



Original Article

Ultrastructural effect on mastitis pathogens by extract of endophytic fungi associated with ethnoveterinary plant, *Hibiscus sabdariffa* L.



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ABSTRACT

Three endophytic fungi isolated from different parts of *Hibiscus sabdariffa* L. were identified using morphological and molecular approaches. Ethanolic extract of endophytic fungi as well as plant extracts were evaluated for *in vitro* antibacterial activity by using well diffusion method and their minimum inhibitory concentration estimated. The culture extract of one endophytic fungus *Glomerella acutata* EF15 was found to be potent antibacterial agent against pathogenic coliform bacteria *Klebsiella pneumoniae* and *Escherichia coli* responsible for causing clinical mastitis. Scanning electron microscopy (SEM) revealed the ultrastructural alteration in the cells of *K. pneumoniae* and *E. coli* when treated with crude ethanolic extract of *G. acutata*. The ethanolic extract of the endophytic fungus revealed potential to be bioprospected as antibacterial agent against pathogens causing coliform mastitis, the existing havoc of dairy industries.

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1. Introduction

Medicinal plants constitute a source of both traditional and modern medicines [1]. About 80% of rural population depends on herbal medicine as their first line curative medicine. There are reports on the antimicrobial activity of the ethnoveterinary plants [2,3] but no reports are available on the antibacterial activity of the fungal endophytes isolated from these ethnoveterinary plants. Rosselle (*Hibiscus sabdariffa* L.; Family Malvaceae) also known as Ranga tengamara in Assamese is mostly used as an ethnoveterinary plant by the local people of Northeast India to treat various ailments of the livestock [4]. The leaf juice is mostly fed to the animal in empty stomach for the treatment of

dysentery. Several reports on the antimicrobial activity of the plant *H. sabdariffa* have also been reported [5,6].

Escherichia coli and *Klebsiella pneumoniae* are characterized under Enterobacteriaceae family that normally inhabit the digestive tract of human and animals but can be transmitted through contaminated food and water and become a cause for enterobacterial infections [7]. The coliform bacteria, *E. coli* and *K. pneumoniae* are also the most common gram-negative causes of clinical mastitis [8–10]. *K. pneumoniae* is the most common *Klebsiella* species found to cause mastitis. *Klebsiella* mastitis causes more losses in livestock industry in comparison to the losses made by *E. coli* mastitis in terms of milk production and survival [11,12]. *E. coli* infections respond well toward the treatment and take a shorter time to recover as compared to *Klebsiella* infections [8,13].

Endophytic fungi are fungi that colonize living, internal tissues of various parts of the plants without harming them [14]. Antimicrobial activity of the natural

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products from plants have been extensively studied against newly emerged antibiotic resistant pathogens in a view that these new inhibitory agents from natural products are effective, have low toxicity and have a low environmental impact [15]. Recently endophytes are viewed as outstanding source of secondary metabolites and bioactive antimicrobial natural products [16]. The aim of the present study was to assess the antibacterial effect of endophytic fungi isolated from the *H. sabdariffa* plant. This study reports the isolation and antimicrobial potential, against mastitis pathogens, of endophytic fungi associated with ethnoveterinary plant *H. sabdariffa* used in treatment of cows, buffaloes and goats. The antimicrobial effect on the ultrastructure of the pathogens was evaluated using scanning electron micrographs.

2. Materials and methods

2.1. Plant collection and isolation of endophytic fungi

Fresh plants (stem, root and leaves) of *H. sabdariffa* L. were collected from Assam, India under sterilized condition in polythene bags. The plant parts were separated and cut into small pieces and washed thoroughly with tap water. They were surface sterilized in 70% ethanol for 3 min, 4% sodium hypochlorite for 1 min and again in 70% ethanol for 3 min and rinsed twice in sterile distilled water. Samples were blotted dry with sterile paper towels, then dried in laminar air flow and placed on potato dextrose agar (PDA) containing 100 µg/ml of streptomycin sulphate to suppress bacterial growth. Plates were incubated at 25 °C until the outgrowth of endophytic fungi from the explants was observed. The outgrowths were subcultured to produce pure culture on PDA plates [17]. All isolates were maintained in PDA slants and kept at 4 °C.

2.2. Morphological features

The endophytic fungal isolates were identified initially by their morphological characteristics such as hyphal feature, arrangement of spores and reproductive structures.

2.3. Molecular characterization of the endophytic fungi

Fungal mycelia grown in potato dextrose broth (PDB) for more than one week were taken and fungal genomic DNA was extracted using the HiPurA fungal DNA isolation kit (Himedia) following manufacturer's instruction. Fungal rDNA-ITS region was amplified from the extracted genomic DNA by using fungal domain specific primer ITS1F and ITS4R [18]. PCR was performed in a GeneAmp 9700 Thermal Cycler (Applied Biosystems, USA) under the following conditions, initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 30 s, and 72 °C for 1 min, with a final extension step of 72 °C for 10 min. The PCR amplicons were excised and purified using a QIA Quick Gel Extraction Kit (Qiagen, Germany). The amplicons were sequenced in Applied Biosystems 3700 Genetic Analyser (Applied Biosystems, USA) with Big Dye Terminator ver. 3.1. The sequences were then compared with the GenBank database by the BLASTN program. Sequences obtained

were then searched for similarity with other deposited sequences in GenBank. Alignments and phylogenetic analysis were performed using MEGA 4.0 software [19].

2.4. Preparation of plant extract

The collected plants were first washed properly with distilled water and then dried in an oven at 50 °C for 72 h with forced air, after which the dried plant were ground into a fine powder using a clean pestle and mortar. Dried sample (approximately 100 g) was soaked in ethanol (300 mL) for 4 days at room temperature (25 ± 2 °C) with an intermittent stirring to allow the powder to fully dissolve in the ethanol. The mixture was then filtered through Whatman No. 1 paper and then the filtrate was concentrated under vacuum using a rotary evaporator. The dry crude extracts were then finally dissolved in dimethylsulfoxide (DMSO) to make a final concentration of 1 mg/ml and then sterilized by filtration using a 0.22 µm membrane for antimicrobial assay [20]. The resultant extract was kept at 4 °C for further analysis.

2.5. Fungal broth fermentation and preparation of culture extract

Endophytic fungi were cultured in potato dextrose broth (PDB) at 25 °C for 4 weeks. The mycelia of fungi were separated from the fermentation broth by filtration. The mycelia filtrates were extracted thrice with an equal volume of 70% ethanol. Ethanol was then evaporated to dryness under reduced pressure at 45 °C using a rotary evaporator to obtain the crude extract [21] of the mycelial product. The dry crude extracts were then finally dissolved in dimethylsulfoxide (DMSO) to make a final concentration of 1 mg/ml of stock solution which was further used for antimicrobial assay.

2.6. Antimicrobial assay using well diffusion method

The crude ethanolic fungal extracts were screened for antimicrobial activity against two pathogenic coliform bacteria *K. pneumoniae* and *E. coli* isolated from bovine mastitis samples which were collected from College of Veterinary Science, Assam. The test organisms were grown overnight at 37 °C in brain heart infusion broth. The antimicrobial assay using the crude extract was carried out on Mueller Hinton agar medium plates. One milliliter of inoculum was swapped on molten agar plates. Using the sterile cork borer 7 mm wells were made in the plates in which 50 µL of fungal extract was loaded. The control well was loaded with DMSO. The plates were incubated for 24 h at 37 °C and then were observed for zone of inhibition [22].

2.7. Determination of minimal inhibitory concentration

Minimum inhibitory concentration of all the fungal extract and plant extract were done by using microtiter broth dilution technique of Keskin et al. [23] with minor modification. A sterile 96 round-bottom well plate was used and was labeled properly. Crude extract of volume 100 µL was added into the first row of the plate.

To rest of the wells, 50 μ L of potato dextrose broth was added and then serial dilutions were performed using a micropipette (A1–A10). Then, 50 μ L of broth containing bacterial suspension (10^6 cfu/mL) was added to each well. Single antimicrobial extract in progressive dilutions was added in each column of wells. Each plate had a set of both growth (A11) and sterility control (A12). Plates were sealed with parafilm and placed in an incubator at 37 °C for 24 h. After incubation, 10 μ L of 0.2% 2,3,5-triphenyl tetrazolium chloride (TTC) solution was added to each well. The plates having TTC were then incubated at 37 °C for 1 h. A visible color changes from purple to pink, indicated growth of microorganism and were recorded as negative. The MIC value of the extract was taken as the lowest concentration that showed no microbial growth.

2.8. Scanning electron microscopic analysis

Mastitis pathogens (*K. pneumoniae* and *E. coli*) having susceptibility to growth in presence of fungal extract were analyzed under scanning electron microscope (JSM-6360, Jeol) for their ultrastructural deformities implicated on the cells. Test organisms were treated with 4 \times concentration of the MIC of the fungal extracts and kept under overnight incubation at 37 °C. Then the cells were pelleted after centrifugation at 8000 rpm for 15 min. The pellets were then washed with PBS pH 7.3 several times to remove the debris and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 4 h. Glutaraldehyde was drained carefully and placed in three consecutive 1 h washes of 0.1 M cacodylate buffer. The samples were dehydrated with acetone series (50%, 70%, 80%, 90%, 95% and 100%) and the drying was done with TMS (trimethyl silane). Cells were immersed in TMS for 10 min in two changes at 4 °C and are brought to room temperature for drying. Finally, samples were sputter coated with a thin layer of gold–palladium and scanned under SEM.

3. Results

3.1. Isolation and identification

A total of eight isolates were isolated from different parts of the plant *H. sabdariffa* L. most being isolated from the leaf segments. The colony morphological trait of the isolate such as hyphal features and arrangement of spores were used for the tentative identification. Molecular characterization was considered for confirmatory identification of the isolate in which rDNA-ITS region was amplified, the sequence data was aligned using BLAST and the endophytic fungi were found to be closest homolog of *Aspergillus niger* (EF13), *Corynespora cassicola* (EF14) and *Glomerella acutata* (EF15). The ITS sequences for the fungi were deposited to NCBI and accessions obtained as KF928291, KF928292 and KF928293 respectively (Fig. 1).

3.2. Antimicrobial screening

The crude ethanolic extract of different endophytic fungi along with the plant extract was evaluated for the antimicrobial efficacy against two clinically significant

Table 1

Antimicrobial activity of endophytic fungal plant and the host plant extract by well diffusion method measured as zone of inhibition (mm).

Fungal isolates and the host plant	Test organisms with growth inhibition zone	
	<i>Klebsiella pneumoniae</i>	<i>Escherichia coli</i>
<i>Aspergillus niger</i> EF 13	++	+
<i>Corynespora cassicola</i> EF14	+	++
<i>Glomerella acutata</i> EF15	+++	+++
<i>Hibiscus subdarifa</i> L.	++	++

+ indicates inhibition zone between 12 and 14 mm; ++ indicates inhibition zone between 14 and 18 mm; +++ indicates inhibition zone between 18 and 22 mm.

Table 2

MIC values of the crude ethanolic extract of endophytic fungi and the host plant.

Fungal isolates and the host plant	MIC (mg/ml)	
	<i>Klebsiella pneumoniae</i>	<i>Escherichia coli</i>
<i>Aspergillus niger</i> EF 13	0.083 \pm 0.021	0.33 \pm 0.08
<i>Corynespora cassicola</i> EF14	0.25 \pm 0.00	0.20 \pm 0.041
<i>Glomerella acutata</i> EF15	0.021 \pm 0.005	0.016 \pm 0.00
<i>Hibiscus subdarifa</i> L.	0.032 \pm 0.00	0.16 \pm 0.041

animal pathogens. Among the eight isolates, only three isolates showed potent antimicrobial activity. Crude ethanolic plant extract also showed the antimicrobial activity. The fungal isolate EF15 showed the highest antimicrobial activity against both the tested animal pathogen followed by plant extract and the fungal isolates EF13 and EF14 (Table 1). The control well with DMSO did not show any zone of inhibition.

3.3. Minimum inhibitory concentration (MIC)

All active extracts showing potent antimicrobial activity were further determined for their MIC by a microtiter broth dilution technique. The ethanolic extract of the fungal isolate EF15 showed MIC of 0.021 \pm 0.005 mg/ml and 0.016 \pm 0.00 mg/ml for *K. pneumoniae* and *E. coli* respectively (Table 2), which showed its efficacy as a potent antimicrobial. The findings of the MIC corroborated with the results obtained for well diffusion assay during primary screening.

3.4. Scanning electron micrographs

The antibacterial effect of the isolate EF15 on *K. pneumoniae* and *E. coli* was prominent on the cellular ultrastructure as revealed by scanning electron micrographs. The treatment of the mastitis pathogens with 4 \times concentrations of the MIC value of the fungal extract of EF15, the cells became sticky and shrunken when compared to control cells (Figs. 4 and 5). The normal pathogenic bacterial cells without any treatment revealed normal rod shape cell structure with smooth and regular surface without any shrinkage or cavity formation (Figs. 2 and 6). The treated bacterial cells showed multiple deformities and cells

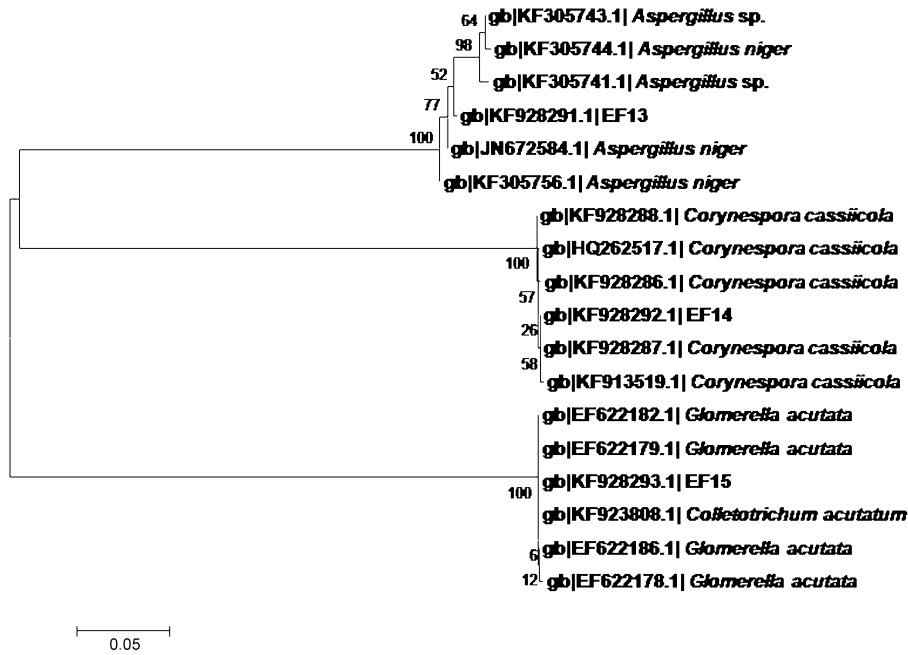


Fig. 1. Evolutionary positions of the three endophytic fungal isolates with other related fungal species based on rRNA-ITS sequence similarity.

exhibited crumpled or shrunken surfaces with large number of cavity formation (Figs. 3, 4, 7–9).

4. Discussion

Drug resistance among microbes has become a global concern and there is an urgent need to develop new antimicrobial agents [20]. Endophytes are known to be a source of bioactive and pharmacologically active metabolites and an increasing number of novel compounds are being isolated from endophytic fungi [24]. The traditional ethno-veterinary medicinal practices are mostly followed by the rural folk for the management of most of the veterinary diseases. Our study on *H. sabdariffa* L. showed the presence of the fungi *A. niger*, *C. cassicola* and *G. acutata*

which are first time report and were not reported from the same plant by Kumaresan et al. [25]. Mastitis is one of the most serious problems creating havoc in dairy industry causing great economic losses in terms of decreased milk production [26]. Coliform bacteria are responsible for many cases of acute clinical mastitis. They also cause sub-clinical infections that persist for longer period of time. Coliform bacteria like *K. pneumoniae*, *E. coli*, *Enterobacter aerogenes* and *Serratia marcescens* are four coliform bacteria that cause mastitis. Among them *K. pneumoniae* and *E. coli* are more common [27]. Research has shown that contaminated bedding is the main cause of udder infection and clinical mastitis. In the present study, the endophytic fungi *G. acutata* EF15 showed potent activity against both the test



Fig. 2. Scanning electron micrograph of normal *Klebsiella pneumoniae* cells showing normal morphology.

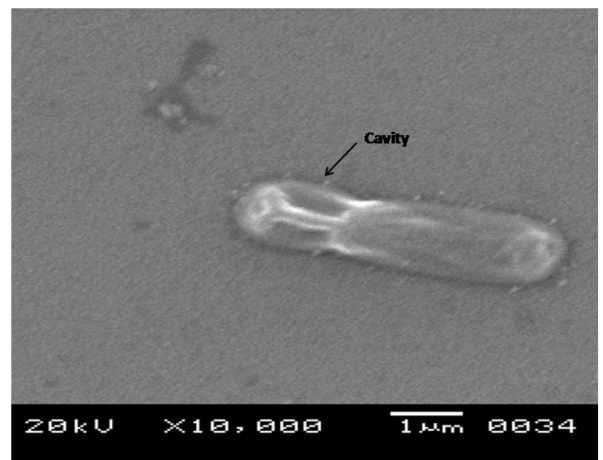


Fig. 3. Cavity formation in *Klebsiella pneumoniae* cell treated with endophytic fungi extract.

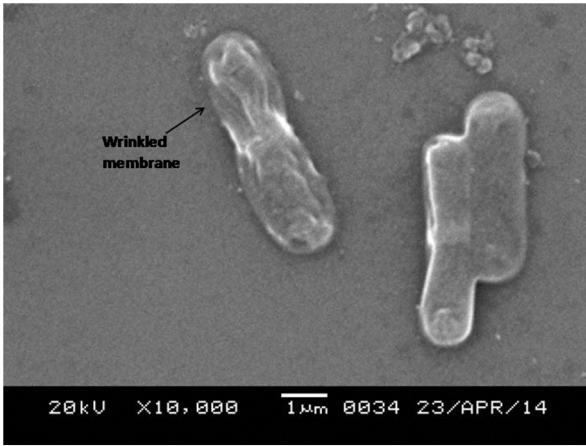


Fig. 4. Micrograph showing shrunken and wrinkled *Klebsiella pneumoniae* bacterial cell.

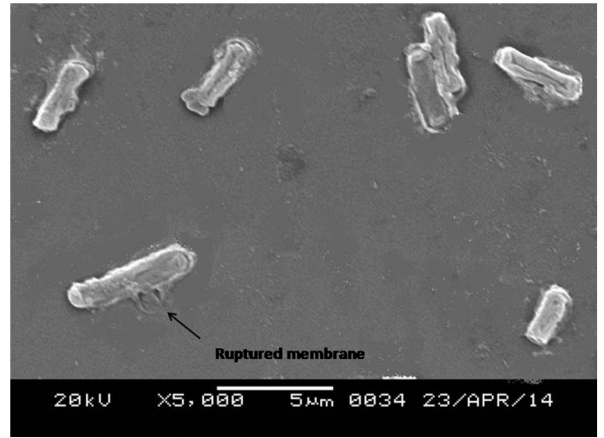


Fig. 7. Micrographs showing rupturing of the cell membrane of *Escherichia coli*.

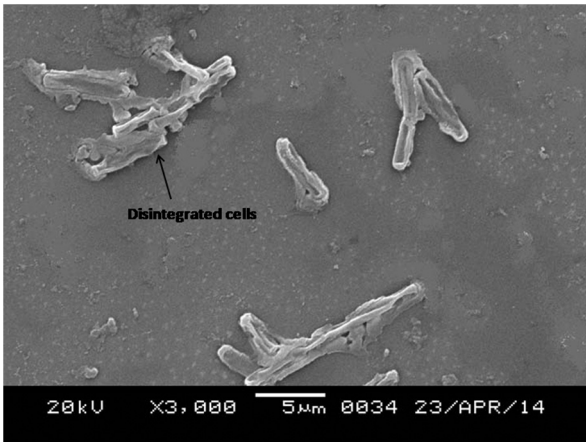


Fig. 5. Collapsed and disintegrated *Klebsiella pneumoniae* bacterial cells losing their normal morphology.

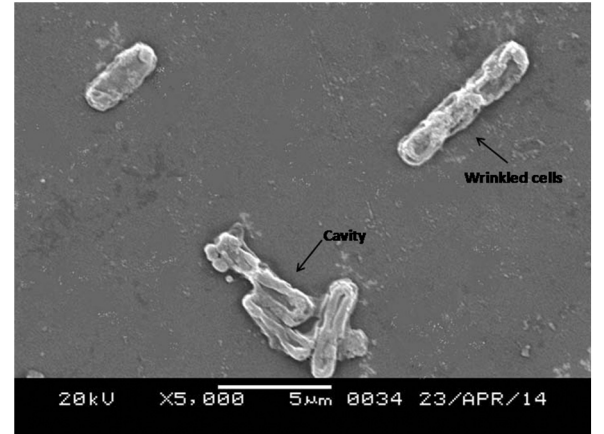


Fig. 8. Cavity formation in the bacterial cells of *Escherichia coli* treated with endophytic fungi extract.

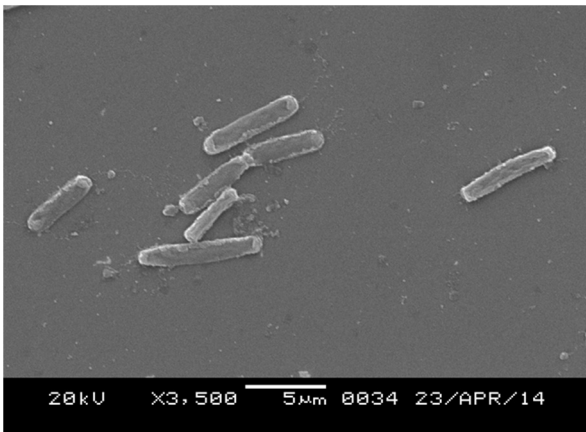


Fig. 6. Scanning electron micrograph of *Escherichia coli* having normal rod shaped cells.

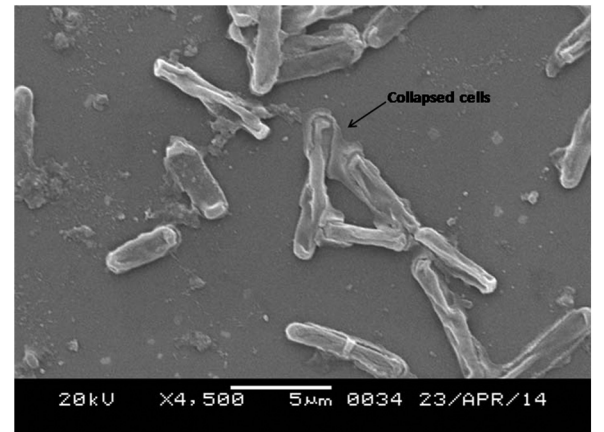


Fig. 9. Deformed *Escherichia coli* bacterial cells losing their normal cell morphology after treatment.

pathogens *K. pneumoniae* and *E. coli* which is in agreement with earlier reports [28]. The study indicated the ethanolic extract of endophytic fungi showing better antimicrobial activity when compared to the ethanolic plant extract of the host plant. One of the mechanisms involved in bacterial cell destruction is the action of antimicrobial agents on the cell membrane of the bacteria [29,30] leading to complete damage of the cells. The scanning electron micrographs clearly indicated that the bacterial cells crumpled, shrunken and cavities formation were prominent on the cell membrane of the bacteria which lead to complete damage of the cells corroborating earlier findings on bacterial cell damage [30]. This finding confirms the potential for investigating endophytic fungi from ethnoveterinary plant, *H. sabdariffa* L. as a potent antimicrobial agent against coliform bacteria causing mastitis.

Conflict of interest

None declared.

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