

RESEARCH ARTICLE

Determination and production of antimicrobial compounds by *Aspergillus clavatonanicus* strain MJ31, an endophytic fungus from *Mirabilis jalapa* L. using UPLC-ESI-MS/MS and TD-GC-MS analysis

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Abstract

Endophytic fungi associated with medicinal plants are reported as potent producers of diverse classes of secondary metabolites. In the present study, an endophytic fungi, *Aspergillus clavatonanicus* strain MJ31, exhibiting significant antimicrobial activity was isolated from roots of *Mirabilis jalapa* L., was identified by sequencing three nuclear genes i.e. internal transcribed spacers ribosomal RNA (ITS rRNA), 28S ribosomal RNA (28S rRNA) and translation elongation factor 1- alpha (EF 1 α). Ethyl acetate extract of strain MJ31 displayed significant antimicrobial potential against *Bacillus subtilis*, followed by *Micrococcus luteus* and *Staphylococcus aureus* with minimum inhibitory concentrations (MIC) of 0.078, 0.156 and 0.312 mg/ml respectively. In addition, the strain was evaluated for its ability to synthesize bioactive compounds by the amplification of polyketide synthase (PKS) and non ribosomal peptide synthetase (NRPS) genes. Further, seven antibiotics (miconazole, ketoconazole, fluconazole, ampicillin, streptomycin, chloramphenicol, and rifampicin) were detected and quantified using UPLC-ESI-MS/MS. Additionally, thermal desorption-gas chromatography mass spectrometry (TD-GC-MS) analysis of strain MJ31 showed the presence of 28 volatile compounds. This is the first report on *A. clavatonanicus* as an endophyte obtained from *M. jalapa*. We conclude that *A. clavatonanicus* strain MJ31 has prolific antimicrobial potential against both plant and human pathogens and can be exploited for the discovery of new antimicrobial compounds and could be an alternate source for the production of secondary metabolites.

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Introduction

Escalating risk of drug resistance by pathogenic microorganisms to available commercial drugs has become a global concern around the world [1, 2]. Inappropriate use of antibiotics, poor hygienic conditions and delay in diagnosis of the disease are among some of the important factors that favored these circumstances. Methicillin resistant *Staphylococcus aureus*, penicillin resistant *Streptococcus pneumoniae* and vancomycin resistant *Enterococcus faecium* are few such examples [3]. This has led to the investigation, to look for an alternative source of new and efficient antimicrobial agent having broad range of antimicrobial activity. Investigating new and specialized ecological niches and habitats may enhance the chances of finding novel bioactive compounds [4]. One such habitat is plant endosphere that harbors microorganisms which reside in inter or intracellular spaces without causing apparent symptoms [5–7]. These organisms with endophytic lifestyle appeared to be associated with plants in all ecosystems and believed to play diverse indispensable functions in their natural habitat [8, 9].

Endophytic fungi are the most frequently encountered endophytes that form a multifarious group of microbes and have a potential to synthesize several bioactive compounds [10–12]. They are considered as a reservoir of new bioactive compounds, with reported antimicrobial, anticancer, antioxidant, insecticidal, antiparasitic, antiviral, antitubercular and immunomodulatory activities having wide scope in pharmaceutical and agrochemical industries [13, 14].

Many antimicrobial compounds such as phenone, trichodermin, cryptocin, altenuin, dihydroxycadalene, ambuic acid and nodulosporins from fungal endophytes have been reported to protect plants against phytopathogens [3, 15]. Occasionally, endophytic fungi also produce host plant secondary metabolites having therapeutic potential like paclitaxel, camptothecin, podophyllotoxin, hypericin and azadirachtin [12, 16].

The bioactive compounds produced by endophytic fungi belong to diverse structural groups such as alkaloids, terpenoids, polyketides, steroids, quinones, phenols, coumarins and peptides originate from different biosynthetic pathways [15]. Bioinformatics analysis has predicted presence of two highly conserved genes polyketide synthases (PKS) and non ribosomal peptide synthases (NRPS) in genome of endophytic fungi which are involved in biosynthesis of various secondary metabolites [17]. Polyketides are structurally diverse group of compounds with application in human and veterinary medicine including antibiotics erythromycin and tetracycline, antiparasitic compound avermectin and anti tumour compound daunorubicin [18].

Many well known volatile organic compounds (VOCs) of microbiological origin exhibit biological activity. Fungi are known to produce a large number of VOCs produced by fungi have received limited attention in terms of their relationship to plant pathogenesis or growth promotion. Fungi emit cocktails of dozens to hundreds of unique VOCs that fall into many chemical classes including alcohols, aldehydes, acids, ethers, esters, ketones, hydrocarbons, terpenes and sulfur compounds [19]. Therefore, an investigation of the VOCs produced by fungal species provides fertile ground for developing a new understanding of the mechanisms involved in both the triggering of the mycoparasitism response and of the phenomenon of plant growth promotion. Since the fungal strains under investigation possess significant antimicrobial property and growth promotion, this research has significant implications for exploiting VOCs as a info chemical for crop protection.

Mirabilis jalapa (Nyctaginaceae) commonly known as a four o'clock plant and a Marvel of Peru, is an ornamental flowering plant. The plant is been used as traditional medicine throughout the world for the treatment of various diseases and disorders including sexually transmitted diseases, urinary and kidney infections, acute arthritis, anesthesia, inflammation, irregular menstruation and cancerous growths [20–24]. The phytochemical constituents and medicinal property of this plant are well characterized and rich in many active compounds

including triterpenes, flavonoids, alkaloids, and steroids [20, 24]. For example, an antiviral ribosome-inactivating protein (RIP) and rotenoids showed an effective inhibition of HIV-1 reverse transcriptase activity isolated from *Mirabilis jalapa* [22, 25]. Considering the medicinal attributes of *M. jalapa*, the present study was designed to explore the antimicrobial potential of endophytic fungi associated with the plant for their ability to produce bioactive substances endowed with antimicrobial potential.

Materials and methods

Collection of plant samples

Healthy plant (*Mirabilis jalapa* L.) was collected from Dampa Tiger Reserve Forest [DTRF] (23° 44' N 92° 39' E), Mizoram, Northeast India during February, 2014. The cut ends were sealed with wax and were brought to the laboratory. The samples were processed for isolation of endophytic fungi within 12 hours of collection.

Isolation of endophytic fungi

Surface sterilization of the tissues was done by following the protocol of Cannon and Simmons [26]. Sterilized tissue fragments were incubated on three nutritional media [Potato Dextrose Agar (PDA), Malt Yeast Extract Agar (MYE) and Czapek Dox Agar (CDA)] supplemented with streptomycin sulphate (60 $\hat{1}$ / $\hat{4}$ g/mL) and chloramphenicol (60 $\hat{1}$ / $\hat{4}$ g/mL) to suppress bacterial growth. Efficacy of surface sterilization was tested by tissues fingerprinting method; briefly surface sterilized tissue fragments were imprinted on same set of nutritional media to monitor any fungal growth [27, 28]. Alternatively, aliquot from last wash was spread on PDA plate and growth was monitored to cross check the epiphytic microbial growth [29]. All plates were incubated at $26 \pm 2^\circ\text{C}$ for 5 $\hat{1}$ 7 days under 12 h white lights: 12 h dark cycles [30]. Petri plates were monitored every day upto 3 $\hat{1}$ 4 weeks for any hyphae emerging from the tissues. The hyphal tips coming out from the sterile tissues were sub cultured on to a fresh nutritional media to obtain a pure culture for identification and enumeration. Obtained cultures were preserved in 30% glycerol at -80°C .

Morphological and microscopic identification of endophytic isolates

The recovered pure isolates were identified according to their micro and macroscopic structures. The isolates were identified upto genus level using the keys given in standard manuals [31 $\hat{1}$ 34]. All identified isolates were designated with a unique code and were maintained by repeated sub-culturing on respective nutritional media.

Dual culture antagonistic bioassay

Antagonistic potential of the obtained isolates was performed by using dual culture technique against five fungal plant pathogens [*Fusarium oxysporum* f. sp. *ciceri* (NBAIMCC-F-02211), *Fusarium oxysporum* f. sp. *pisi* (MTCC-2480), *Fusarium culmorum* (MTCC-2090), *Fusarium graminearum* (MTCC-1893), *Aspergillus flavus* (MTCC-9064)] and one dermatophyte [*Trichophyton mentagrophytes* (MTCC-8476)] [35]. Five mm diameter mycelial disc of actively grown pathogen and tested endophytic isolate were inoculated on petriplates containing PDA in opposite direction at equal distance from the periphery. The petriplates inoculated with mycelial plugs of pathogen alone was used as a control. Plates were incubated at $26 \pm 2^\circ\text{C}$ for six days and the percent of inhibition (PI %) was calculated and expressed as $\text{PI} \% = \{(C \hat{1} T)/C\} \times 100$, where, C is the growth of the pathogen and T is the growth of pathogen in presence of endophytic isolate.

Genomic DNA extraction and identification using nuclear genes (ITS rRNA, 28S rRNA and EF 1 α)

Aspergillus clavatonanicus strain MJ31, exhibiting significant antimicrobial activity was isolated from roots of *Mirabilis jalapa* L., was identified by sequencing three nuclear genes i.e. internal transcribed spacers ribosomal RNA (ITS rRNA), 28S ribosomal RNA (28S rRNA) and translation elongation factor 1- alpha (EF 1 α). The total genomic DNA was isolated as described by Ceniz [36]. ITS rRNA gene was amplified using universal primers ITS1 (5'-TCCGTAGGTG AACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as per White et al. [37]. Whereas the primers LROR (5'-ACCCGCTGAACTTA AGC -3') and LR7 (5'-TACTACCACCAAGATCT-3') were used to amplify partial 28S rRNA gene, similarly the primers EF1-728F (5'-CATCG AGAAGTTCGAGAAGG-3') and EF1-986R (5'- TACTTGAAGGAACCCTTACC-3') were used to amplify a part of translation elongation factor 1 α [38, 39]. A negative reaction without DNA template was used in every batch of PCR. The amplified PCR products (2 μ l) were visualized on 1.5% (w/v) agarose gel prepared in 1X TBE buffer by using gel documentation system (Bio-Rad Gel Doc XR+ gel documentation system, California, USA). The amplicons were purified by using HiPurA PCR product purification kit (HiMedia, India) according to manufacturer's instructions. Sequencing was done commercially at Sci Genome Pvt. Ltd. Kochin, India.

The obtained sequences were subjected to BLAST analysis with the deposited sequences in the NCBI database to find the homology with the closest related organisms. The maximum likelihood phylogenetic trees for ITS rRNA gene, 28S rRNA gene and translational elongation factor 1 α gene were constructed using MEGA 6.0 to estimate the taxonomic placement of MJ31 [40]. The reference sequences with highest scores from BLASTn result were retrieved from NCBI database and multiple sequence alignment was performed using the Clustal W software packaged in MEGA 6.0 [40, 41]. The evolutionary model was selected based on highest AIC (Akaike Information Criterion) values and lowest BIC scores (Bayesian Information Criterion). The significance of the branching order was determined by bootstrap analysis of 1000 replicates.

Preparation of fungal extract of strain MJ31

The endophytic fungal strain MJ31 which showed the highest percentage of inhibition was selected for the preparation of fungal extract by small scale fermentation. Five mycelial agar plug of grown strain was inoculated in 2 L Erlenmeyer flasks containing 700 ml potato dextrose broth (PDB) media and incubated at 26 \pm 2°C for three weeks. The culture broth was separated from mycelial mat by filtration using whatman no.1 filter paper. Both the culture filtrate and mycelial mat were separately extracted thrice by using ethyl acetate. The filtrate was evaporated to dryness in a rotary evaporatory system (BUCHI, Switzerland) to obtain a crude extract.

Poisoned food bioassay of endophytic fungal strain MJ31

Antifungal bioassay of crude ethyl acetate extract of MJ31 was evaluated against same set of filamentous fungal pathogens used earlier in dual culture antagonistic assay by biometric agar dilution method [42, 43].

A stock solution of 50mg/ml was prepared by dissolving the crude extract in ethyl acetate. Different concentrations of the extract (1000 μ g/ml, 500 μ g/ml and 250 μ g/ml) were added into the autoclaved PDA media. The pathogenic fungi were inoculated at the centre of

the plates containing different concentrations of extracts and control plate was used without any with the used solvent alone. The percent of inhibition (PI %) was calculated as $PI\% = \{(C - T)/C\} \times 100$, where PI is inhibition of radial mycelial growth; C is radial growth measurement of the pathogen in control; T is radial growth of the pathogen in the presence of fungal isolates. IC_{50} was calculated by regression equation analysis using PI% at different concentrations ($\hat{1}/4$ g/ml) by Graph Pad Prism software version 5.03.

Antibacterial and anti-yeast potential of endophytic fungal strain MJ31

Test microorganisms. The test microorganisms used in antibacterial and anti yeast assay were obtained from Microbial Type Culture collection (MTCC), Chandigarh, India. Ethyl acetate extract of strain MJ31 was tested against a yeast (*Candida albicans*, MTCC-3017), three Gram positive bacteria (*Bacillus subtilis*, MTCC- 2097; *Staphylococcus aureus*, MTCC-96; *Micrococcus luteus*, NCIM-2097) and two Gram negative bacteria (*Pseudomonas aeruginosa*, MTCC-2453; *Escherichia coli*, MTCC-739). The bacterial pathogens were cultured in nutrient agar (NA) and maintained at 37°C whereas the yeast pathogens was cultured in saboraud dextrose agar (SDA) media and was maintained at $26 \pm 2^\circ\text{C}$.

Evaluation of antibacterial and anti yeast activity by disc diffusion assay. All bacterial and yeast pathogens were inoculated in Mullerâ Hinton broth and the suspension was adjusted to 1.5×10^8 colony forming units (CFU) (0.5 Mcfarland scale) and finally diluted to 1×10^8 CFU for bacterial cells and 1×10^6 for yeast cells [44, 45]. The antimicrobial activities of endophytic fungi were evaluated using paper disc diffusion assay [46â 48]. The culture suspensions were evenly spread out on Mullerâ Hinton agar for bacterial and yeast pathogens with help of sterile L-shaped spreaders. Eight standard antibiotic discs [ampicillin (10 $\hat{1}/4$ g), streptomycin (10 $\hat{1}/4$ g), erythromycin (10 $\hat{1}/4$ g), gentamycin (10 $\hat{1}/4$ g), clindamycin (10 $\hat{1}/4$ g), nalidixic acid (30 $\hat{1}/4$ g) chloramphenicol (10 $\hat{1}/4$ g) and vancomycin (10 $\hat{1}/4$ g)] were placed on the periphery of each plate containing bacterial suspension at equal distance from each other whereas disc containing ethyl acetate extract of strain MJ31 was placed at the centre. For plate containing yeast suspension, six standard antibiotic discs [itraconazole (10 $\hat{1}/4$ g), voriconazole (1 $\hat{1}/4$ g), miconazole (30 $\hat{1}/4$ g), ketokonazole (50 $\hat{1}/4$ g), fluconazole (25 $\hat{1}/4$ g) and nystatin (50 $\hat{1}/4$ g)] were placed at the periphery of the plate and ethyl acetate extract of strain MJ31 disc was placed at the centre. Crude ethyl acetate extract of MJ31 was tested at a concentration of 5 mg/ml per disc along with standard antibiotic discs as positive control. The plates were incubated at 37°C for 24 h and 48 h for bacterial and yeast pathogens respectively. The diameters of inhibition zones were measured and the mean of triplicate readings were recorded.

Estimation of minimum inhibitory concentration (MIC) and minimum microbicidal concentrations (MMC). Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MBC) of crude ethyl acetate extract of strain MJ31 was determined against tested bacterial and yeast pathogen by using broth micro dilution method [45]. Chloramphenicol and Nystatin were used as positive control. Uninoculated broth was kept for sterility checks whereas wells with only fungal extracts served as negative control. The plates were incubated at 37°C for 24 h and 28°C for 48 h for bacterial and fungal pathogens respectively. Antimicrobial activity was determined by adding 10 $\hat{1}/4$ l of 0.5% aqueous solution of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] as microbial growth indicator and incubated for additional 30 mins. MIC was observed as the concentration of extract where no visible growth occurred, as indicated by the MTT staining. 50 $\hat{1}/4$ l of culture broth was transferred on the Muller-Hinton agar plates and incubated in the same conditions as mentioned above. The complete absence of growth on the agar surface with the concentration of extract is defined as minimum microbicidal concentration (MMC).

PCR amplification and sequencing of antimicrobial biosynthetic genes (PKS and NRPS)

Strain MJ31 was subjected to amplification of polyketide synthase (PKSI) gene and nonribosomal peptide synthetase (NRPS) gene to evaluate the ability to produce polyketides and oligomeric peptides. Ketosynthase domain (KS) of PKSI (polyketide synthase I) gene was amplified using 3 sets of degenerate primers, LC1 (GAY CCI MGI TTY TTY AAY ATG) and LC2c (GTI CCI GTI CCR TGC ATY TC), LC3 (GCI GAR CAR ATG GAY CCI CA) and LC5c (GTI GAI GTI GCR TGI GCY TC) [49], KS3 (TTY GAY GCI GCI TTY TTY AA) and KS4c (RTG RTT IGG CAT IGT IAT ICC) [50]. The PCR reaction was performed in a total volume of 25 μ l containing 100 ng of template DNA, 1X PCR buffer, 4mM MgCl₂, 0.2 mM dNTPs, 10 pmole of each primer and 2U of *Taq* polymerase. PCR amplification was performed in a thermal cycler (Applied Biosystems, Singapore). The thermal cycler conditions were as follows: initial denaturation at 94°C for 5 min and 35 cycles of denaturation at 94°C for 1 min., primer annealing for 1 min 20 sec at 55°C for LC1/2 and LC3/5 primers and 1 min 20 sec at 50.5°C for KS3/4 primers and extension at 72°C for 3 min followed by final extension at 72°C for 10 min.

NRPS gene was amplified using degenerate primers RJ016-F (TAYGGNCCNACNGA) and RJ016-R (ARRTCNCCNGTYTTRTA) [51]. The amplification was carried out in a final volume of 25 μ l containing 100 ng of template DNA, 1X PCR buffer, 4mM MgCl₂, 0.2 mM dNTPs, 10 pmole of each primer and 2U of *Taq* DNA polymerase. PCR condition involved initial denaturation at 94°C for 5 min., followed by 35 cycles of denaturation at 94°C for 1 min., annealing of primers at 51°C for 1 min., extension at 72°C for 1 min. and final extension at 72°C for 10 min. A negative control of PCR reaction mixture without DNA template of strain MJ31 was also included with each set of primer. The amplified bands were visualized on 1.5% agarose gel, purified and were sequenced commercially at Sci-Genome, Cochin, India. The sequences were identified using BLASTx analysis (<https://blast.ncbi.nlm.nih.gov/blast/Blast.cgi>) and deposited in NCBI GenBank.

Quantitative determination of standard antibiotics

Preparation of standard solution. A mixed standard stock solution containing antibiotics streptomycin, ampicillin, chloramphenicol, rifampicin, fluconazole, ketoconazole and miconazole was prepared in methanol. The working standard solution (0.5 μ g/1500 ng/ml) was prepared by diluting stock solution with methanol for plotting calibration curve. Both the mixed standard stock and working solutions were stored at -20°C until use and vortexed preceding injection. The internal standards curcumin and palmatine were used as internal standards for negative and positive mode respectively and were spiked to each concentration at a final concentration of 50 ng/mL (i.e. 50 μ l of internal standards mixture of 1000 ng/mL of palmatine and curcumin in methanol).

Instrumentation and analytical conditions. Quantitative analysis of antibiotics was carried out by an Acquity ultra-performance liquid chromatography (UPLCTM) system coupled with an auto sampler and a binary pump (Waters, Milford, MA). Compounds were separated using an Acquity BEH C18 (2.1 mm \times 50 mm, 1.7 μ m; Waters, Milford, MA) analytical column. The mobile phase consisted of two solvents: 0.1% (v/v) formic acid in water (A) and acetonitrile (B) with the gradient program performed of a linear increase from 0 to 0.8 min, 15% B; 0.8 to 2 min, 15 to 50% B; 2 to 3.5 min, 50 to 90% B, 3.5 to 5.5 min, 90% B and 1 min post-run, 15% B. The flow rate was set at 0.25 mL/min and the injection volume was 5 μ L.

The UPLC system was attached to triple-quadrupole linear ion trap mass spectrometer (API 4000 QTRAP \hat{c} MS/MS system from AB Sciex, Concord, ON, Canada) equipped with electrospray (Turbo VTM) ion source was operated in negative and positive ionization mode.

The optimized parameters for negative mode were as follows: the ion spray voltage was set to 4200 V, the turbo spray temperature, 450°C; nebulizer gas (gas 1), 20 psi; heater gas (gas 2), 20 psi; collision gas, medium; the curtain gas (CUR) was kept at 20 psi. The optimized parameters for positive mode were as follows: the ion spray voltage was set to 5500 V; the turbo spray temperature, 450°C; nebulizer gas (gas 1), 50 psi; heater gas (gas 2), 50 psi; collision gas, medium; the curtain gas (CUR) was kept at 20 psi.

Mass spectrometric conditions were optimized by infusing 100 to 500 ng/ml solutions of each analytes dissolved in methanol at 10 μ l/min using a Harvard 22' syringe pump (Harvard Apparatus, South Natick, MA, USA). For the MRM quantitation, highest abundance of precursor-to-product ions for each compound was chosen. Analyst 1.5.1 software package (AB Sciex) was used for instrument control and data acquisition. For, full scan ESI-MS analysis, the spectra covered the range from m/z 100 to 1000. All the MS parameters for reference analytes i.e., precursor ion (Q1), product ion (Q3), declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) were optimized in negative and positive ESI mode, by flow injection analysis (FIA). MRM parameters were optimized to achieve the most abundant, specific and stable MRM transition for each compound.

Determination of volatile compounds by using thermal desorption-gas chromatography mass spectrometry (TD-GC-MS) analysis

For headspace volatile analysis, the fungal isolates MJ31 was grown in 500 ml glass flasks containing 150 ml of PDB (Potato Dextrose Broth) and incubated at $30 \pm 2^\circ\text{C}$ for 5 days. VOCs were capture and analysis were conducted as described previously using a trap method by Lee et al. [52]. Headspace samples taken from sterile PDB served as negative controls. The VOCs were adsorbed on 9 cm Tenax TA coated stainless steel desorbing columns (Perkin Elmer HO 244966) inserted into the headspace through a rubber cork in to the culture medium. The volatile compounds were analyzed with a GC-MS customized with Thermal Desorber turbomatrix 150 (Perkin Elmer, USA). The GC conditions were: 10:1 split, helium carrier at 20 psi, oven temperature from 50 to 250°C at 10°C per min. The MS conditions were: positive ion mode, electron impact spectra at 70 eV and the analyses were done using 30 m X 250 μ m capillary column with 5% phenyl-methyl siloxane (Elite 5MS). The detected compounds were identified by mass spectral database (NIST 2014). All volatiles showing mass spectra with match factors >90% were put on a positive list of tentative volatile compounds.

Statistical analysis

The data (expressed as the mean of three replicates \pm standard deviation) were calculated using Microsoft Excel XP 2007 to analyze significant difference between antimicrobial activities of MJ31 isolate against bacterial and fungal pathogens.

Results

Isolation and identification of strain MJ31

In total 53 endophytic fungi which showed significant antimicrobial activity were obtained and identified from various tissues of *M. jalapa* (Unpublished data). Among all the isolates strain MJ31 showed maximum antimicrobial activity (S1 Table) and was identified by sequencing the rDNA ITS region (ITS1-5.8S-ITS2), partial 28S rRNA gene and translation elongation factor 1 alpha (EF 1 α) as *Aspergillus clavatonanicus* (KM203598, MF596170 and MF598999) in BLAST search analysis. The phylogenetic trees of ITS region, partial 28S rRNA

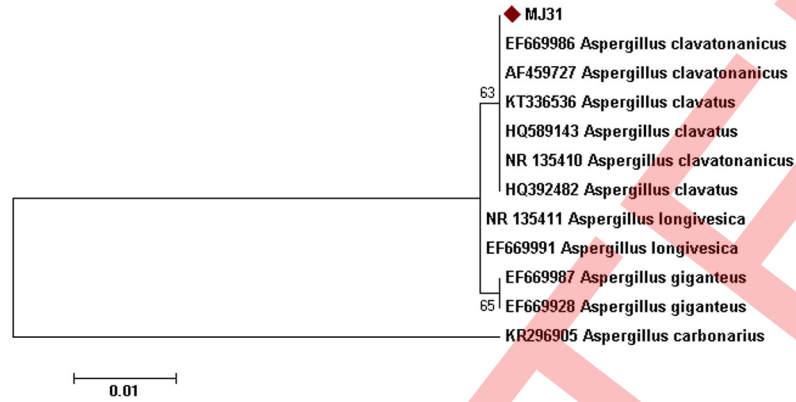


Fig 1. A neighbor joining tree revealing the evolutionary relationship among the endophytic fungus MJ31 and the closest BLAST sequences in the NCBI database based on ITS1-5.8S-ITS2 rDNA sequences. The bootstrap values were depicted based on 1000 replications, whereas *Aspergillus carbonarius* (KR296905) was taken as an out-group.

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gene and translation elongation factor $\hat{I}\hat{\pm}$ were constructed to define the molecular taxonomic position of the strain.

The maximum likelihood phylogenetic tree generated based on ITS analysis was based on Tamura 3-parameter model as per lowest BIC and highest AIC values using MEGA 6.0. For phylogenetic analysis the sequence was aligned with the reference sequences from the closest strains retrieved from NCBI Genbank database. All the gaps and missing data were eliminated and the estimated transition/transversion bias (R) was 1.08. Phylogenetic analysis revealed that MJ31 was classified into a distinct clade consisted of *A. clavatonanicus* and *Aspergillus clavatus* with 63% bootstrap support whereas *Aspergillus longivesica* and *Aspergillus giganteus* formed a separate clade. *Aspergillus carbonarius* was used as an outgroup (Fig 1).

The maximum likelihood tree of MJ31, generated by 28S rRNA gene sequences based on Kimura 2-parameter model according to lowest BIC and highest AIC values using MEGA 6.0. All positions containing gaps and missing data were eliminated. Gaps were treated by pair wise deletion and the estimated transition/transversion bias (R) was 17.37. The maximum Log likelihood (lnL) for substitution computation was -835.422. The phylogenetic tree clearly showed that strain MJ31 was closely related to *Aspergillus clavatonanicus* isolate NRRL 4741 (EF669986), *Aspergillus clavatonanicus* isolate NRRL 4741 (AF459727) and *Aspergillus clavatonanicus* isolate NRRL 4741 (U28397) under a bootstrap support value of 64% whereas *Aspergillus fumigatus* strain ATCC MYA-4896 was used as an out group (Fig 2).

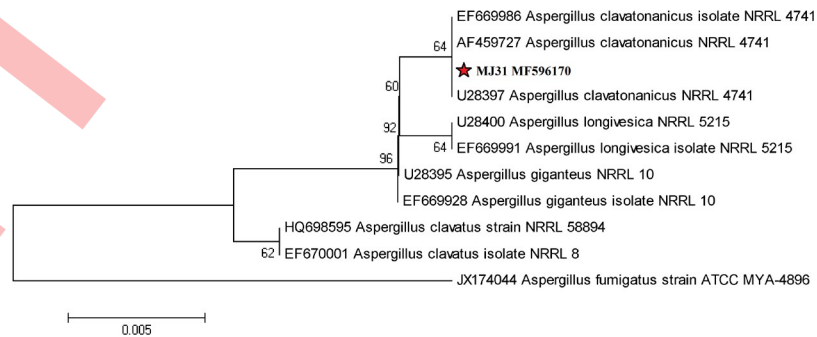


Fig 2. A neighbor joining tree revealing the evolutionary relationship among the endophytic fungus MJ31 and the BLAST analysis tool along with the nine type strains retrieved from NCBI GenBank databases based on 28S rRNA sequences. The bootstrap values were depicted based on 1000 replications, whereas *Aspergillus fumigatus* strain ATCC MYA-4896 was taken as an out-group.

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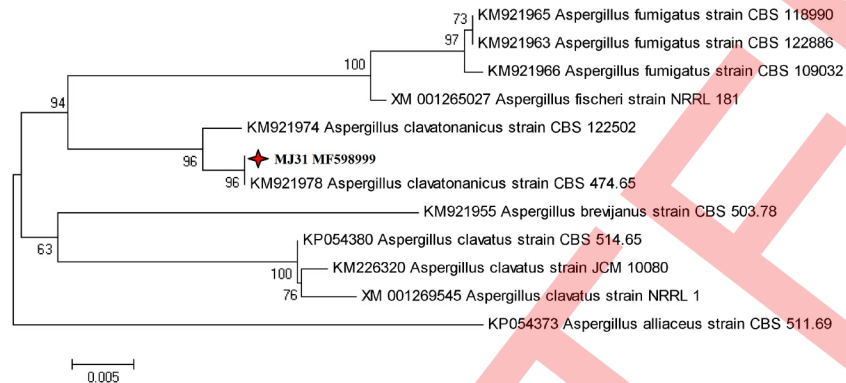


Fig 3. A neighbor joining tree revealing the evolutionary relationship among the endophytic fungus MJ31 and the BlastN analysis along with the ten type strains retrieved from NCBI GenBank databases based on elongation factor 1 α (EF-1 α) sequences. The bootstrap values were depicted based on 1000 replications, whereas *Aspergillus alliaceus* strain CBS511.69 was taken as an out-group.

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The maximum likelihood tree of MJ31, generated by gene sequences of partial elongation factor 1 α (EF-1 α) was based on Tamura-Nei model according to lowest BIC and highest AIC values using MEGA 6.0. All positions containing gaps and missing data were eliminated. Gaps were treated by pair wise deletion and the estimated transition/transversion bias (R) was 2.2. The phylogenetic tree exhibited that strain MJ31 was clustered with *Aspergillus clavatonanicus* strain CBS 474.65 (KM921978) and *Aspergillus clavatonanicus* isolate CBS 122502 (KM921974) under a bootstrap support value of 96% whereas *Aspergillus alliaceus* strain CBS 511.69 was used as an out group (Fig 3).

Dual culture antagonistic bioassay of *Aspergillus clavatonanicus* strain MJ31

In dual antagonistic bioassay, observations were made to gain insight into antagonistic capability of *Aspergillus clavatonanicus* strain MJ31 against six phytopathogens. Macroscopic evaluation of the interaction revealed significant percent of growth inhibition (% PI) of five tested pathogens, *F. oxysporum* f. sp. *ciceri* (NAIMCC-F-02211), *F. oxysporum* f. sp. *pisi* (MTCC-2480), *F. culmorum* (MTCC-2090), *F. graminearum* (MTCC-1893) and *Trichophyton mentagrophytes* (MTCC-8476). The percentage of growth inhibition ranged from 50% upto 74%. The least inhibition was observed against *Aspergillus flavus* (MTCC-9064) as compared with the control. Strain MJ31 showed the most prominent antagonistic activity against *F. graminearum* (74.59%) and *F. culmorum* (70.36%), followed by *F. oxysporum* f. sp. *pisi* (61.25%) (Table 1; Fig 4)

Poisoned food bioassay of *Aspergillus clavatonanicus* strain MJ31

Poison food bioassay of the crude extract was evaluated against fungal phytopathogens to confirm the presence of antifungal compounds. Highest percentage of inhibition (% PI) was recorded against *Fusarium graminearum* with IC₅₀ value of 492.9 μ g/ml followed by *Fusarium culmorum* (496.1 μ g/ml), *Fusarium oxysporum* f. sp. *pisi* (501.1 μ g/ml), *Fusarium oxysporum* f. sp. *ciceri* (502.8 μ g/ml) and *Trichophyton mentagrophytes* (504.4 μ g/ml) (Table 1; Fig 5).

Antibacterial and anti yeast activity of *Aspergillus clavatonanicus* strain MJ31

Ethyl acetate extract of strain MJ31 was evaluated for its antibacterial and anti yeast activity against five human bacterial pathogens (*S. aureus*, *B. subtilis*, *M. luteus*, *P. aeruginosa*, *E. coli*)

Table 1. Antimycotic activity of *Aspergillus clavatonanicus* strain MJ31.

Test microorganisms	Percent of inhibition (% PI±SD) by dual culture antagonism assay	Percent of inhibition (% PI) by ethyl acetate extract			IC ₅₀ (ng/ml)
		250 ng/ml	500 ng/ml	1000 ng/ml	
<i>F. oxysporum</i> f. sp. <i>ciceri</i> (NAIMCC-F-02211)	58.90±1.34	36.42±1.33	44.95±1.34	59.68±1.34	502.8
<i>F. oxysporum</i> f. sp. <i>pisi</i> (MTCC- 2480)	61.25±1.56	44.44±2.22	59.66±2.22	67.77±1.11	501.1
<i>F. culmorum</i> (MTCC-2090)	70.36±1.28	59.62±1.28	74.81±1.28	86.29±0.64	496.1
<i>F. graminearum</i> (MTCC-1893)	74.59±1.37	70.16±1.19	81.34±0.68	88.09±1.19	492.9
<i>T. mentagrophytes</i> (MTCC-8476)	55.85±1.55	29.72±2.70	39.63±1.56	53.15±1.55	504.4
<i>A. flavus</i> (MTCC-9064)	50.36±1.28	28.14±1.28	34.07±1.28	46.66±1.28	512.5

<https://doi.org/10.1371/journal.pone.0186234.t001>

and a yeast pathogen (*C. albicans*). The results showed that the strain MJ31 inhibited the growth of all tested pathogens (Table 2). The highest zone of inhibition was observed against gram positive bacteria *B. subtilis* (24.5±0.5) followed by *M. luteus* (22.66±0.57) and *S. aureus* (21.83±0.28) (Table 2; Fig 6). However, the observed zone of inhibition was comparatively less against *C. albicans* (17±0.5) and least against *P. aeruginosa* (14.5±0.5).

Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC)

The ethyl acetate extract of strain MJ31 was subjected to broth micro dilution method in order to determine MIC and MMC against tested pathogens. The results revealed a strong antimicrobial activity of the extract against Gram positive bacteria *B. subtilis* (0.078 mg/ml) followed by *M. luteus* (0.156 mg/ml), *S. aureus* (0.312 mg/ml) and *E. coli* (0.625 mg/ml). The ethyl acetate extract of strain showed comparatively less MIC values against yeast *C. albicans* (1.25 mg/ml) and Gram negative bacteria *P. aeruginosa* (10 mg/ml) (Table 3).

Detection and sequencing of PKS and NRPS gene

Ketosynthase (KS) domain of polyketide synthases (PKS) type I and adenylation (A) domain of non-ribosomal peptide synthetases (NRPS) were detected in strain MJ31 which might play a role in antimicrobial activity. An expected 700 bp band of KS domain was detected by LC3 and LC5_C primers which were responsible for the synthesis of partially reducing (PR) type

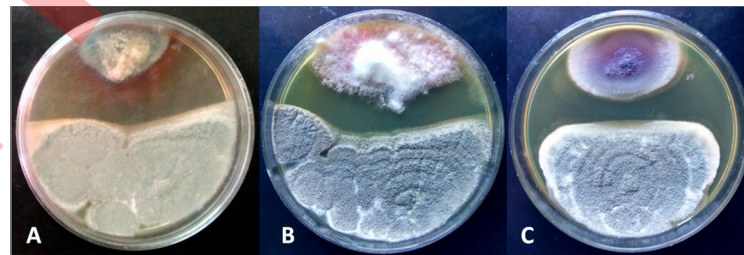


Fig 4. Endophyte- pathogen interactions observed in dual culture antagonism bioassay of strain MJ31 against A: *F. graminearum*, B: *F. culmorum* and C: *F. oxysporum* f. sp. *pisi*.

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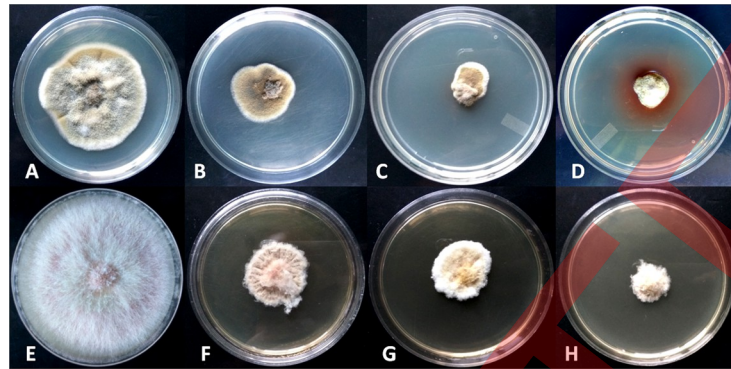


Fig 5. Antifungal activity of ethyl acetate extract of *Aspergillus clavatonanicus* strain MJ31 against fungal phytopathogens. A: *F. graminearum* (control), B- D: antifungal activity of ethyl acetate extract against *F. graminearum* at 250 ng/ml, 500 ng/ml and 1000 ng/ml, E: *F. culmorum* (control), F-H: antifungal activity of ethyl acetate extract against *F. culmorum* at 250 ng/ml, 500 ng/ml and 1000 ng/ml.

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PKSs. NRPS gene was also detected with the amplified product size of 300 bp (Fig 7). Annotation of the sequences using ORF finder and BLASTx analysis of PKS1 revealed the sequence similarity of 92% and 81% sequence similarity for PKS1 and NRPS genes respectively. The sequences were deposited in GenBank with accession numbers KY114487 and KY114488.

Detection and quantification of antibiotics using UPLC-MRM method

Analytical method validation. The proposed UPLC-MRM method for quantitative analysis was validated according to the guidelines of international conference on harmonization (ICH, Q₂R₁) by linearity, LOQs and LODs, precision, solution stability and recovery.

Linearity, limits of detection (LOD) and quantification (LOQ). A series of concentrations of standard solution were prepared for the establishing calibration curves. The peak areas were plotted against the corresponding concentrations to obtain the calibration curves. LOD and LOQ were determined by calibration curve method. LOD and LOQ were calculated by using following equations. $LOD = (3.3 \times Sy.x)/S$; $LOQ = (10 \times Sy.x)/S$ (Where, $Sy.x$ is standard deviation of residuals from line; S is slope). The results were listed in Table 4. All the calibration curves indicated good linearity with correlation coefficients (r^2) from 0.9989 to 0.9999

Table 2. Antibacterial and antiyeast activity of endophytic fungus *Aspergillus clavatonanicus* strain MJ31 against human pathogens.

Test pathogens	Diameter of inhibition zone [mean (mm) ± SD]														
	MJ31*	A*	S*	E*	G*	CD*	NA*	C*	VA*	I*	V*	M*	K*	F*	NY*
<i>S.aureus</i>	21.83 ±0.28	24.33 ±0.57	12.5±0.5	17±1.0	14.16 ±0.28	15.0 ±0.5	10.33 ±0.57	20.66 ±0.57	14.5 ±0.5						
<i>B. subtilis</i>	24.5±0.5	20.66 ±1.15	11.33 ±0.57	16.33 ±0.57	14.16 ±0.28	-	11.83 ±0.76	20.66 ±0.57	-						
<i>M. luteus</i>	22.66 ±0.57	24.83 ±0.76	10.5±0.5	16.5±0.5	12±0.0	16.5 ±0.5	11.33 ±0.76	19.66 ±0.76	13±0.5						
<i>P. aeruginosa</i>	14±0.5	12.5±0.5	10±0.5	-	13.33 ±0.76	-	15.33 ±0.57	18±0.5	-						
<i>E. coli</i>	21±1.0	22.66 ±0.76	12.16 ±1.40	-	17.83 ±0.76	-	-	18.5±0.5	-						
<i>C. albicans</i>	17±0.5									18.16 ±0.28	9.5 ±0.5	11.33 ±0.28	17.33 ±0.28	8.5 ±0.5	-

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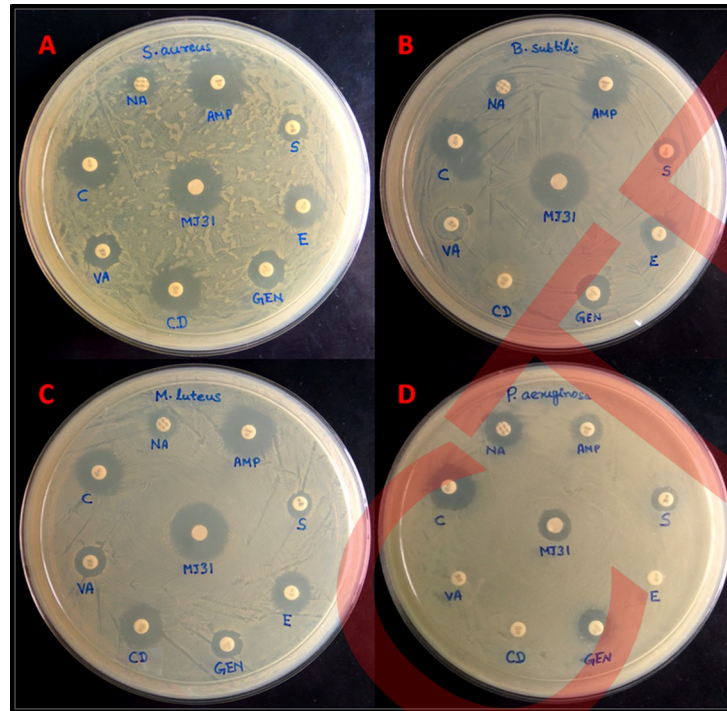


Fig 6. Antimicrobial activity of endophytic fungus *A. clavatonanicus* strain MJ31, showing zone of inhibition (mm± SD) along with reference standard antibiotics against A) *Staphylococcus aureus*; B) *Bacillus subtilis*; C) *Micrococcus luteus* and D) *Pseudomonas aeruginosa*.

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within the test ranges. The LOD and LOQ for each reference analyte were less than 0.52 ng/mL and 0.78 ng/mL, respectively (Table 4).

Precision, stability and recovery. The Precision was measured by relative standard deviation (RSD) with intra-day and inter-day variations were evaluated by determination of analytes in six replicates on a single day and by duplicating the experiments over three successive days. The overall intra-day and inter-day precision was not more than 2.01%. Replicate injections at 0, 2, 4, 8, 12 and 24 h. were performed to examine stability of samples. The stability RSD% value of analytes is \approx 2.45%. To evaluate the accuracy, recovery test was applied by spiking three different concentration levels of the standards at low (80% of the known amounts), medium (100% of the known amounts), and high (120% of the known amounts) of the analytical standards into the samples. At each level three replicates were performed and were calculated using the following equation:

$$\text{Recovery(\%)} = 100 \times \frac{\text{found amount}}{\text{original amount/spiked amount}}$$

Table 3. Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) of methanol extract of *Aspergillus clavatonanicus* strain MJ31 against bacterial and yeast human pathogens.

Concentration (mg/ml)											
<i>S. aureus</i> (MTCC-96)		<i>B. subtilis</i> (NCIM-2097)		<i>M. luteus</i> (NCIM-2170)		<i>P. aeruginosa</i> (MTCC-739)		<i>E. coli</i> (MTCC-739)		<i>C. albicans</i> (MTCC-3017)	
MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
0.312	2.5	0.078	0.625	0.156	1.25	10	-	0.625	2.5	1.25	10

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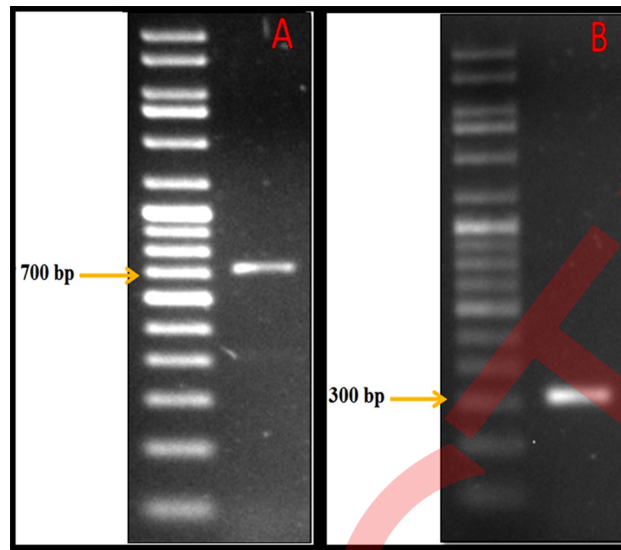


Fig 7. PCR based detection of (A) ketosynthase domain of PKS I gene and (B) adenylation domain of NRPS gene.

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The developed analytical method had good accuracy with overall recovery in the range from 97.98% -102.12% (RSD $\hat{=}$ 1.45%) for all analytes (Table 4).

Quantitative analysis. In this study seven standard antibiotics i.e. ampicillin, streptomycin, chloramphenicol, rifampicin, ketoconazole, fluconazole and miconazole were detected in the methanolic extract of strain MJ31 and quantified using UPLC-ESI-MS/MS. In the quantitative analysis miconazole (900 η g/g) was detected in highest amount followed by ketoconazole (63.67 η g/g), rifampicin (45.33 η g/g), ampicillin (37.33 η g/g) and streptomycin (30 η g/g). MS/MS spectra and MRM extracted ion chromatogram of reference analytes are shown in Fig 8 and Fig 9 respectively.

TD-GC-MS analysis of *Aspergillus clavatonanicus* strain MJ31

A total of 28 unique volatile compounds were detected at least twice (Table 5) which encompassed hydrocarbons, alcohols, ketones, aldehydes, alkanes, alkenes, esters, aromatic compounds,

Table 4. Validation parameters of UPLC-MRM method for quantitative analysis of standard antibiotics and MJ31 crude extract.

Analytes	Regression Equation	r^2	Linear range ng/ml	LOD ng/ml	LOQ ng/ml	Precision RSD (%)		Stability RSD (n = 5)	Recovery RSD (%)	Quantitative analysis of sample (η g/g)
						Intra-day (n = 6)	Inter-day (n = 6)			
Ampicillin	$y = 3961.5x - 20987$	0.9996	10–500	3.11	7.54	1.16	2.21	1.52	0.54	37.33
Fluconazole	$y = 15101x + 3474.3$	0.9996	0.5–100	0.14	0.38	0.22	1.45	2.25	1.26	1.65
Chloramphenicol	$y = 447.14x + 135.85$	0.9998	1–250	0.44	0.85	0.44	0.62	1.39	1.63	9.20
Ketoconazole	$y = 884.99x - 11510$	0.9999	10–250	4.33	8.11	1.52	1.59	2.11	1.37	63.67
Rifampicin	$y = 2394x - 15083$	0.9988	1–250	0.31	0.73	2.66	1.63	0.7	1.05	45.33
Miconazole	$y = 2117.3x + 45116$	0.9999	1–100	0.37	0.83	1.23	0.73	1.08	1.15	900
Streptomycin	$y = 1791.7x - 3466.7$	0.9991	1–100	0.41	0.85	1.11	0.48	1.12	1.03	30

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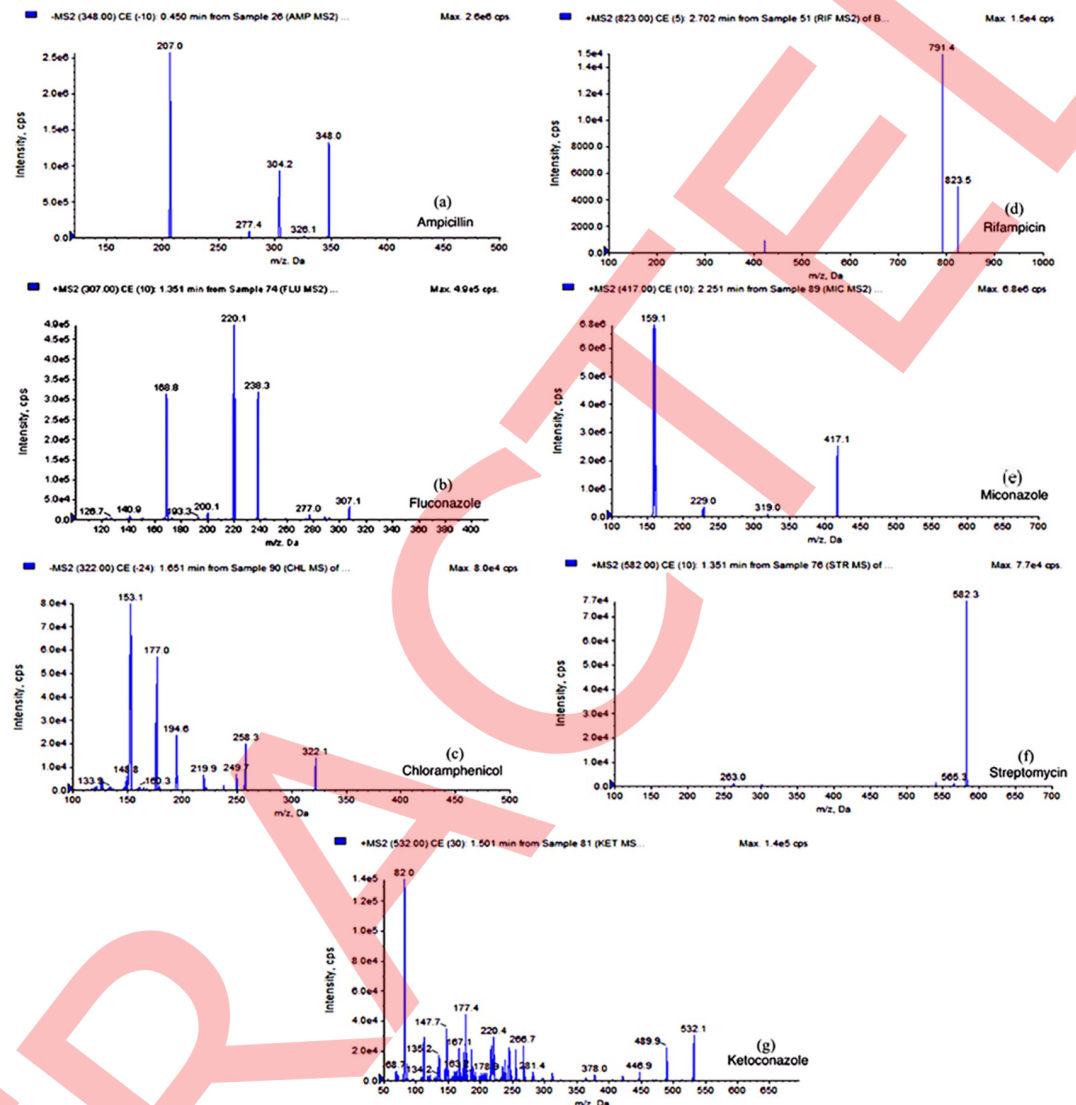


Fig 8. MS/MS spectra of reference analytes: (a) Ampicillin; (b) Fluconazole, previously reported in [90, 91, 92 retracted in 93], (c) Chloramphenicol, previously reported in [90, 91], (d) Rifampicin, previously reported in [90, 91, 92 retracted in 93], (e) Miconazole, previously reported in [91], (f) Streptomycin, previously reported in [90], and (g) Ketoconazole, previously reported in [91, 92 retracted in 93]. For Fig 8B, 8C, 8D, 8F the following copyright limitations apply: Copyright © 2016 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.

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heterocyclic compounds, and various other compounds like furan and pyran derivatives. The TD-GC-MS data was deconvoluted using the NIST software and the measured mass spectra were matched to entries in the compound library. The details about the volatiles and their retention time are presented in Table 5. The most abundant volatile metabolites identified were Pentadecane, Naphthalene, Tetradecane, Hexadecane, Octanol and Eicosane. The fungal isolate MJ31 showed unique compounds like 6-PP (6 Pentyl-2H Pyrone-2-one), disulphide dimethyl, 1, 2 butadiene, m-camphorene, 3-Thietanol, Thiopivalic acid, Pthalic acid, Heneicosane, pyrazol and benzene derivatives (Fig 10). According to PCA analysis, most of the unique compounds were produced by MJ 31 whereas the other compounds like carbonic acid, sulphurous acid and few alkanes were found in the control. The Biplot analyses and comparison with the control was given in Fig 11 and it showed that the VOCs produced by the strain MJ 31 differed significantly.

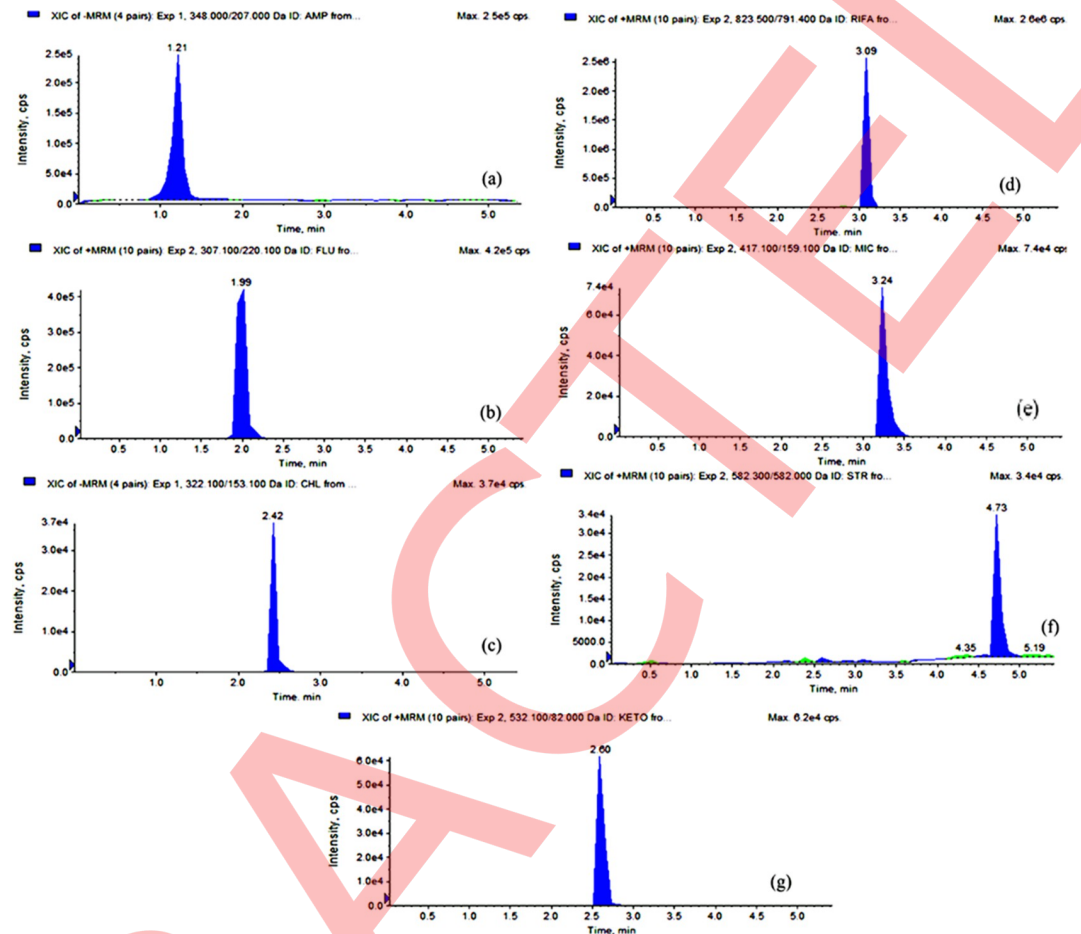


Fig 9. MRM extracted ion chromatogram of reference analytes: (a) Ampicillin; (b) Fluconazole; (c) Chloramphenicol; (d) Rifampicin; (e) Miconazole; (f) Streptomycin and (g) Ketoconazole.

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Discussion

Endophytes live within the plant tissues in an imperceptible manner without causing any detectable infection to the host. The most significant attribute is to provide protection against plant pathogens and pests to the host plant [8]. Recently, several researchers stated that endophytic microorganisms isolated from plants belong to unusual locations have greater chances to get novel isolates having capability to produce novel bioactive compounds [8, 61]. Moreover, a range of bioactive compounds have been reported from fungal endophytes associated with medicinal plants having ethnobotanical history [62, 63]. Additionally, in recent years the endophytic fungi associated with medicinal plants from north east India have been explored as potential source for the discovery of antimicrobial compounds [64–67]. The promise shown by the previous researchers encouraged us to search the antimicrobial potential of fungal endophytes associated with *M. jalapa* L., an ethnomedicinal plant of Mizoram.

In the present study, an endophytic fungal strain MJ31 having antimicrobial activity isolated from root tissues of *M. jalapa* was identified as *Aspergillus clavatonanicus* based on sequencing and phylogenetic analysis of ITS rRNA gene, 28S rRNA gene and translation elongation factor 1 alpha (EF 1 α). This findings was in agreement with Devaraju and Satish [68] who reported antimicrobial potential of endophytic fungi associated with *M. jalapa*. A.

Table 5. Volatile compounds detected by GC-MS analysis of ethyl acetate extract of *Aspergillus clavatonanicus* strain MJ31.

Sl. No	Retention Time	Compound Name (IUPAC)	Potential functions	References
1	2.429	6 Pentyl 2H Pyran 2-one	Antibiotic	Vinale et al. [53]
2	2.614	1,2-butadiene	Plant growth promotion	Lee et al. [54]
3	3.49	3-Thietanol	Alarm pheromones and predator scent analogues	Luntz [55]
4	3.664	Disulfide dimethyl	Fumigant and volatile indicator	Mason et al. [56]
5	4.12	Ethane Sulfonyl Chloride Disulfide dimethyl	Mason et al., 2010	Mason et al. [56]
6	4.48	Cyclopropyl Carbonyl oxy tridecane	Analogs of plant hormone	-
7	7.95	Thiopivalic acid	Organosulfur compound	-
8	9.13	Dimethyl trisulfide	Fumigant	Mason et al. [56]
9	9.346	2,3-Epoxy octane	Oxylipin that mediates epoxidation reactions in resting cells; involved in morphogenesis	Chen et al. [57]
10	10.06	Benzene ethanamine 4-methoxy	Plant growth promotion	Lee et al. [52]
11	14.158	2-Furancarbodithioic acid methyl ester	Inhibitory volatile	Lee et al. [54]
12	14.949	2-Hexyl 1-Octanol	Semiochemical-Insect deterrent	Aldrich [58]; Kishimoto et al. [59]
13	15.189	Dodecane	Inhibitory volatile	Lee et al. [54]
14	15.769	Benzene,4-(2-butenyl)-1,2-dimethyl	-	-
15	16.12	1-Decanol	Inhibitory volatile	Lee et al. [54]
16	16.574	Hexadecane	Inhibitory volatile	Lee et al. [54]
17	16.72	Decahydro-1,1 4a,5-6-penta Methyl Naphthalene	Fumigant	-
18	17.194	Tetradecene	Inhibitory volatile	Lee et al. [54]
19	17.885	Pentadecane	Inhibitory volatile	Lee et al. [54]
20	18.395	Ethanol	Inhibitory volatile	Lee et al. [54]
21	19.030	Carbonic acid	-	-
22	20.501	Furan derivatives	Fumigant	Lee et al. [54]
23	21.091	Eicosane	Antimicrobial	Lee et al. [54]
24	21.672	Phthalic acid, hept -3yl isobutyl ester	Precursor of benzoic acid derivatives that involves in plant growth promotion	Lee et al. [54]
25	22.512	Nonadecane	Antimicrobial	Nemcovic et al. [60]
26	22.932	m-Camphorene	Fumigant	Mason et al. [56]
27	24.468	2(6-Hydroxynaphthyl) propionic acid methyl ester	-	
28	25.818	Heneicosane	Aggregation Pheromones	Luntz [55]

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clavatonanicus was earlier reported as an endophyte of *Taxus mairei* [69]. Although, *Aspergillus* are mostly isolated from soil; a number of species under genus *Aspergillus* such as *A. fumigatus*, *A. niger*, *A. clavatus*, *A. awamori*, *A. parasiticus* and *A. terreus* are reported as endophytes which suggests frequent occurrence of this fungus as endophyte [70, 71].

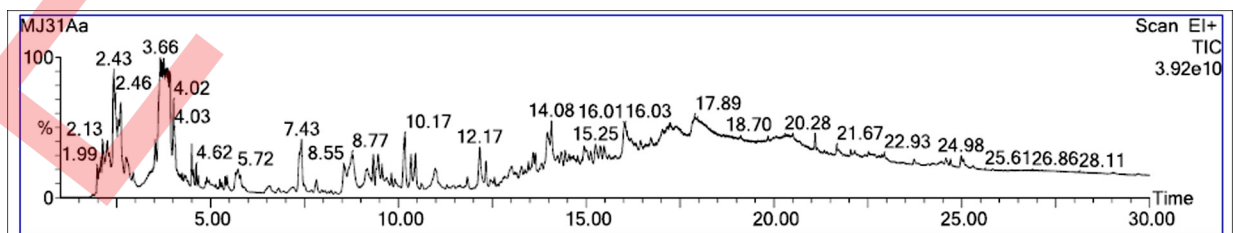


Fig 10. GC-MS profiling detected volatile compounds from fungal isolate *Aspergillus clavatonanicus* strain MJ31 compared with NIST library.

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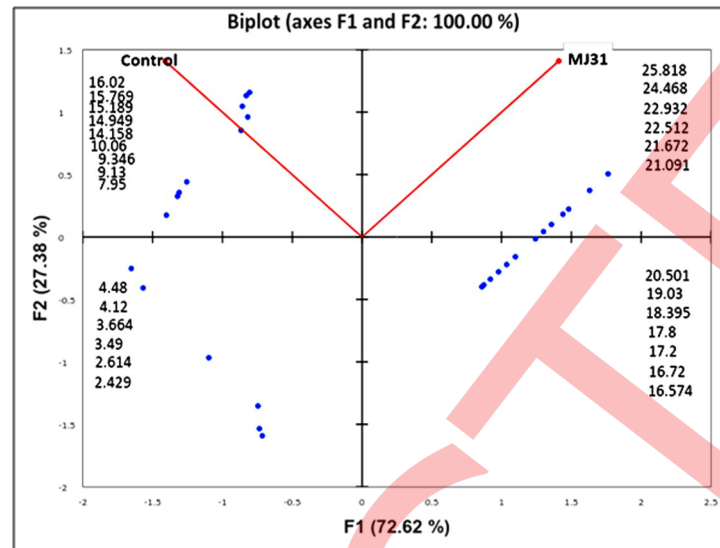


Fig 11. PCA Biplot based on the retention time (Each retention time corresponds to each detected volatile compound (VOC)).

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A. clavatonanicus strain MJ31 was evaluated for its antiphytopathogenic activity by dual confrontation assay against six fungal phytopathogens and displayed significant antifungal activity against all tested pathogens with percent of inhibition (% PI) ranges from 50% to 74%. Qadri et al. [72] reported antiphytopathogenic activity of endophytic fungi associated with *Pinus wallichiana* against seven fungal pathogens of different genera. The highest antagonistic activity of strain MJ31 was recorded against *Fusarium oxysporum* f. sp. *pisi* followed by *Fusarium graminearum* and *Fusarium culmorum*. Wicklow et al. [73] obtained similar result with endophytic fungi *Acremonium zeae* against *Aspergillus flavus* and *Fusarium verticillioides* in antagonism cultural tests. The ethyl acetate extract of strain MJ31 showed significant percentage of growth inhibition against *F. graminearum*, followed by *F. culmorum* and *F. oxysporum* f. sp. *pisi*, at the concentration of 250ng/ml with IC₅₀ values of 492.9 ng/ml, 496.1 ng/ml and 501.1 ng/ml respectively. The results obtained are in accordance with the findings of Zhang et al. [69] who has reported potent antifungal activity of isolated metabolites clavatul and patulin from endophytic fungi *A. clavatonanicus* associated with *T. mairei*. Li et al. [74] has also reported potent antifungal activity of metabolites isolated from fungal endophyte *A. fumigatus* against three fungal pathogens belonging to genera *Fusarium*. These findings showed the capability of the isolated endophytic strain to produce potent antimycotic metabolites.

The ethyl acetate extract of *A. clavatonanicus* strain MJ31 exhibited wide spectrum antimicrobial activity against all tested bacterial pathogens. Previous studies on fungal endophytes have already established them as reservoir of antimicrobial compounds [12, 75, 76]. The highest zone of inhibition was observed against Gram positive bacteria *Micrococcus luteus* followed by *Staphylococcus aureus* and *Bacillus subtilis*. Comparatively lesser activity was observed in case of Gram negative *Escherichia coli*, *P. aeruginosa* and yeast *C. albicans*. The result obtained are in agreement with findings of Santos et al. [2] who has reported potent activity of endophytic fungi against Gram positive bacteria obtained from leaves of *Indigofera suffruticosa* and found less antimicrobial activity against *P. aeruginosa*. Devaraju and Satish [68] demonstrated antimicrobial activity of endophytic *Fusarium* sp. associated with *Mirabilis jalapa* L. which further support our findings. The highest MIC (0.078 mg/ml) and MMC (0.625 mg/ml) values of ethyl acetate extract was also achieved against Gram positive bacteria *B. subtilis* followed by *M.*

luteus and *S. aureus*. However, significant MIC was obtained against Gram negative bacteria *E. coli*, *Pseudomonas aeruginosa* and yeast pathogen *C. albicans*. The broad spectrum antimicrobial activity displayed by strain MJ31 supports the hypothesis that the strain can serve as an alternative source of potent antimicrobial compounds which has been reported by several researchers [77-79]. Ola et al. [78] has isolated an antimicrobial compound neosartoinin from endophytic fungi *A. fumigatus* having wide spectrum activity against *Staphylococci*, *Streptococci* and *Bacillus subtilis* with MIC in the range of 4 ng/ml-32 ng/ml. More recently, a cyclic pentapeptide isolated from *A. tamarii* has displayed potent antimicrobial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, *C. albicans*, and *F. solani* and *P. chrysogenum* at relatively low concentration [80]. The ethyl acetate extract significantly inhibited all the tested gram positive bacteria whereas it exhibited comparatively lesser activity against gram negative bacteria. The results thus obtained with ethyl acetate extract followed the same pattern as the antibiotics i.e. better bioactivity against gram positive bacteria and comparatively lesser activity against gram negative bacteria.

The isolate *A. clavatonanicus* strain MJ31 was further screened for the presence of Polyketide synthases (PKS) and Non ribosomal peptide synthetase (NRPS) genes to figure out its polyketide and peptide synthesis capability. Various structurally diverse bioactive metabolites with applications in medicine and agriculture have been isolated from these two families of natural products [18]. In a similar study, Wang et al. [81] estimated the potential of fungal endophytes to produce bioactive natural products based on detection of betaketosynthase domain in the polyketide synthase (PKS) gene cluster. PCR amplification reveals presence of both the PKS-I and NRPS genes in the isolate, suggesting their role in its antimicrobial activity. Similarly, Miller et al. [82] used genetic screening by amplifying PKS and NRPS genes to evaluate the bioactive potential of endophytes associated with traditional herbs. Peptides synthesized by NRPS have significant application in medicine as antibiotic and antifungal agents. Peptides produced by endophytes in the recent past have received attention of researchers owing to their promising bioactive potential [83].

Based on antimicrobial activity against plant fungal as well as human bacterial pathogen, ethyl acetate extract of *A. clavatonanicus* strain MJ31 was observed for the detection and quantification of known standard antibiotics that were used in the antimicrobial assay using UPLC-ESI-MS/MS method. Seven known antibiotics i.e. ampicillin, streptomycin, chloramphenicol, rifampicin, ketokonazole, fluconazole and miconazole were detected and quantified. Antibiotic miconazole was found in highest amount (900 ng/g) followed by ketokonazole (63.67 ng/g), rifampicin (45.33 ng/g), ampicillin (37.33 ng/g) and streptomycin (30 ng/g) respectively. Miconazole is an imidazole antifungal agent that commonly used to cure vaginal, skin and nail infection caused by yeast and dermatophytes and also reported to possess activity against *S. aureus* and *S. pyogenes* [84]. Ketoconazole is also used for the treatment of skin dermatophytosis and infection caused by several species of *Candida* [85, 86]. Detection of such antifungal antibiotics further implies their possible role in antifungal activity of *A. clavatonanicus* strain MJ31. Among the antibacterial antibiotics, rifampicin and ampicillin were detected in highest amount. Rifampicin considered exhibiting bactericidal effect against *S. pneumoniae* while having bacteriostatic effect against *E. coli* and *N. gonorrhoea*. Clearly, detection of these antibiotics in the extract is one of the reasons behind antibacterial potential of the strain.

Volatile-mediated interactions between plants and microbes have been gaining increased attention in agriculture. Fungi produce a large number of VOCs as mixtures of alcohols, ketones, esters, small alkenes, monoterpenes, sesquiterpenes, and derivatives [19]. Moreover the VOCs also serve as an electronic noose by acting as a signal molecule. The relative abundance of CCOT (Cyclopropyl carbonyl oxy tri decane) is 100%, 3-Thietanol (40%), disulphide dimethyl (50%) and 6-PP (90%) in the strain MJ31. This shows the abundance of these unique

compounds respectively. Octenol is one of the most significant compounds identified in the strain MJ31 with potential role in plant immunity. Moreover, exposure to 1-octen-3-ol induced expression of the defense genes that are associated with wounding or ethylene and jasmonic acid signaling in *Arabidopsis thaliana* and inhibited growth of the pathogen *Botrytis cinerea* on infected leaves [59]. Similarly Wilkins et al. [87] reported that *Trichoderma viride* produced volatile metabolites such as 2-propanol, 3-methylfuran, methyl-1-propanol, 1-pentanol, and 2-hexanone for pathogen inhibition. Furthermore compounds like pentanones, octanones, nonanones and undecanones have been described in cultures of *T. atroviride* [60] and heptanone by *T. viride* [88]. A massive collection of volatiles in the group of cyclohexane, cyclopentane, alcohols, esters, sulfur containing compounds were already reported in *T. harzianum* [89]. Disulfide, furanyl derivatives and naphthalene identified in this investigation has fumigant, insecticidal as well as nematocidal activity. Of these, octadecane, heptadienal, and pent-2-ynol are known to emit by plants under stress. The compound 6-pentyl-2H-pyran-2-one (6PP), a lactone with a coconut-like odor, is commonly produced by *Trichoderma* and has been shown to both improve and inhibit plant growth and health at different concentrations [53]. Since the chromatogram provided is a single time point for GC-MS analysis, it is recognized as a snapshot that does not capture the full range of VOCs likely to have been produced by the two fungal strains. Hence, it can be concluded that direct growth promotion using VOCs as signaling compounds should be added to the already known mechanisms (e.g. antibiotic production, competition with plant pathogens, to enhance sustained plant vigor).

Conclusions

The present study demonstrates broad spectrum antimicrobial activity of *Aspergillus clavatonanicus* strain MJ31, against both plant and human pathogens. The antimicrobial activity of strain MJ31 was established by detection and production of known antibiotics and antimicrobial volatile compounds. To the best of our knowledge, this is the first report for detection and quantification of known antibiotics like ampicillin, streptomycin, chloramphenicol, rifampicin, miconazole, ketokonazole and fluconazole from a single strain of endophytic fungi. We reported significantly high amount of miconazole in the crude ethyl acetate extract which again supports its ability to synthesize antimicrobial bioactive compounds. Moreover, detection of betaketosynthase (KS) domain in PKS gene cluster and adenylation domain (A) in NRPS gene clusters suggests that this strain can serve as a resource for bioactive natural products. We conclude that, the endophytic fungus *A. clavatonanicus* strain MJ31 associated with root tissues of *M. jalapa* showed the ability to resist fungal pathogens which can be exploited not only in developing biocontrol agents in crop disease management, but also as sustainable and alternative resource for the discovery of potent antimicrobial metabolites.

Supporting information

S1 Table. Antifungal activity of endophytic fungi associated with *Mirabilis jalapa* against fungal phytopathogens by dual culture antagonistic bioassay.
(DOC)

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