Antibacterial effect of an extract of the endophytic fungus *Alternaria alternata* and its cytotoxic activity on MCF-7 and MDA MB-231 tumour cell lines

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Abstract: There is a growing need for new and effective antimicrobial agents to treat life-threatening diseases. Fungal endophytes are receiving increasing attention by natural product chemists due to the diverse and structurally unprecedented compounds, which make them interesting candidates for drug discovery. The present study evaluates the antibacterial activity of ethyl acetate extract of the endophytic fungus *Alternaria alternata* VN3 on multi-resistant clinical strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, as well as its cytotoxicity on MCF-7 and MDA MB-231 tumour cell lines of breast cancer. The maximum inhibition zone of 21.4±0.07 mm and 21.5±0.25 mm was observed for *S. aureus* strain 10 and *P. aeruginosa* strain 2, respectively. The ethyl acetate extract showed minimal inhibitory concentration ranging from 100 to 900 μg/ml for *S. aureus* and *P. aeruginosa*. Further, the ethyl acetate extract of *A. alternata* VN3 exhibited moderate anticancer activity against MCF-7 and MDA MB-231 cell lines. At 30 μg/ml the cell viability was decreased to 75.5% and 71.8% for MCF-7 and MDA MB-231 cells, respectively. These results clearly indicate that the metabolites of *A. alternata* VN3 are a potential source for production of new drugs.

Keywords: endophytic fungus, *Alternaria alternata*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, anticancer activity, antibacterial activity

INTRODUCTION

The treatment of bacterial infections is increasingly complicated by the ability of bacteria to develop resistance to antimicrobial agents (TENOVER 2006). There is a growing need for new and effective antibiotic agents due to the emergence of life-

threatening, multidrug-resistant (MDR) bacterial infections (Weight 2007; Demain & Sanchez 2009). *Staphylococcus aureus* is a major pathogen causing a diversity of infections, including bacteraemia, pneumonia, skin, soft tissue, and osteo-articular infections (Lowy 1998). Resistance of *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), continues to be a problem for clinicians worldwide. The growing prevalence of MRSA as a cause of these infections has increased the use of the glyco-peptide antibiotic vancomycin over the past 3 decades (NNIS 2002).

MRSA is one of the most important nosocomial pathogens worldwide, but recently it is increasingly identified as the aetiological agent of infections acquired in the community. Molecular epidemiological studies indicate that community-associated (CA)-MRSA and healthcare-associated (HA)-MRSA may have distinctive phenotypic and genetic features (Mammina et al. 2012). These days, MRSA infections acquired outside of the hospital setting have been increasingly reported. The resistance of MRSA to various antimicrobials is globally increasing at an alarming rate. As a result, treatment of MRSA infections has become more challenging. This is a disturbing revelation and a major concern among health care professionals.

Over the past few years, a notable increase in antibiotic resistance among Gram-negative bacteria recovered from hospitalized patients has been reported, especially for critically ill patients (Fridkin & Gaynes 1999). *Pseudomonas aeruginosa* is an invading Gram-negative opportunistic pathogen that causes nosocomial infections and is responsible for 10% of all hospital-acquired infections (Nagaveni et al. 2011). The increasing prevalence of nosocomial infections produced by multidrug-resistant (MDR) *P. aeruginosa* strains, including isolates with resistance to broad-spectrum β-lactams, aminoglycosides, and fluoroquinolones, severely compromises the selection of appropriate treatment (Wang et al. 2009).

New methods of treatment are needed also in cancer, which is a major killer disease all over the world, as more than 6 000 000 new cases are reported every year. Cancer still remains one of the most serious health problems, and breast cancer is the second most universal cause of cancer deaths in women (Ravikumar et al. 2010).

Nature is an attractive source of new therapeutic compounds, as a tremendous chemical diversity is found in millions of species of plants, animals, and microorganisms. Plant-derived compounds have played an important role in the development of several clinically useful anti-cancer agents. Bioactive natural compounds produced by endophytes have also been promising potential usefulness in safety and human health concerns, although there is still a significant demand of the drug industry for synthetic products due to economic and time-consuming reasons (Strobel et al. 2004). Recently, endophytes have been recognized as important sources of a variety of structurally novel and biologically active secondary metabolites, including terpenoids, steroids, alkaloids, and isocoumarin derivatives (Ya-Tuan et al. 2010). Endophyte-derived enzymes and drugs find applications in medicine, agriculture, and industry (Zaferanloo et al. 2014).

Alternaria spp. are also endophytes and their metabolites exhibit a variety of biological activities, such as phytotoxic, cytotoxic, and antimicrobial properties, which have drawn the attention of many chemists, pharmacologists, and plant pathologists in research programs as well as in application studies (Brase et al. 2009; Tsuge et al.

2013). In recent years, more and more metabolites with bioactivities from *Alternaria* fungi have been isolated and structurally characterized. In this study, the endophytic fungal extract of *A. alternata* VN3 was screened for its antibacterial and anticancer activity against multidrug-resistant *Staphylococcus aureus, Pseudomonas aeruginosa*, and human breast cancer cells.

MATERIALS AND METHODS

Isolation of endophytic fungi

Healthy leaves of the medicinal plant *Vitex negundo* L. were collected in the Botanical Garden of our college in Virudhunagar. All the leaf samples were washed twice in distilled water and then surface-sterilized by immersion for 1 min in 70% (v/v) ethanol, 4 min in sodium hypochlorite [3% (v/v) available chlorine], 30 s in 70% (v/v) ethanol, and next washed 3 times in sterilized distilled water for 1 min each time. After surface sterilization, the samples were cut into 5–7-mm pieces and aseptically transferred to Petri plates containing malt extract agar with 50 μg/ml of streptomycin to suppress bacterial growth. These Petri plates were incubated at 30°C with normal photoperiod (12 h light and 12 h dark).The plates were examined daily for up to one month for the development of fungal colonies growing out from the leaf segments (ARNOLD et al. 2000)**.** The fungi growing out from the leaf tissue were subsequently transferred onto fresh malt extract agar plates without antibiotics. The identification of endophytic fungal strains was confirmed by morphological and 18S rRNA sequence analysis (ALTSCHUL et al. 1990).

18S rRNA gene sequencing

To verify identification of the fungal strains, total genomic DNA was extracted using the method of DOYLE & DOYLE (1987). The ITS1 5.8s and ITS2 regions of nrDNA were amplified using the primers ITS1 and ITS2 (WHITE et al. 1990) and the 18S region was amplified using the 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3' primers (WHITE et al. 1990). Isolates of 18s rRNA fungal sequences obtained were submitted to GenBank (NCBI, USA) (accession number JF 795288). The 18S rRNA gene sequencing was done at Synergy Scientific Services, Chennai, India.

Extraction of bioactive compounds

The selected endophytic fungal isolates of *Alternaria alternata* were grown on potato dextrose agar at 30°C for 5 days. Three pieces (0.5×0.5 cm^2) of actively growing mycelial agar plugs were inoculated into 500-ml Erlenmeyer flasks containing 200 ml of potato dextrose broth and incubated at 30°C for 3 weeks under stable conditions. After the incubation period, fungal mycelium was separated from the culture medium by vacuum filtration through Whatman no. 1 filter paper. The dried mycelia and culture filtrate were extracted 3 times with ethyl acetate alone. The solvent was removed by evaporation under reduced pressure at 50°C, using rotary vacuum evaporator. After evaporation, the dried fungal extract was dissolved in 10% dimethyl sulphoxide (DMSO) and we studied the antibacterial and anticancer activity.

Determination of the sensitivity of pathogenic bacteria

The Kirby–Bauer disc diffusion test was used to determine the antibiotic resistance of clinical strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The strains of *S. aureus* (1–10) and *P. aeruginosa* (1–8) were obtained from Bose Clinical Laboratory and X-ray (Madurai, Tamilnadu, India). The nutrient broth was prepared and well-isolated colonies of the same type from a culture agar plate were inoculated into it. The culture broth was incubated at 37°C until the culture equalled 0.5 McFarland standards. A McFarland 0.5 turbidity standard corresponded to an inoculum of 1×10^8 CFU/ml (Acar & Goldstein 1991). Usually 2–8 h was required to reach this standard. A sterile cotton swab was dipped in the inoculum and the excess was removed by rotating the swab several times against the inside wall of the tube above the level of the fluid. The surface of this plate was inoculated by streaking the swab over the surface. Antibiotic discs with penicillin (10 units/disc), methicillin (5 μg/disc), and vancomycin (30 μg/disc), were used for *S. aureus*. Ciprofloxacin (5 μg/disc), cefotaxime (30 μg/disc), ofloxacin (5 μg/disc), and amikacin (30 μg/disc) were used for *P. aeruginosa* strains. Ethyl acetate extract (100 μg/ml) of *Alternaria alternata* was used to study the antibacterial activity against the *S. aureus* and *P. aeruginosa* stains. In the control, the DMSO solvent alone was used (10 μl). To ensure complete contact of the discs with the agar surface, the discs were pressed down slightly. The experiments were carried out in triplicates. Inoculated plates were inverted and incubated at 37°C for 18 h. After the incubation period, the diameter of the inhibition zone was measured and results were interpreted according to the standards of the Clinical Laboratory Standards Institute (CLSI 2005).

Determination of minimum inhibitory concentration (MIC)

MIC of the ethyl acetate extract was determined based on a broth micro-dilution method in a 96-well microplate (Al-Bayati 2008). Briefly, the clinical strains of *Staphylococcus aureus* (1–10) and *Pseudomonas aeruginosa* (1–8) were cultured overnight at 37°C on Muller Hinton (MH) broth (Himedia Laboratories, Mumbai, India) and adjusted to a final density of 10^8 colony-forming units (CFU)/ml by 0.5 McFarland standards. The crude ethyl acetate extract (1 mg/ml) was dissolved in 10% DMSO. In 96-well plates, each well had 90 μl of MH broth supplemented with 2% NaCl, 10 μl of bacterial inoculum, and 10 μl of different concentrations of the fungal extract. The plate was incubated at 37°C for 18 h. After incubation, the bacterial growth was visually inspected and the lowest concentration of fungal extract at which no observable bacterial growth or turbidity was observed, was considered as the MIC value. Experiments were performed independently 3 times and each sample was assayed in duplicate.

Cell culture

Human breast cancer cells MCF-7 and MDA MB-231 were obtained from the American Type Culture Collection (Rockville, MD) and grown in minimum essential eagle medium (MEM) supplemented with 10% foetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) in 24-well tissue culture plates (Costar, Cambridge, MA).

MTT reduction cytotoxic assay

A yellow tetrazole, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was taken up by cells and was reduced to a coloured formazan product that could be detected by spectrophotometry (k_{max} = 562 nm). Reduction of MTT was dependent on the mitochondrial respiratory function, and thus it reflected the relative number of viable cells in the culture. MCF-7 and MDA MB-231 cells were treated with various concentrations (0, 10, 20 and 30 µg/ml) of the ethyl acetate extract of *Alternaria alternata* VN3 for 48 h. DMSO alone was used in the control. At the end of the treatments, media were aspirated, the cells were washed with DPBS buffer, and again incubated with 5 mg/ml of MTT for 1 h at 37°C. DMSO was used to dissolve the formazan. The absorbance was measured at 562 nm, using an automated TECAN multimode reader. The results represented are from 3 independent experiments.

RESULTS AND DISCUSSION

In total, 30 endophytic fungi were isolated from the medicinal plant *Vitex negundo*. Among them, *Alternaria alternata* was selected, based on its antimicrobial and anticancer activity. The fungus grows rapidly on potato dextrose agar and the colony was flat, downy to cottony, and eventually was covered by greyish, short, aerial hyphae. The reverse side was typically brown to black, due to pigment production. *Alternaria* is septate, with dark hyphae. They bear simple or branched large conidia (8–16 µm × 23–50 µm), which have both transverse and longitudinal septa. These conidia may be observed singly or in acropetal chains and may produce germ tubes. They were ovoid to obclavate, darkly pigmented muriform, smooth or roughened. Morphological characteristics allowed the identification of the endophytic fungus as *A. alternata*, which was confirmed by the sequence of its 18S rRNA gene that gave a 95% sequence similarity to those accessible at the BLASTN of *A. alternata*. The endophytic fungus was designated as *A. alternata* VN3. The fungal sequence was deposited as GenBank accession no. JF 795288.

The antibiotic activity of *Staphylococcus aureus* strains (1–10) was determined using commercial antibiotics. All the *S. aureus* strains were slightly or moderately inhibited by penicillin. Strain 7 was moderately inhibited by methicillin, in contrast to all the other strains (1–6 and 8–10), with low inhibition by methicillin. Strains 6, 8, and 9 were moderately sensitive to vancomycin, whereas the other strains (1–5, 7, and 10) were highly inhibited by vancomycin. The ethyl acetate extract of *Alternaria alternata* VN3 showed effective antibacterial activity against *S. aureus* strains (1–10). At a concentration of 100 µg/ml, a maximum inhibition zone of 21.4±0.07 mm was observed for *S. aureus* strain 10 and a minimum inhibition zone of 12.6±0.04 mm for strain 8 (Table 1).

To determine the antibacterial profile of *Pseudomonas aeruginosa* strains, ciprofloxacin, cefotaxime, ofloxacin, and amikacin were used. Strains 1 and 3 were moderately sensitive, whereas strains 2, 5, and 6 reacted more strongly to the 4 antibiotics. Strain 4 reacted strongly to ciprofloxacin and ofloxacin, whereas moderately to cefotaxime and amikacin. Strain 7 was highly inhibited by cefotaxime and only slightly or moderately by all the other antibiotics. Ciprofloxacin was highly effec-

Table 1. Antibacterial activity and minimum inhibitory concentration (MIC) of ethyl acetate extracts of *Alternaria alternata* VN3 against *Staphylococcus aureus* strains

S. aureus strains	Zone of inhibition (mm)				MIC (µg/ml)
	Penicillin (10 unit/disc)	Methicillin (10 µg/disc)	Vancomycin (30 µg/disc)	Ethyl acetate extract (100 µg/ml)	
1	12.8±0.15	10.8±0.14	16.5±0.08	20.4±0.04	200
2	12.8±0.14	11.2±0.31	15.4±0.28	18.2±0.25	300
3	14.2±0.28	12.1±0.16	16.0±0.22	17.6±0.31	500
4	11.5±0.35	10.2±0.31	17.2±0.14	13.5±0.14	600
5	10.8±0.14	8.9±0.22	18.5±0.28	18.6±0.35	300
6	10.3±0.15	9.5±0.35	12.4±0.35	17.4±0.15	600
7	13.8±0.16	15.5±0.07	16.9±0.14	15.8±0.22	400
8	10.5±0.23	8.8±0.14	13.5±0.21	12.6±0.04	900
9	14.0±0.21	12.2±0.10	14.8±0.22	16.5±0.35	800
10	14.9±0.1	8.9±0.18	17.4±0.35	21.4±0.07	100

tive, while the other antibiotics were moderately effective for *P. aeruginosa* strain 8. The ethyl acetate extract showed significant antibacterial activity against clinical strains of *P. aeruginosa*. A maximum inhibition zone of 21.5±0.25 mm was observed for *P. aeruginosa* strain 2, while a minimum inhibition zone of 13.8±0.22 mm, for strain 3 (Table 2).

Similarly, Damajan et al. (2007) reported antibacterial activity of extracts of *Amanita virosa* (Fr) Bertill (Amanitaceae) and *Cortinarius praestans* Cordier (Cortinariaceae) against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Xanalteric acids I and II as well as 11 known secondary metabolites were obtained from the endopytic fungus *Alternaria* sp. isolated from the mangrove plant *Sonneratia alba*. Xanalteric acids I and II exhibited weak antibacterial activity against multidrug-resistant *Staphylococcus aureus*, whereas alternusin displayed broad antimicrobial activity against several additional multidrug-resistant bacterial and fungal strains (Kjer et al. 2009).

The lowest concentration of the fungal extract at which no growth of microorganism observed upon visual observation after incubation at 37°C for 18 h was considered as the MIC value. Pellets formed at the bottom of wells were considered bacterial growth even if the wells were clear of turbidity. The MIC value ranged from

Table 2. Antibacterial activity and minimum inhibitory concentration (MIC) of ethyl acetate extracts of *Alternaria alternata* VN3 against *Pseudomonas aeruginosa* strains

P. aeruginosa strains	Zone of inhibition (mm)					MIC (μg/ml)
	CI (5 μg/disc)	CE (30μg/ disc)	OF (5 g/disc)	AK (30μg/disc)	Ethyl acetate extract (100 μg/ml)	
1	14.5±0.4	12.4±0.4	12.5±0.35	15.5±0.17	17.6±0.31	600
2	21.6±0.1	23.5±0.14	20.3±0.28	20.5±0.14	21.5±0.25	200
3	15.4±0.28	14.9±0.14	13.5±0.4	12.9±0.28	13.8±0.22	700
4	23.7±0.23	16.8±0.1	18.6±0.31	14.5±0.31	15.3±0.14	400
5	24.3±0.04	24.8±0.15	19.5±0.14	19.4±0.22	20.8±0.1	200
6	22.3±0.15	25.4±0.07	18.4±0.28	18.8±0.07	20.3±0.07	300
7	16.5±0.22	23.7±0.18	13.9±0.4	10.5±0.28	14.6±0.22	500
8	18.7±0.14	16.5±0.35	16.5±0.14	14.4±0.23	18.3±0.14	500

CI = ciprofloxacin; CE = cefotaxime; OF = ofloxacin; AK = amikacin.

100 to 900 μg/ml for clinical strains of *S. aureus*. The lowest MIC value of 100 μg/ml was observed for *S. aureus* strain 10 (Table 1). The MIC value ranged from 200 to 700 μg/ml for *P. aeruginosa* strains (1–8). The lowest MIC value of 200 μg/ml was observed for strains 2 and 5 (Table 2).

The recorded MIC values tended to be higher than earlier reported for *Alternaria* spp. Three new cerebrosides, alternarosides A–C, and new diketopiperazine alkaloid alternarosin A were isolated from ethyl acetate extract of *Alternaria raphani*. Alternarosides A–C and alternarosin A showed weak antibacterial activity against *Escherichia coli, Bacillus subtilis,* and *Candida albicans*, with MIC values ranging from 70 to 400 μM (WANG et al. 2010). The bioactive compound altechromone was isolated from endophytic fungi from *Alternaira brassicicola* ML-P08, which displayed antimicrobial activity against *B. subtilis, E. coli, Pseudomonas fluorescens*, and *C. albicans*, with MIC values of 3.9, 3.9, 1.8, and 3.9 μg/ml, respectively (GU 2009).

The anticancer activity of the ethyl acetate extract of *Alternaria alternata* VN3 was also investigated, against the malignant MCF-7 and metastatic MDA MB-231 human breast cancer cell lines for 48 h. The ethyl acetate extract of *A. alternata* VN3 dose dependently (0–30 μg/ml) increased cytotoxicity in both MCF-7 and MDA MB-231 breast cancer cells (Figs. 1–2). At 30 μg/ml, cell viability decreased to 75.5% and 71.8% for MCF-7 and MDA MB-231 cell, respectively. Similarly, LIU et al. (2007)
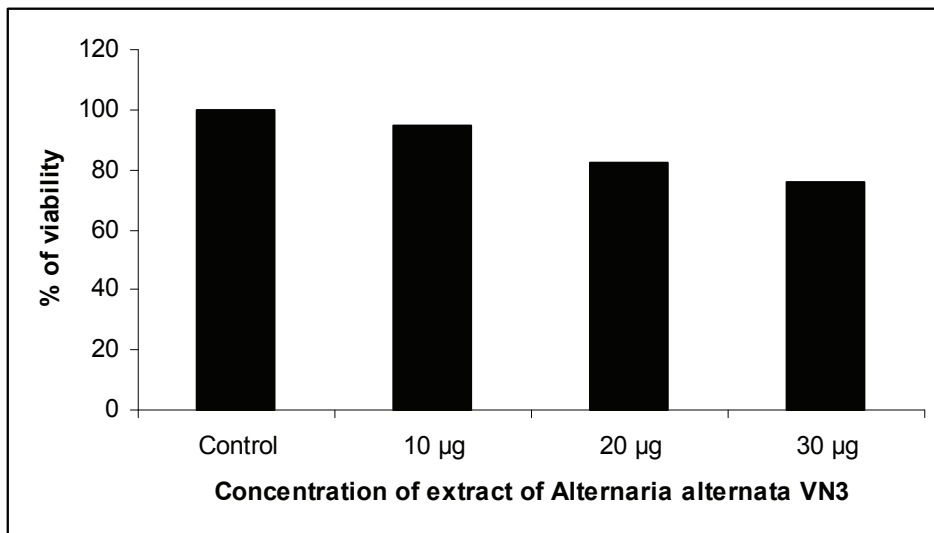
Fig. 1. Anticancer activity of ethyl acetate extract of *Alternaria alternata* VN3 against MCF-7 cells
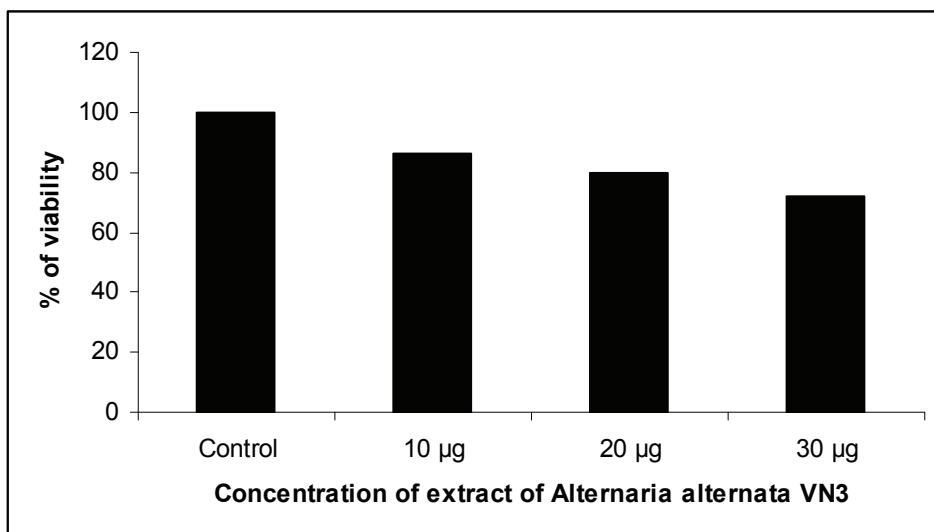

Fig. 2. Anticancer activity of ethyl acetate extract of *Alternaria alternata* VN3 against MDA MB-231 cells

demonstrated for the first time that alternol, a new compound purified from the fermenting products of *A. alternata* var. *monosporus* from the bark of yew (*Taxus* sp.), was found to inhibit proliferation and induce apoptosis in mouse lymphocyte leukaemia L1210 cells. FERNANDES et al. (2009) studied the antitumour activity of fermentation extract of the endophytic fungus *A. alternata* isolated from *Coffea arabica* L. At a dilution of 400 μg/ml, the extract displayed a cytotoxic activity of approximately 50% towards HeLa cells in vitro. In comparison, *A. alternata* VN3 had effective anticancer activity at a lower concentration. The control DMSO had no significant effect on MCF-7 and MDA MB-231 breast cancer cells.

CONCLUSIONS

The endophytic microorganism *Alternaria alternata* seems to produce bioactive and probably chemically novel compounds bearing pharmaceutical potential. The ethyl acetate extract of *A. alternata* VN3 had the maximum inhibition zone of 21.4±0.07 mm and 21.5±0.25 mm for *Staphylococcus aureus* strain 10 and *Pseudomonas aeruginosa* strain 2, respectively. Also, *A. alternata* VN3 showed moderate anticancer activity against MCF-7 and MDA MB-231 cell lines. At 30 μg/ml, cell viability was decreased to 75.5% and 71.8% for MCF-7 and MDA MB-231 cells, respectively. Our results indicate that the ethyl acetate extract of endophytic fungus *A. alternata* VN3 is a potential source for production of new metabolites for treatment of drug-resistant bacteria and cancer cell lines. However, further chemical characterization is needed to identify the compounds and their mechanism of action in the endophyte extracts with the observed biological activity against human pathogenic bacteria and cancer cell lines.

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