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Original Article

Investigation of Antimicrobial Activity and Identification of Bioactive Volatile Metabolites of Jute Endophytic Fungus *Aspergillus flavus*

Farhana Tasnim Chowdhury[1], Mrinmoy Sarker[1], Muhammad Saiful Islam[2], Husna Parvin Nur[2], Mohammad Riazul Islam[1], Haseena Khan[1]*

[1]Molecular Biology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka-1000; [2]Fibre and Polymer Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka-1205.

ABSTRACT: Jute, a well-known tropical plant with high fibre quality and medicinal importance, is extensively used in south Asian region. The present study was intended to isolate, identify, and evaluate the biological properties of an endophytic fungus of jute and also to decipher the plethora of volatile organic compounds it produces. The target endophyte *Aspergillus flavus* was identified by morphological analysis and by sequencing the internal transcribed spacer (ITS) region. Both extracellular and intracellular fungal extract exhibited broad spectrum antibacterial activity against six opportunistic human pathogenic bacteria (*Lactococcus lactis* NCTC 497, Bacillus subtilis168, *Staphylococcus aureus* SG511, Staphylococcus carnosusTM300, *Bacillus pseudomycoides* DSM 12442 and Escherichia coli BL21 (DE3)). The ethyl acetate extract displayed greater antimicrobial activity against all the tested pathogens than the methanolic extract. Further, profiling of total volatile organic compounds of the fermented extract using Gas Chromatograph-Mass Spectrometry (GC-MS) confirmed the presence of a variety of bioactive components. The major compounds identified in GC-MS analysis included 4-nitrobenzoic acid, 3-chlorophenyl ester (27.23%) and (+)-salsolidine (21.82%) which are nitrobenzoate and alkaloid class of compounds respectively and are known to have antimicrobial potential. In conclusion, the endophytic A. flavus isolated from jute can be seen as a commercial and potential natural resource with varied therapeutic and biological activities. However, the features of these compounds and the mechanisms of action should be further studied to understand their specific activity.

Key words: Jute, endophyte, *Aspergillus flavus*, antimicrobial activity, GC-MS.

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Corresponding author
Haseena Khan
University of Dhaka
Email: haseena@du.ac.bd

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INTRODUCTION

Jute is well recognized for its golden fibre. Due to its excellent fibre quality, it is popular among farmers of south Asia. Moreover, recent work of lignin reduction in the jute fibre has opened new possibilities for jute in textile industries [1]. Publishing the genome of the extraordinary plant has an enormous impact on the study of jute at a greater detail in molecular level [2].

Over the years, study of jute remained focused only on the plant itself, overlooking the immense potentiality of millions of its benevolent residents, called endophytes. Endophytes essentially comprise of bacteria and fungi that reside within

plant tissues without causing any harm. Endophytes also help plants in growth, reproduction, senescence and other miscellaneous physiological processes [3-5]. Endophytes are believed to contribute to host plant's adaptation to biotic and abiotic stress by increasing their resistance capacity to drought, salinity, low temperature etc. due to their colonization inside the host [4].The mutualistic co-evolution of plants and their endophytes have also created a vast array of complex biological compounds termed secondary metabolites. Many endophytes have been reported to produce compounds having antimicrobial potential, thereby raising the potential of using endophytes as alternative sources for these metabolites [6]. Secondary metabolites such as terpenoids, esters and polyketides are responsible for these antifungal, and antibacterial activities [7]. With increasing occurrences of drug resistance, the most important challenge today is the discovery of novel and effective antimicrobial molecules.

One class of molecules produced by endophytic microorganisms that are of specific interest is low molecular weight volatile organic compounds (VOCs) [8]. These are organic compounds comprising of alcohols, esters, alkanes, alkenes, ketones, acids and hydrocarbons. They are mostly derived from different metabolic pathways. There is increasing evidence that these organic volatile compounds play an important role in the antimicrobial capacity of microorganisms and thus protect the host plant from various pathogens. A number of endophytic fungi that belong to *Muscodor* and *Trichoderma* genera are reported to produce volatile antibiotics [9]. But the overall metabolic profiling of many of these inhabitants are yet to be deciphered by researchers.

It is apparent that endophytic fungi are now the most captivating and promising source of economically and biologically beneficial products or metabolites. So, in this study, we set out to isolate new potent endophytes producing active metabolites which may be beneficial in therapeutic applications. Here, we report strong antimicrobial properties of jute endophyte *Aspergillus flavus* against six opportunistic human pathogens and it was found that it's ethyl acetate extract is more potential than the methanolic extract. Finally, we characterized the volatile profiles released by the isolate using Gas Chromatograph–Mass Spectrometry (GC–MS).

METHODS AND MATERIALS

Isolation of endophytic fungi

The fungal endophyte used in this study was isolated from jute (*Corchorus olitorius*, O-9897) leaves. The jute leaves were collected from mature healthy plants growing in the botanical garden of University of Dhaka. Plant material was washed with tap water initially and then surface sterilized with 0.5 % sodium hypochlorite (2 min) and 70 % v/v ethanol (2 min), respectively. The plant material was cut into 1 cm pieces, placed on potato dextrose agar (PDA) plates and incubated at 28°C for 7 days. After incubation, the fungal hyphae were moved to another PDA plate and continuous subculture was performed to get the pure plates [10]. Pure culture plates were preserved at 4°C and glycerol stock of fungi were stored at -80°C.

Identification of endophytic fungi

The isolated fungi were at first identified by morphological analysis. For molecular identification, total genomic DNA of the fungus was isolated according to the sodium dodecyl sulphate (SDS) method [11]. The fungal ITS, a 5.8S ribosomal gene sequence region was amplified by PCR using universal fungal specific primer ITS1 (5′-TCC GTA GGT GAA CCT GCG-3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC-3′) [12]. PCR conditions used were as follows: denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 40 sec at annealing temperature 58.4°C, and 40 sec at 72°C , with a final extension of 5 min at 72°C. The 15µL reaction mixture contained 1.5µL of 10X PCR buffer, 1.2 µL of 25mM $MgCl_2$, 0.3 µL of 10 mM dNTPs mix, 0.5 µl of 5 U/µLTaq polymerase (Invitrogen, life-technology, USA), 0.5 µL of sample DNA (50 ng/µL), 0.5 µL each primer (10 mM) and nuclease free water to make the final volume. The PCR product was separated by gel electrophoresis and purified by a Gel extraction Kit (Qiagen, USA). The purified products were delivered to 1st BASE, Malaysia for sequencing. The ITS sequences so obtained were aligned by BLASTn tool (http://www.ncbi.nlm.nih.gov/BLAST).

Extraction of fungal crude extracts using different solvents

Culture of *Aspergillus flavus* was grown in 500 mL conical flask containing 200 mL of PDB media for secondary metabolite analysis. Conical flasks containing fungal inoculum were incubated in a shaker incubator at 28°C at 180 rpm for 2 weeks. To determine the ideal solvent for extraction, two different solvents, methanol and

ethyl acetate were used for metabolite extraction. Both fungal mycelium and extracellular media were mixed separately with both the solvents to identify the metabolic source of the bioactive compounds. The fungal mycelia were homogenized and mixed with 100 mL solvent. Another 100 mL of solvent was added to 200 mL extracellular PDB media and both were kept in the shaker incubator for 4 hours at 180 rpm at 28˚C. Then the solvents were separated and vacuum evaporated using a rotary evaporator. Dry weight of the extracts was measured. Dry weight of methanolic intracellular and extracellular extracts was respectively 31.2 mg and 57.5 mg. Dry weight of ethyl acetate intracellular and extracellular extracts was respectively 46.3 mg and 112.7 mg. Methanolic and ethyl acetate extracts were then dissolved in respective HPLC grade solvents in room temperature and were further assayed for antibacterial activity.

Determination of the antibacterial activity of the crude extracts

Antimicrobial activities of the crude extracts were done by the agar well diffusion method [13]. 100 µL of cell suspension (≈10^8 cell) of each indicator strains (*Bacillus subtilis*168, *Escherichia coli* BL21 (DE3), *Lactococcus lactis* NCTC 497, *Staphylococcus aureus* SG511, *Staphylococcus carnosus* TM300 and *Staphylococcus simulans 22*) were spread over the Tryptic Soy Agar (TSA) surface of the plates. Spaced wells of 6 mm diameter were made in the agar. 50 µL of 10 mg/mL concentrated crude extracts were poured into the wells. Ampicillin (0.2 mg/mL) and kanamycin (0.2 mg/mL) were used as positive controls. Methanol and ethyl acetate were used as blank and ddH_2O was used as negative control. Plates were incubated at 37˚C for 24 hours. Experiments were repeated three times for statistical significance.

Complete volatome analysis

Ethyl acetate extract of extracellular components showed the highest antimicrobial activity and for this reason extracellular ethyl acetate extract was subjected to GC-MS analysis using Perkin Elmer Clarus@6890 gas chromatograph with a Perkin Elmer Clarus@SQ8C mass detector connected with a capillary column Perkin Elmer, Elite-5 MS (60 m ×0.25 mm, film thickness 0.25 µm). 1 μL of the sample with a split ratio of 20:1 was injected. Helium was used as the carrier gas and the flow rate was 1 mL/min. The oven temperature was set at 80°C (held for 3 min), raised at 3°C per min to 272°C (held for 10 min).The analysis was carried out in the EI (electron impact) mode with 70 eV of ionization energy. The compounds were detected after analyzing the mass spectrum of each component using the NIST98 library.

RESULTS AND DISCUSSION

The most vital asset of endophytic fungi is their potential to produce a huge variety of bioactive molecules [6, 14, 15]. Natural products produced by endophytic fungi have been reported to have antimicrobial properties against various opportunistic human pathogens [16, 17]. The characterization of endophytic microorganisms residing within plants is essential for the search of new potential therapeutics. Moreover, the therapeutic potential of a host plant can be responsible for the capability of its endophytes to produce biologically active compounds through the activation of different genes due to the influence of the surrounding environment [18]. Till date various plants have been reported for the presence of endophytic fungi capable of producing metabolites with antimicrobial potential. For example, extracts of fungal endophytes isolated from *Dioscorea zingiberensis* were previously studied and found to have huge antimicrobial activity [19]. Endophytes isolated from different aerial tissues of *Argyrosomus argentatus* have also been reported for their potent antibacterial activity [20]. Similarly, almost 25 fungal isolates were identified from *Coffea arabica* and *Coffea robusta* with varying antimicrobial activities [16, 21].

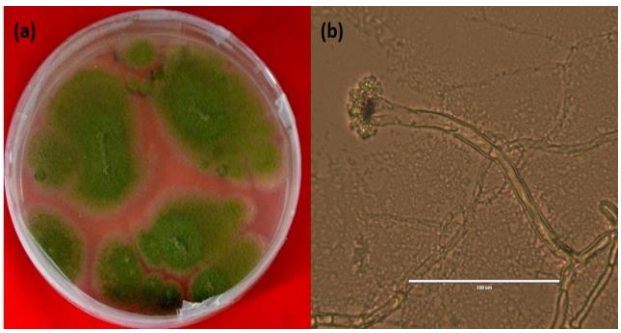

Figure 1: (a) *Aspergillus flavus* on PDA plate; (b) Microscopic photograph of *Aspergillus flavus*

The endophytic fungus isolated in our study from the leaves of a jute plant elicits no symptoms of infection hence establishing it as an endophyte. The isolated strain was identified and its bioactive properties as well as secondary metabolites were characterized. Molecular identification of the isolate was done by performing ITS region sequencing followed by BLASTn analysis. It showed 100% similarity with *Aspergillus flavus* and macroscopic as well as microscopic analysis

also confirmed this fungus to be *Aspergillus flavus*. The *A. flavus* was morphologically identified based on parrot green colonies with small conidia, globose to sub-globose vesicles (Figure 1). The fungus was cultivated in conical flasks for the production and collection of secondary metabolites to analyze their antimicrobial potential and total volatome. The amount of total extract was determined in both methanolic solvent and ethyl acetate solvent. They were calculated to be 212.8 mg and 150.3 mg respectively.

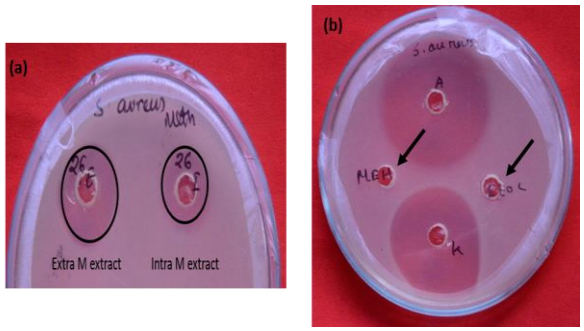

Figure 2: (a) Antimicrobial activity of Extracellular and intracellular methanolic extracts of *Aspergillus flavus* against *Staphylococcus aureus* SG511, indicated by black circles; (b) No activity was found for both blanks methanol and ethyl acetate, indicated by black arrows and positive control ampicillin (A) and kanamycin (K) showed prominent zones of inhibition

The antimicrobial activity of the extracts was assessed by agar well diffusion method against six common opportunistic bacteria (Table 1 and Figure 2). The fungal extracts showed antibacterial activities with the zones of inhibition ranging between 7.2 and 25.4 mm which are highly significant when compared with controls. Extracellular ethyl acetate extract showed maximum activity against the pathogens. This could be possibly explained as endophytes compete to thrive in either in tissue spaces or in natural habitats they tend to produce bioactive molecules to ensure their survival amidst of other microorganisms. Most of these substances are essentially needed to be of secretary types and released extracellularly by microbes. Hence, it can be assumed that the extracellular components will unambiguously have more antimicrobial agents compared to that of intracellular metabolites which coincided with our obtained results. This implies the fungus could be a potential source of natural bioactive compounds. Moreover, the production of bioactive compounds using microbial source is easier and cost effective, facilitating accessibility and decrease in the price of the product [16].

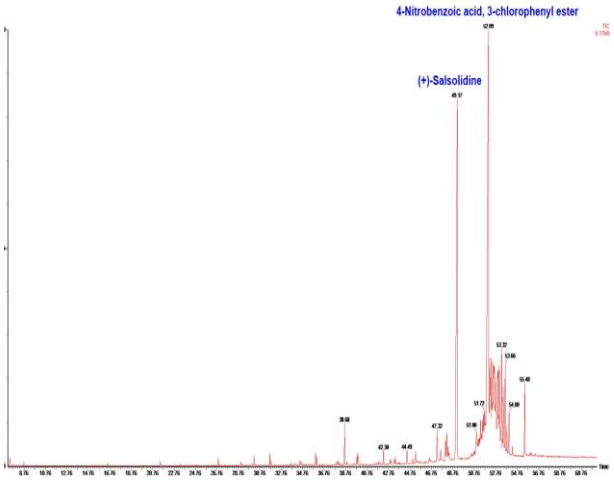

Figure 3: Gas Chromatograph-Mass Spectrometry of crude extracellular ethyl acetate extract of *A. flavus*

Having established the strong antimicrobial activity of the extracellular fungal extract, further chromatographic and spectrometric experiments were carried out to identify and quantify the corresponding bioactive metabolites. GC-MS results of our strain showed the presence of various metabolites. A total of 17 major components which were found to be present in more than 1% abundance in the extract were analyzed using NIST (National Institute of Standards and Technology) library (Figure 3). Their molecular weight, abundance and CAS (Chemical Abstracts Service) no. are shown in

Table 1: Antibacterial activity of different solvent extracts of *A. flavus*

Test organisms	Zone of inhibition in diameter (mm) (mean±SD)			
	Ethyl acetate extract		Methanolic extract	
	Intracellular extract	Extracellular extract	Intracellular extract	Extracellular extract
Bacillus subtilis 168	13.4±0.4	15.7±0.3	9.4±0.1	12.4±0.7
Escherichia coli BL21 (DE3)	14.3±0.3	15.0±0.4	7.2±0.2	11.7±0.9
Lactococcus lactis NCTC 497	17.9±0.9	23.5±0.5	14.7±0.3	21.3±0.1
Staphylococcus aureus SG511	15.1±0.2	25.4±0.2	12.3±.04	17.1±0.1
Staphylococcus carnosus TM300	15.8±0.3	22.4±0.4	12.2±02	19.6±0.6
Staphylococcus simulans 22	18.6±0.6	21.8±0.1	13.1±0.6	15.5±0.5

Table 2: Volatile collection of the ethyl acetate extract of extracellular components of endophyte, *A. flavus* isolated from jute

Peak no.	Retention time (min)	Compound name	Percentage (%)	Formula	Molecular weight(M.W)	CAS no.
1	47.32	Methyl-8-methyl decanoate	1.12	$C_{12}H_{24}O_2$	200	900336-49-1
2	49.17	(+)-Salsolidine	21.82	$C_{12}H_{17}O_2N$	207	54193-08-7
3	51.00	Isoquinoline, 1,2,3,4-tetrahydro-6,7-dimethoxy-1-methyl-	1.04	$C_{12}H_{17}O_2N$	207	5784-74-7
4	51.34	Trans-2-methyl-4-N-pentylthiane, S, S-dioxide	1.69	$C_{11}H_2O_2S$	218	900215-75-3
5	51.55	8-Heptadecene	1.13	$C_{17}H_{34}$	238	2579-04-6
6	51.63	1-Hexadecanol	0.99	$C_{16}H_{34}O$	242	36653-82-4
7	51.74	Ethyl 4,8,12-trimethyl-tridecanoate	1.49	$C_{18}H_{36}O_2$	284	900336-61-3
8	52.09	4-Nitrobenzoic acid, 3-chlorophenyl ester	27.23	$C_{13}H_8O_4NCl$	277	900325-68-6
9	52.21	Benzoic acid, 2,6-dihydroxy-4-methyl-, octyl ester	4.48	$C_{16}H_{24}O_4$	280	900397-17-0
10	52.37	Heptadecanoic acid, methyl ester	2.83	$C_{18}H_{36}O_2$	284	1731-92-6
11	52.56	Heneicosane	5.43	$C_{21}H_{44}$	296	629-94-7
12	52.75	1H-Pyrazole-4-sulfonamide,N-(2,3-dihydro-1,4-benzodioxin-6-yl)-1-	2.44	$C_{12}H_{13}O_4N_3S$	295	900319-53-3
13	52.94	Heptadecanoic acid, 16-methyl-, methyl ester	3.06	$C_{19}H_{38}O_2$	298	5129-61-3
14	53.10	Hexadecanoic acid, 2-methoxy-, methyl ester	2.23	$C_{18}H_{36}O_3$	300	16725-36-3
15	53.32	4-Nitrobenzoic acid, 6-ethyl-3-octyl ester	2.86	$C_{17}H_{25}O_4N$	307	900282-70-7
16	53.46	Docosanal	1.98	$C_{22}H_{44}O$	324	57402-36-5
17	53.66	Erucic acid	1.88	$C_{22}H_{42}O_2$	338	112-86-7

Table 2. They essentially, comprise of hydrocarbons, alcohols, esters, fatty acids, alkanes, alkenes, aromatic compounds, heterocyclic compounds and various other organic compounds. 4-Nitrobenzoic acid, 3-chlorophenyl ester and (+) salsolidine were the dominant ones, making up 27.23% and 21.82 %, respectively, of the VOCs identified in this study. These compounds have also been reported to have potent antimicrobial capacity which may have resulted in positive antimicrobial activity [22-26]. Major and most vital groups of industrially important chemicals extensively used now a days are nitroaromatic compounds like nitrobenzoates, nitrotoluene, nitrophenol etc. which have great antimicrobial potential. They are mostly chemically synthesized. However, numerous naturally produced nitroaromatic compounds and its derivatives from microorganisms have been identified and found to have bioactivity [27]. For example, chloramphenicol, a well-known nitroaromatic antibiotic is produced by the bacterium *Streptomyces venezuelae* [28]. Nitrobenzene and their derivatives are known as initial substances in the production of a huge range of antimicrobials. Derivative of nitrobenzenes, especially different esters of nitrobenzoates known to possess bioactive properties which makes them potential candidates for pharmaceutical use as well as agrochemicals [27, 29]. The derivative of nitrobenzote, 4-Nitrobenzoic acid, 3-chlorophenyl ester which is found in the highest amount in our extract can be a possible candidate responsible for the antimicrobial activity exhibited by the fungus. The alkaloid salsoline, derivative of methyltetrahydroquinoline was also identified in the fungal extract. Different isoquinoline alkaloids and their derivatives have been established to have antibacterial and cytotoxic effect. Numerous studies have also ascribed a very high antimicrobial activity to this specific compound [23, 30]. Thus, the high level of salsolidine (21.82%) in ethyl acetate extract of extracellular component of *A. flavus* may justify the

antimicrobial activity of this fungal isolate against all the six pathogens tested.

The outcomes of our experiments, propose that endophytic fungus *A. flavus* could be a potential source of bioactive secondary metabolites and this is the first report of jute endofungus, *A. flavus* exhibiting antimicrobial activity. This evidently ensures that endophytes harbor a natural capacity to produce bioactive compounds.

CONCLUSION

The present study leads to the conclusion that the fungal endophyte, *A. flavus* isolated from the leaves of a jute plant has the ability to produce bioactive volatile compounds, which are known to possess antimicrobial potential. A total of 17 major volatile compounds were identified by GC-MS. This fungus also showed significant antimicrobial activity against opportunistic human pathogenic bacterial strains, representing a potential alternative source for the production of the antimicrobial products, which can have therapeutic potential. The production of biologically active volatile compounds by endophytic fungus is of huge industrial and academic significance. Moreover, with the emergence of an increasing number of multidrug resistant bacteria, identification of new antimicrobial compounds from natural sources creates immense opportunities of therapeutics.

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Compliance with Ethical Standards

Conflict of interest Authors declared no conflict of interest.

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