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In Vitro Antibacterial and Cytotoxicity Evaluation of Aloe Vera Associated Endophytic Fungi

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Abstract

Objective: The present study was aimed to evaluate *in vitro* antimicrobial and cytotoxicity of endophytic fungi and its metabolites. **Methods:** against *Escherichia coli, Stapylococcus aureus, Klebsiella* sp*, Salmonella* sp*, Proteus* sp*, Shigella* sp*, Candida albicans, Cryptococcus* sp*, Trychophyton* sp*,* and *Penecillium* sp, as well as their cytotoxicity on MOLT -3 cell line. **Results:** Isolated endophytic fungi showed significant inhibition against test microorganisms by the method of dual culture techniques. The crude secondary metabolite of isolate showed highest zone of inhibition against test organisms and the enzyme showed moderate activity. Aloe Vera associated *Aspergillus* sp produce amylase enzyme which showed slight to mild antiproliferative activity on MOLT-3 cell line. **Conclusion:** The results of the present study, suggest that Aloe Vera associated *Aspergillus* sp was potential antimicrobial and antiproliferative agents.

Keywords
Aloe Vera, Antimicrobial activity, Enzymes, Cytotoxic reactivity, Endophytic fungi.

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INTRODUCTION

All over the world cancer one of the major killer disease and more than six million new cases are reported every year. 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. These include Vinblastine, Vincristine, Camptothecin, Podophyllotoxin and Taxol.

Production of a plant-based natural drug is always not up to the desired level. It is produced at a specific developmental stage or under specific environmental condition, stress, or nutrient availability; the plants may be very slow growing taking several years to attain a suitable growth phase for product accumulation and extraction.

Considering the limitations associated with the productivity and vulnerability of plant species as sources of novel metabolites, microorganisms serve as the ultimate, readily renewable, and inexhaustible source of novel structures bearing pharmaceutical potential.

Endophytic microbes have symbiotic association, residing asymptomatically in internal tissues of all higher plants and are of growing interest as promising source of biologically active agents. The

term endophytic microbes refer to an organism which lives within the photosynthetic plant tissue by forming symbiotic relationship with host.[1] And has no harmful effect to the plant. They develop special mechanisms to penetrate inside the host tissue, residing in mutualistic association and their biotransformation abilities opens a new plat-form for synthesis of novel secondary metabolites.[2]

Endophytic fungi are reported for producing antimicrobial and antioxidant, anti-diabetic, anti-arthritis, anti-inflammatory, immunomodulatory, immunosuppressive and anticancer compounds such as Taxol and yet others produce compounds that can be utilized industrially, such as enzyme and solvents. Aloe Vera can serve as good model plants for studying the effect of fungal endophytes colonization on secondary metabolism. It contains at least 6 natural ingredients which act as anti-inflammatory, one of the precursors to cancer. The US Department of Agriculture has however approved Aloe for the treatment of soft tissue cancer in animals (1992) as well as feline leukaemia; and Aloe is approved as an important component where it has a powerful effect on the immune system and complements certain drug therapies.

With the above information, the present investigation was aimed to isolate antibacterial endophytic fungi from Aloe vera and evaluated *in vitro* cytotoxicity efficacy.

MATERIALS AND METHODS

Sample collection

Healthy Aloe vera was collected in the month of December 2017 from Dr.N.G.P. Arts and Science College campus, Coimbatore, Tamil Nadu and used for further process.

Isolation of endophytic fungi

Isolation of endophytic fungi from Aloe vera was carried out using the protocol.[3] Fresh leaves were cut into small pieces with 1 Cm long and sterilized with 70% ethanol for 1min, 1.0% sodium hypochlorite (NaCl) (v/v) for 1min and further cleaned by passing through two sets of sterile distilled water. The sterile sample were placed on plate containing Potato Dextrose agar (PDA) media with 200mg/L concentration of streptomycin to suppress the bacterial contamination. The parafilm wrapped Petri dishes were incubated at 25 ±2°C till the transferred into a new agar slants and stored at 4°C for the further studies.

Identification of endophytic fungi

The isolated endophytic fungi were identified at the Microbiology Laboratory of Dr.N.G.P. Arts and Science College by the method of macroscopic and microscopic colony characteristics of fungi.

Test Microorganisms

Lyophilized 6 test bacteria and 4 fungi cultures were acquired from Kovai Medical Centre and Hospital (KMCH), Coimbatore. Bacterial culture *Escherichia coli, Staphylococcus aureus, Klebsiella* sp*, Salmonella* sp*, Proteus* sp and *Shigella* sp and fungal culture *Candida* sp*, Penicillium* sp*, Trichophyton* sp *and Cryptococcus* sp were used for the antimicrobial evaluation. Each culture was used in the form of 0.5 McFarland identical form.

Preliminary antimicrobial assay

Isolated endophytic fungi were assessed antimicrobial activity by the standard procedure.[4] The respective media was prepared for the growth of bacteria and fungi and 100µL of 0.5 McFarland test organism was spread over the agar media using sterile cotton swab. Nine millimetre diameter of the actively growing fungal culture disc from PDA plates were cut using a sterile cork borer and placed on the surface of the respective plate seeded with the test organism. These plates were sealed with parafilm and kept in refrigerator at 4°C for 12 hours for complete diffusion of antimicrobial compound, thereafter they were incubating at 37°C for 18 hours for bacteria and room temperature for 48-60 hours for fungi respectively. After incubation the zone of inhibition was measured by using scale. Each experiment was carried out in triplicates.

Extraction of secondary metabolites

Secondary metabolites extraction was carried out by the method.[5] Positive endophytic fungal isolates were inoculated into 250mL Erlenmeyer flasks containing 100mL potato Dextrose broth and incubated at room temperature for 21 days under stationary conditions with intermittent shaking. The broth culture was filtered to separate the mycelia and filtrate equal volume of ethyl acetate was added, mixed well for 10 minutes and kept for 5 minutes till the two clear immiscible layers formed. The upper layer of ethyl acetate containing the extracted compounds was separated using separating funnel. The mycelium was grinded properly in a pestle mortar using ethyl acetate as solvent and then it was filtered using cheese cloth. Both mycelia and culture filtrate extracts were pooled together and evaporated to dryness in hot air oven and stored at 4°C for bio assay.

Antimicrobial activity of secondary metabolites

Antimicrobial activity of secondary metabolites attained from endophytic fungi was tested by standard method.[6,7] The sterile growth media plate specific for test organisms were prepared and

inoculated with the test organism. Twenty microliters of crude extract were added on to a sterile 6mm paper disc (Hi-Media) using a micropipette and allowed to dry. Compounds incorporated discs were placed on the surface of the medium and gently pressed for proper adhesion. In the same plate 6mm diameter wells were made using a sterile cork borer and 20µL of secondary metabolites was added to each well. Fluconazole (5 mg/ mL) and chloramphenicol (30 µg/ml) was used as a positive control for fungi and bacteria respectively. Similarly, 20 µl DMSO as well as ethyl acetate were used as negative controls. The plates were incubated at 30 ± 2°C for 12-24 hours for bacteria as well as *Candida albicans* and 40-60 hours for *Penicillum chrysogenum*. The zone of inhibition was measured. The experiment was carried out in triplicates.

Enzyme production by endophytic fungi

The production of enzyme by Aloe vera associated fungi was qualitatively determined by the method.[8] Amylase enzyme activtity was assessed by growing the fungi on glucose yeast extract peptone (GYP) agar medium (glucose-1g, yeast extract 0.1g, peptone 0.5g, agar 16g, distilled water 1000mL and pH 6) containing 1% soluble starch. After 5 days incubation, the plates with fungal colony were flooded with 1% iodine in 2% potassium iodide. The appearance of clear zone surrounding the colony was considered positive for amylase enzyme.

***In vitro* cytotoxicity of enzyme**

The 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT) is a colorimetric assay (Mosmann *et al*, 1983). An *in vitro* cytotoxicity effect of endophytic enzymes was evaluated against MOLT-3. Cells were grown in micro titer 96 well flat bottom plates in a final volume of 100 µL culture medium per well and incubated for at 37°C and 5% CO_2 for 24 h. At the end of the 24 h incubation upon formation of a partial monolayer, the supernatant was flicked off, cells were washed once and endophytic enzyme (5, 25,50,75 and 100 µg/mL) was added into the respectively labelled wells. After 24 h incubation period, 10 µL of 5 mg/mL MTT solution in PBS was added to each well and incubated for 2 h. Thereafter, medium was removed and 100 µL of DMSO was added to all wells to dissolve the formazan crystals. Plates were read at 570 nm using a microplate reader and results were expressed as viability percentage against the untreated control cells (100% of viability). Triplicate wells were assayed for each condition and standard deviations were determined.

Cytotoxicity and cell viability were calculated by below formula.

Cytotoxicity = [(Control – Treated)/ Control] * 100

Cell viability = (Treated / Control) * 100

Statistical analysis

Experimental results were expressed as mean ±SEM. All measurements were replicated three times. The data were analysed by an analysis of variant ($P < 0.05$). The IC_{50} values were calculated from linear regression analysis.

RESULTS

The endophytic fungi were isolated from internal tissues of Aloe vera (Plate 1). The isolates were subcultured on PDA agar plates to remove the adherent plants metabolite from the mycelia and stored at 4°C for the further studies. The isolated endophytic fungi was identified as *Aspergillus* Sp., by macroscopic colony appearance as well as color of the organism and microscopic observation were mentioned in palte 2.

Plate 1: Growth of Aloe vera associated fungi on PDA.

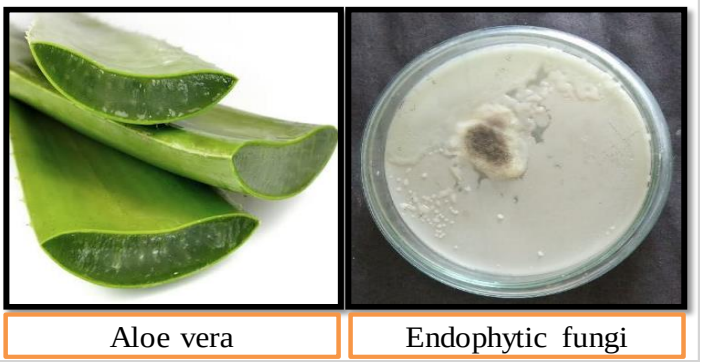
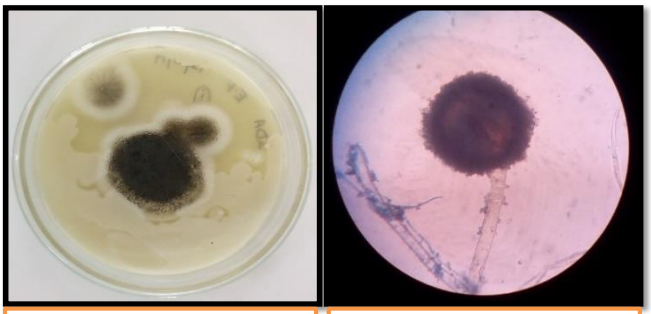


Plate 2: Macroscopic and microscopic observation of endophytic fungi



Macroscopic view | Microscopic view

Antimicrobial activity of endophytic fungi

The anti-microbial activity of isolated endophytic fungi was tested against Gram positive and Gram negative bacteria, and a filamentous fungi respectively by dual culture and the zone of inhibition was mentioned in Table 1.The endophytic fungal isolate of *Aspergillus* sp significantly inhibited test microorganisms (*Escherichia coli, Klebsiella* sp, *Salmonella* sp, *Proteus* sp, *Staphylococcus aureus, Shigella* sp, *Cryptococcus* sp, *Trichophyton* sp, *Candida* sp and *Penicillium* sp) in all tested methods and the zone of inhibition was in the range of 10 to 40mm diameter.

Table 1: Antibacterial activity of Alo vera associated fungi

S.No.	Test Organisms	Zone of Inhibition in mm
1	*Escherichia coli*	30
2	*Staphylococcus aureus*	20
3	*Klebsiella* sp	19
4	*Salmonella* sp	20
5	*Proteus* sp	25
6	*Shigella* sp	20
7	*Candida* sp	40
8	*Cryptococcus* sp	35
9	*Trychophyton* sp	37
10	*Penecillium* sp	30

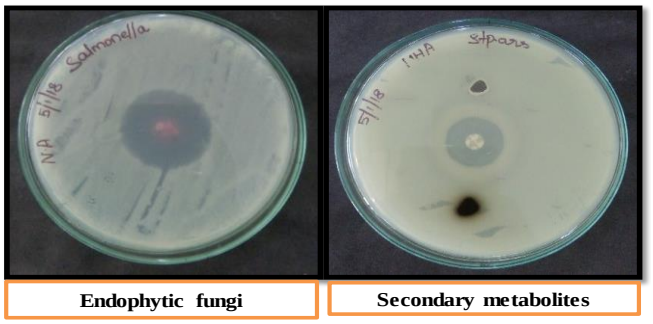
Antimicrobial activity of secondary metabolites of endophytic fungi

The antimicrobial activities of crude ethyl acetate extract of the secondary metabolite isolated from the endophytic fungi showed a broad spectrum and effective antibacterial and antifungal activity (Table 2).

Table 2: Antimicrobial activity of secondary metabolites of endophytic fungi

S.No.	Test organisms	Zone of inhibition (mm)
1	*Escherichia coli*	05.0±10
2	*Staphylococcus aureus*	03.0±9.0
3	*Klebsiella* sp	01.1±9.0
4	*Salmonella* sp	01.0±9.5
5	*Proteus* sp	03.1±9.0
6	*Shigella* sp	02.4±9.1
7	*Candida* sp	09.1±13
8	*Cryptococcus* sp	07.3±11
9	*Trychophyton* sp	08.0±13
10	*Penecillium* sp	07.1±10

Plate 3: Antibacterial activity of endophytic fungi and its secondary metabolites.



The secondary metabolite showed significant activity against Candida sp., *Cryptococcus* sp and *Trychophyton* sp., moderate activity against *Escherichia coli, Proteus* sp., and *Staphylococcus aureus lowest activity against Shigella* sp*., Klebsiella* sp., and *Salmonella* sp.

ENZYME ASSAY

The fungal culture has the ability to produce amylase enzyme was qualitatively examined (plate 2). The plate was flooded with 1% iodine in 2% potassium iodide plate showing clear zone around the fungal colony indicating the degradation of starch by the amylase enzyme produced by the fungi.

Plate 3: Production of Amylase enzymes by endophytic fungi from Aloe vera.

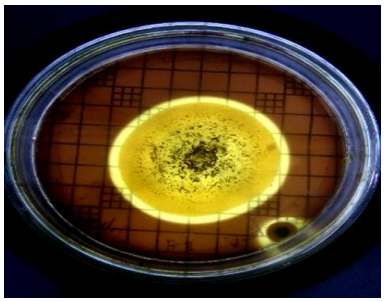


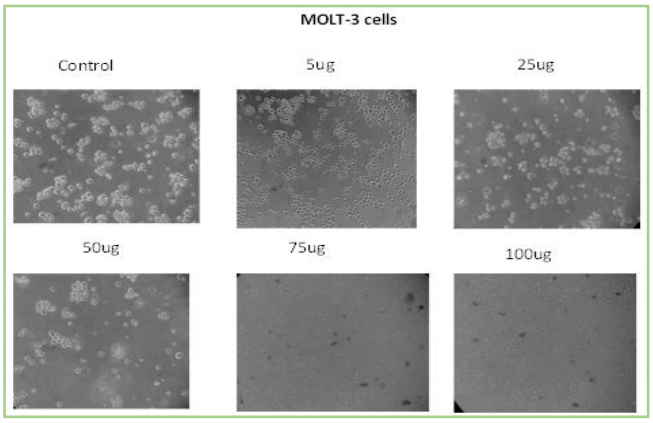
Table 1: Standard for cell viability and cytotoxic reactivity

S.No	Grade (%)	Reactivity
1	0	None
2	1-20	Slight
3	21-50	Mild
4	51-70	Moderate
5	>71	Severe

Table 2: Cytotoxicity assay of amylase enzyme of endophytoic fungi on MOLT-3 cell.

Sample details					
Sample	**Source**	**Conc. (µg)**	**Cytotoxicity (%)**	**Cell Viability (%)**	**Cytotoxicity Reactivity**
		5	0	>99	None
Amylase enzyme	**Endophytic fungi**	25	0	>99	None
		50	0	>99	None
		75	7.2	92.8	Slight
		100	38.2	61.8	Mild

The Amylase of endophytic fungi isolated from the Aloe vera have significantly inhibited Blood cancer MOLT-3 cell line. The endophytic fungi may be responsible for anti-cancer activity. In previous reports, endophytic has been widely studied for its cytotoxic activity. Endophytic of Amylase enzyme has shown cytotoxic action on human blood cancer cells (MOLT-3).



As per ISO 109935:5 the test sample ***Amylase enzyme*** – **Endophytic fungi** showed **Slight to Mild cytotoxic** reactivity to MOLT-3 cells after 24 hours contact, whereas noncytotoxic reactivity was observed in control.

DISCUSSION

Endophytes, the microorganisms that reside in the tissues of living plants, are relatively unstudied and offer potential sources of novel natural products for exploitation in medicine, agriculture and the pharmaceutical industry. They develop special mechanisms to penetrate inside the host tissue, residing in mutualistic association and their biotransformation abilities opens a new plat-form for synthesis of novel secondary metabolites. They produce metabolites to compete with the epiphytes and also with the plant pathogens to maintain a critical balance be-tween fungal virulence and plant defense.

The need for new antimicrobial agents, in general, comes from the increasing rates of resistance to existing antibiotics. This problem extends beyond the clinical application of antimicrobial drugs, such as agricultural microorganisms are also known to have acquired resistance to commonly used antimicrobial chemicals.[9] The endophytic fungi isolates from Aloe vera have significantly inhibited representative Bacteria and Fungi. The zone of inhibition by these fungi are very much comparable to the standard antibiotics.

Eighty percent of the isolates showed positive for amylase activity. The representative petri plates indicating the presence of enzymes from isolated endophytic fungi by qualitative test.[10] The clear zone around the fungal colony indicating the degradation of starch by the amylase enzyme produced by the fungi.

Starch, a complex polymer made of amylose and amylopectin, will be broken down to simple glucose molecule upon action of amylase enzyme. Amylose are long unbranched chain of D-glucose polymers are linked by α-1.4 and in amylopectin highly branched structure containing glucose bounded by α-1, 4-glycosidic bonds, present in human saliva, pancreatic secretion breaks down to produce disaccharides and trisaccharides.[11] α-amylases act randomly than β-amylase. β-amylases are produced by bacteria, fungi and plants. The optimum pH for α-amylase and β-amylases are6.7-7 and 4-5.[12] Amylase enzyme is produced in large quantities by *Bacillus amyloliquifacience, Bacillus substilis, Aspergillus niger, Mucor* sp*, Rhizopus* sp. This extracellular enzyme has large scale applications in the field of textile, cleaning, laundry, brewing and baking industry.[13,14,15]

The major advantage of using microorganisms for production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics.[16] The microbial amylases meet industrial demands; a large number of them are available commercially; and, they have almost completely replaced chemical hydrolysis of starch in starch processing industry.[17] Amylases stand out as a class of enzymes, which are of useful applications in the food, brewing, textile, detergent and pharmaceutical industries. They are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharides mixtures, high fructose syrup and maltotetraose syrup. In detergents production, they are applied to

improve cleaning effect band are also used for starch de-sizing in textiles industry.[18,19]

From the literature and current study carried out Endophytic fungi proves to be an anticancer agent. Secondary metabolites isolated from Aloe vera have been studied extensively for anticancer activity and also for alpha amylase from these fungi. Hence study of enzyme inhibition activity of endophytic fungi and its metabolites responsible for this activity may leads to exploration of this fungus as an alternative source of natural drug.

Further, this study strengthens the concept that if an endophytic fungus from a plant produces antidiabetic and anticancer compounds, exploitation of medicinal plants for the natural medicine can be reduced.[20] The endophytic fungi can be grown and within few weeks one can produce bioactive compounds in industrial scale.

CONCLUSION

Specific activity of Amylase was expressed as the activity of the enzyme (U) per mg protein released. Enzyme yield was expressed as the activity of Amylase. Concentration of amylase was assayed using Lowery *et al*., method. The enzyme in the culture broth was separated and purified by using dialysis membrane.

The present was successfully established the high yield of Amylase with a strain of *Aspergillus* sp. The *in vitro* cytotoxicity test assayed by MTT assay slight to mild activity on MTT-3 blood cancer cell line.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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