initial screening, one of the potent fungal strains RL4 isolated from *Rauvolfia* leaf was inoculated in 1L of potato dextrose broth and incubated at 28°C for 10 days. CFS was taken after filtration followed by centrifugation at 6000 rpm for 10 min and extracted with ethyl acetate (EA). 40 ml of EA was added for every 100 ml of culture filtrate, mixed vigorously and the EA layer was separated using separating funnel. The EA extract was then evaporated to dryness in vacuum evaporator at room temperature (26±2°C), weighed and stored at 4°C for further studies.

Antibacterial study

Antibacterial activity of EA fraction of CFS of RL4 was checked against three Gram-positive bacteria, namely *Bacillus subtilis* MTCC121, *Staphylococcus aureus* MTCC96, and *Listeria monocytogenes* MTCC657 by disc diffusion method [12]. Different concentrations (1–10 mg/ml) of EA fractions were prepared after dissolving in dimethyl sulfoxide (DMSO), sterilized paper discs (Whatman No. 1) were soaked and placed on the lawn of pathogenic bacteria in nutrient agar (NA) plates. Plates were incubated for 24 h at their respective growth temperatures. Zones of inhibition were observed and diameters were measured.

Antibacterial activity of the EA fraction was also checked by counting the colony-forming units (CFUs) against these Gram-positive bacteria. 1% of bacterial culture (OD at 620 nm=0.5) was added to fresh nutrient broth and treated with EA fraction at a concentration of 2 mg/ml. After 24 h of incubation at their respective growth temperatures, CFUs were counted by spreading on NA plates and compared with untreated control.

To determine the mode of action, the EA fraction was added on actively growing cultures of three Gram-positive bacteria mentioned earlier at a concentration of 2 mg/ml followed by counting the CFUs at different time intervals and plotting the values in graphs [13].

Thin-layer chromatographic (TLC) analysis

The EA fraction of the CFS of the endophytic fungus was analyzed by TLC. The dried fraction was redissolved in EA at a concentration of 20 mg/ml and 10 µl of suspension was spotted on TLC plates (MERCK Silica gel F₂₅₄). EA and chloroform in the ratio 1:1 were used as running solvent. Retention factor (R_f) values for all the bands were calculated under ultraviolet light. The bands were then cut and placed on lawn of bacteria on NA plates to check the antibacterial activities. Zones of inhibition were observed after 24 h of incubation.

Antioxidant activity

Free radical scavenging activity of EA fraction was determined using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) by the method described by Lee et al. [14]. The dried EA fraction was dissolved in methanol at a concentration of 10 mg/ml. 100 µl of various concentrations of the methanolic fractions were mixed with 2900 µl DPPH solution and incubated in the dark at room temperature for 30 min. In control set, only 100 µl of methanol was added to the DPPH. The absorbance was

recorded at 517 nm. The percentage of free radical scavenging activity of methanolic extract was calculated as: %I = $[(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$. Where A_{blank}: Absorbance of control; A_{sample}: Absorbance of methanolic extract and DPPH. IC₅₀ was calculated by plotting straight line equation.

Anticancerous activity

The anticancerous study was carried out using human breast cancer cell line MCF7. 1×10⁴ cells were seeded into each well in 96 well plates and allowed to stand for 24 h. After 24 h, complete media was removed

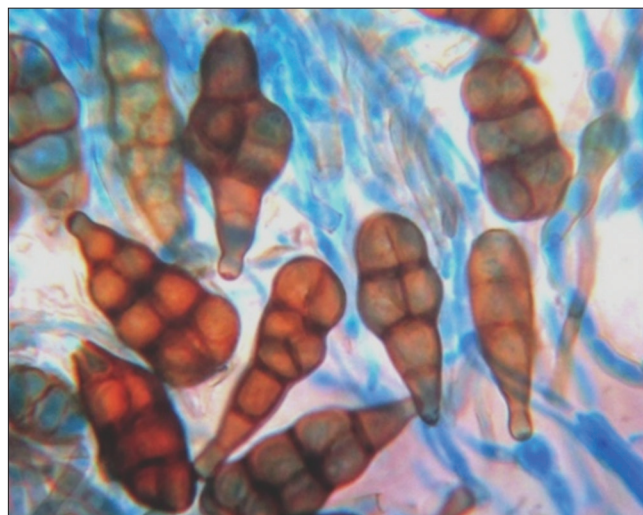


Fig. 1: *Alternaria* sp. RL4 under light microscope

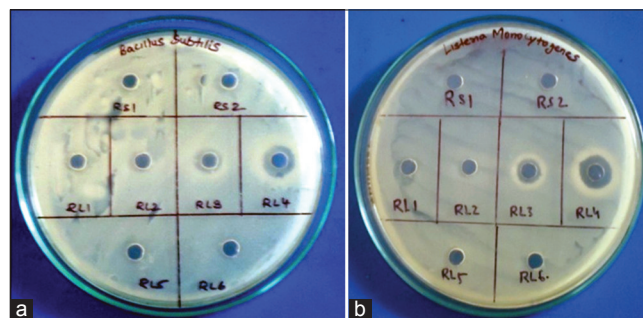


Fig. 2: Zone of inhibition produced by cell-free supernatant of the endophytic fungi against Gram-positive bacteria: (a) *B. subtilis* MTCC121, (b) *L. monocytogenes* MTCC657

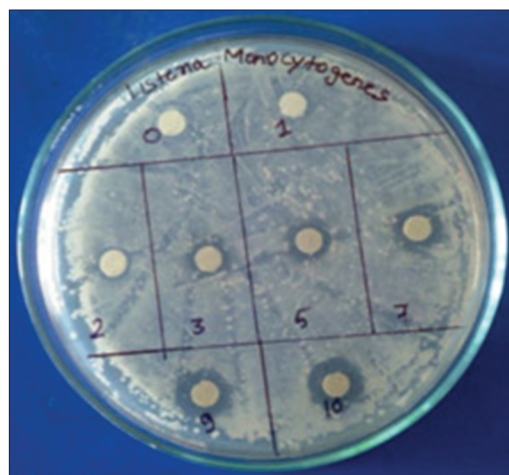


Fig. 3: Zones of inhibition produced by different concentration of ethyl acetate fraction of RL4 against *L. monocytogenes* MTCC657

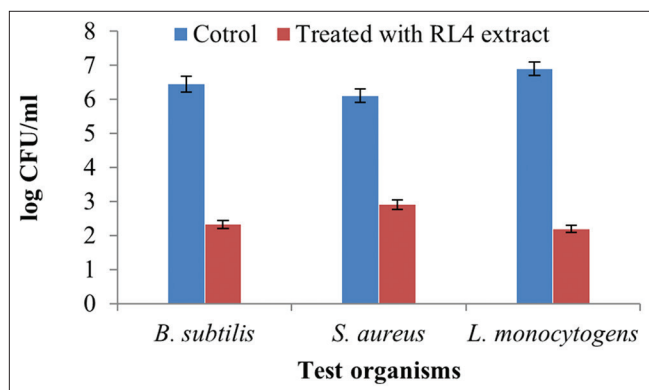


Fig. 4: Effects of ethyl acetate fraction (2 mg/ml) of RL4 on colony-forming units of pathogenic bacteria

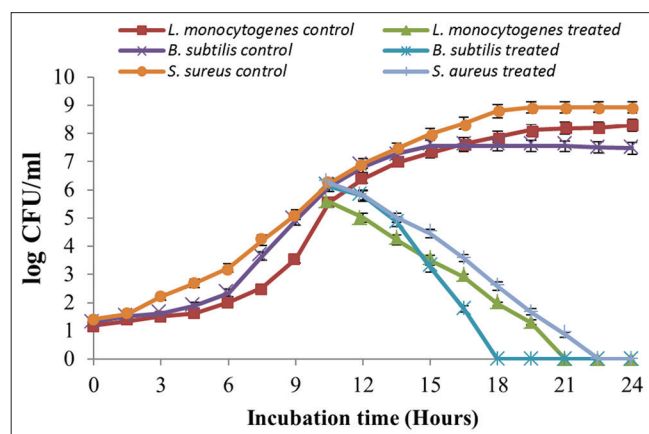


Fig. 5: Cidal activity of ethyl acetate fraction (2 mg/ml) of RL4 on pathogenic bacteria

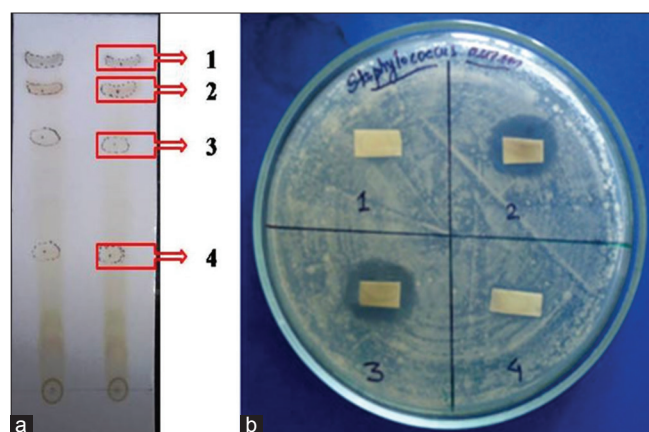


Fig. 6: Antimicrobial activity of ethyl acetate fraction RL4 against *S. aureus* MTCC96 after TLC analysis: (a) separation on TLC plate, (b) zones of inhibition produced by different bands

and the wells were replenished with incomplete media (without FBS) and different concentrations (1, 5, 10, 50, and 100 µg/ml) of the crude extract of the endophytic fungus *Alternaria* sp. Some wells were kept untreated as control. Plates were incubated for 24 h at 37°C in a 5% CO₂ incubator (Thermo Scientific, USA) and after the incubation period, proliferation was estimated by MTT assay method at 595 nm [15]. All experiments were performed and recorded as cell viability percentage in comparison with the control experiment. Cell death percentage was calculated considering 0% death in control experiments. 50 mM Mytomycin C was used as positive control.

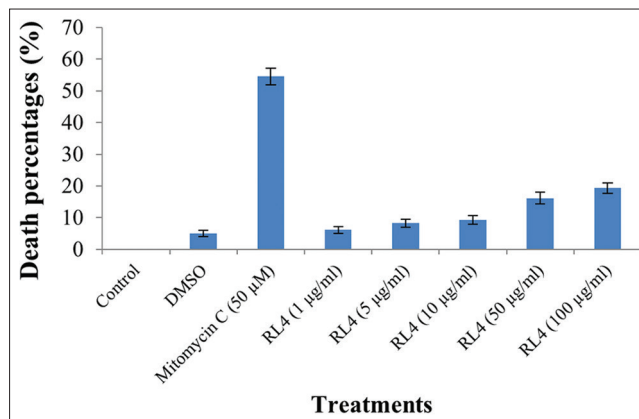


Fig. 7: MTT assay result of MCF7 after treatment with the crude extracts of RL4

Table 1: Zones of inhibition produced by endophytic fungal strains against pathogenic organisms

Pathogenic organisms	Diameter of inhibition zones (mm)	
	RL3 (<i>Alternaria</i> sp.)	RL4 (<i>Alternaria</i> sp.)
<i>B. subtilis</i> MTCC121	8±1.0	10±2.0
<i>L. monocytogenes</i> MTCC657	6±1.5	17±1.0
<i>S. aureus</i> MTCC96	6±1.0	12±0.5
<i>P. aeruginosa</i> MTCC741	-	-
<i>E. coli</i> MTCC1667	-	-
<i>S. typhimurium</i> MTCC98	-	-

B. subtilis: *Bacillus subtilis*, *L. monocytogenes*: *Listeria monocytogenes*, *S. aureus*: *Staphylococcus aureus*, *P. aeruginosa*: *Pseudomonas aeruginosa*, *E. coli*: *Escherichia coli*, *S. typhimurium*: *Salmonella typhimurium*

Statistical analysis

Each data represented is average of at least three replicates. Means and standard deviations were calculated using Microsoft Excel 2007 program.

RESULTS

Endophytic fungi were isolated from disease-free, healthy leaves and stems of *R. serpentina* plants. A total of eight different fungal strains were isolated from 30 leaves and 20 stem segments. During morphological characterization under light microscope, two isolates were identified as *Colletotrichum* spp., four were identified as *Alternaria* spp., and remaining two were unidentified due to the absence of reproductive structures.

Among the eight different isolates, CFS of two fungal strain, namely RL3 and RL4, which were isolated from leaves of *R. serpentina* and identified as species of *Alternaria* (Fig. 1), showed prominent zones of inhibition (Table 1) against all of the Gram-positive bacterial strains (Fig 2), namely *B. subtilis* (10±2.0 mm), *S. aureus* (12±0.5 mm), and *L. monocytogenes* (17±1.0 mm). No inhibition zone was found against any of the Gram-negative bacteria tested. Among these two strains, RL4 was more effective than RL3 to produce zones of inhibitions against pathogenic bacteria.

On the basis of antimicrobial potential, the isolated fungal strain *Alternaria* sp. RL4 was selected for EA extraction of antimicrobial principles. When the antimicrobial potential of the EA fraction was checked by disc diffusion method, it was found that the EA fraction was able to produce zones of inhibition against all of the tested bacteria, namely *B. subtilis*, *L. monocytogenes*, and *S. aureus* at a concentration of 2 mg/ml (Fig. 3). It was also observed that the extract showed maximum zones of inhibition against *L. monocytogenes* which is a foodborne pathogen causing spoilage of different food products (Table 2).

Table 2: Diameter of inhibition zones produced by different concentration of EA fraction of RL4 against pathogenic bacteria

Concentrations of EA fraction (mg/ml)	Diameter of inhibition zones (mm) against pathogenic bacteria		
	<i>B. subtilis</i> MTCC121	<i>L. monocytogenes</i> MTCC657	<i>S. aureus</i> MTCC96
1	0	0	0
2	6±1.0	8±1.5	6±1.0
3	7±2.0	9±1.0	6±1.0
5	8±1.5	10±1.0	7±1.5
7	9±1.5	10±1.5	8±1.5
9	10±2.0	12±2.5	8±2.0
10	10±2.0	12±2.0	9±2.0

EA: Ethyl acetate

Table 3: Percentage of inhibition of free radicals by crude extract of RL4

Concentration of crude extract (µg/ml)	Percentage (%) of inhibition of free radicals
0	0
10	9.34±1.68
20	18.26±1.97
30	28.3±2.71
50	50.20±2.13
75	79.8±3.2
100	98.5±2.43
IC50	49.80±2.11 µg/ml

Antimicrobial activity of the EA fraction of RL4 was also checked against three Gram-positive bacteria by counting the CFUs. 64.04%, 52.38%, and 68.12% inhibition in the number of the CFUs were noticed in case of *B. subtilis*, *S. aureus*, and *L. monocytogenes*, respectively, in the presence of 2 mg/ml of the crude EA fraction (Fig. 4).

To study the mode of action of the antimicrobial principle, the EA fraction of RL4 was added to the mid-log phase culture of *B. subtilis*, *S. aureus*, and *L. monocytogenes*. After addition of the extract, a rapid decrease in the number of CFUs were noticed for all of the bacteria (Fig. 5).

TLC analysis of EA fraction of CFS of RL4 revealed four distinct bands with Rf values of 0.864±0.032, 0.790±0.024, 0.641±0.019, and 0.345±0.027 under UV light. When the antimicrobial properties of individual bands were studied, band 2 (Rf=0.790±0.024) and band 3 (Rf=0.641±0.019) were able to produce prominent zones of inhibition against all of the three Gram-positive bacteria (Fig. 6).

A prominent color changes from pink to yellow during DPPH free-radical scavenging assay in the presence of different concentration of the crude extract of RL4 also suggested very good antioxidant property. When optical densities were measured and percentages of inhibition were calculated, 98.5±2.43% inhibition of free radicals was noticed at a concentration of 100 µg/ml of crude extract (Table 3). From the standard curve of percentage (%) of inhibitions, IC₅₀ value of crude extract of RL4 was calculated as 49.80±2.11 µg/ml. Ascorbic acid, which was used as positive control, showed an IC₅₀ value of 20.23 µg/ml.

Anticancerous activity of EA extract of RL4 was studied at different concentrations (dissolved in DMSO) against human breast cancer cell line MCF7. Death percentages of cells were found to be 16.13±1.84% and 19.36±1.66% in the presence of 50 µg/ml and 100 µg/ml crude extract, respectively (Fig. 7). 54.56±2.61% cell death was found in the presence 50 mM Mitomycin C used as positive control.

DISCUSSIONS

In the present study, eight endophytic fungi were isolated from *R. serpentina* mostly belonging to the genus *Alternaria* and *Colletotrichum*. Nath et al. [8] reported the presence of *Colletotrichum*,

Penicillium, and *Aspergillus* as endophytes in *R. serpentina* whereas Qadri et al. [16] reported the presence of only *Alternaria* spp. Among the different isolates CFS of two strains, namely RL4 and RL3, identified as *Alternaria* spp. were able to inhibit Gram-positive bacteria *in vitro*. As RL4 was more effective than RL3, it was taken for further studies. The EA fraction of the CFS of RL4 showed prominent zones of inhibition against Gram-positive bacteria, namely *B. subtilis*, *S. aureus*, and *L. monocytogenes*, but it was unable to inhibit Gram-negative bacteria. Most of the bioactive metabolites from endophytic fungi reported were more effective against Gram-positive bacteria than Gram-negative bacteria and pathogenic fungi [17]. The reason for the sensitivity of the extract only against Gram-positive bacteria may be due to the differences between cell wall composition with that of Gram-negative microorganisms. In Gram-negative bacteria, the outer wall layer is made up of lipopolysaccharide. This makes the cell wall impermeable to lipophilic solutes. Gram-positive bacteria are more susceptible due to the presence of only an outer peptidoglycan layer which is not an effective permeability barrier [18,19]. Hellwig et al. [20] reported a novel antibacterial antibiotic altersetin from endophytic *Alternaria* sp. which was effective against Gram-positive bacteria. During mode of action studies, a rapid decrease in the numbers of CFUs indicated strong cidal nature of the antimicrobial principles. Production of prominent zones of inhibition by band 2 and band 3 after TLC analysis against all of the Gram-positive bacteria tested, suggested that *Alternaria* sp. RL4 was able to produce at least two different compounds with antimicrobial activities. Antioxidant compounds play an important role as a health-protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart disease [21]. Crude extract of *Alternaria* sp. RL4 was also showed very good antioxidant activities. Antioxidant activities by different endophytes have also been reported by several workers [22-24]. Death of human breast cancer cell MCF7 after treatment with crude extract of RL4 strongly suggested anticancerous property of the extract. Large numbers of secondary metabolites with potent anticancerous activities have already been isolated from endophytic fungi [25-27]. Therefore, secondary metabolites produced by endophytic fungi could be alternative sources for development of novel anticancer drugs [28,29].

CONCLUSIONS

Endophytic fungi *Alternaria* sp. RL4 isolated from *R. serpentina* showed very good antibacterial activities against Gram-positive pathogenic bacterial strains. It can produce at least two different antibacterial compounds with cidal mode of action. In addition, it also showed antioxidant and anticancerous properties. Therefore, *Alternaria* sp. RL4 could be a very good source of bioactive compounds or in development of new drugs.

ACKNOWLEDGMENTS

We are thankful to DST INSPIRE Program and Indian Academy of Sciences for financial support. We are also thankful to Dr. Anshuman Chatterjee, Department of Zoology, Visva-Bharati for his help during the anticancerous study.

AUTHOR'S CONTRIBUTION

R. Ghosh, S. Barman, and Pavan Kumar J G S performed mainly the experimental parts of the work. R. Ghosh written the MS. N.C. Mandal supervised the overall work and also corrected the MS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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