Vol. 14(3), pp. 81-91, July-September 2020
DOI: 10.5897/AJBR2020.1055
Article Number: FA93F1264712
ISSN 1996-0778

http://www.academicjournals.org/AJBR

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African Journal of Biochemistry Research

Full Length Research Paper

Antibacterial activity of endophytic fungi isolated from leaves of medicinal Plant *Leucas martinicensis* L. growing in a Kenyan tropical forest

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Received 22 September, 2019; Accepted 23 January, 2020

Infectious diseases are major threat to public health; a problem that has been exacerbated by emergence of multi-drug resistant (MDR) strains. Finding alternative antimicrobial compounds from natural sources such as fungal endophytes and medicinal plants is crucial for addressing antimicrobial resistance. Thus, in this study search for endophytes with antibacterial activities from leaves of medicinal plant *Leucas martinicensis* was undertaken. Three fungal endophytes were isolated from fresh leaves and characterized using ribosomal Internal Transcribed Spacer (ITS) DNA. Antibacterial activities against five bacterial pathogens were determined using dual cultures and, disc diffusion assay for ethyl acetate extracts and pure compounds. Fungal endophytes isolated were LM-L(1), AD-L(1) and LM-S(6) belonging to genera *Nigrospora*, *Diaporthe* and *Epicoccum,* respectively. Axenic cultures and ethyl acetate extracts displayed antagonistic activity against *Escherichia coli, Staphylococcus aureus*, *Proteus vulgaris, Klebsiella pneumoniae* and *Salmonella typhi* unlike pure compounds. Irrespective of endophyte isolate, increasing the concentration of ethyl acetate fractions from 0.625 to 5.0 mg/ml during minimum inhibitory concentration (MIC) assay increased antibacterial activity; although 2 to 3 folds lower than chloramphenicol at 30 µg/disc. However, ethyl acetate fraction F3 at 5.0 mg/ml obtained from isolate LM-L(1) isolate belonging to genus *Nigrospora* produced activity that was not significantly ($p \geq 0.05$) different from chloramphenicol discs. Failure of pure compounds unlike ethyl acetate and axenic endophyte cultures suggests antibacterial activity observed was due to synergistic interactions of compounds. Nonetheless, the results demonstrate that fungal endophytes isolated from *L. martinicensis* possess antibacterial compounds which can be exploited further as lead compounds towards addressing antimicrobial drug resistance.

Key words: Fungal endophytes, antimicrobial activity, drug resistance, *Leucas martinicensis.*

INTRODUCTION

Antibiotics play a crucial role in human medicine and agriculture production by enabling treatment of infectious diseases and facilitating intensive livestock production. Emergence of antimicrobial resistance is therefore a

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great threat to management of human and livestock diseases (Jasovský et al., 2016). Although resistance to common antimicrobial drugs is occurring at an alarming rate globally (Laxminarayan et al., 2016), there have been attempts to address antimicrobial resistance problem through a number of initiatives. These include development of guidelines on the use of medically important antibiotics in animal production and governance approach (Padiyara et al., 2018). In addition, progressive approaches such as the use of bacteriophages, probiotics and vaccines are promising alternatives (Aslam et al., 2018). However, development of new classes of antibiotics is an attractive approach for addressing antimicrobial resistance (AMR), particularly due to the decline in research and development of new antibiotics by major pharmaceutical companies. Nonetheless, the greatest challenge in drug development is identification of new microbial agents (Holmes et al., 2016). Recently, attention has shifted to finding alternative sources of antimicrobial compounds from natural sources such as saprophytic fungi and endophytes (Arora and Kaur, 2019; Mookherjee et al., 2018).

Endophytes are either fungi or bacteria that inhabit tissues of plants and they do not cause any apparent disease (Sandhu et al., 2014). Fungal endophytes are an under-explored group of micro-organisms which may possess bioactive compounds of pharmacological significance (Selvi and Balagengatharathilagam, 2014). Fungal endophytes are known to protect their host from phytopathogens, herbivory and enable plants to endure adverse environmental conditions (Patle et al., 2018; Radu and Kqueen, 2002). Bioactive compounds from fungal endophytes are known to possess antimicrobial properties such as antiviral, antioxidants antifungal, antibacterial and immune-suppressive among others (Wanga et al., 2018). Antimicrobials currently in the market such as fusidic acid (Elsebai et al., 2014), cephalosporins (Saxena et al., 2019) and penicillin as well as an antifungal drug like Griseofulvin (Lee et al., 2016), have been sourced from fungi. Strikingly, the possibility of fungal endophytes isolated from medicinal plants to produce secondary metabolites of pharmacological importance is very high (Nasimiyu et al., 2018).

Leucas martinicensis L. is an aromatic medicinal plant that is found in Tropical and sub-Tropical Africa and Indian subcontinent (Nondo et al., 2017). It is an annual erect shrub that grows up to a height of 1.5 m (Eze et al., 2013). *L. martinicensis* is commonly known as white wort and 'moetiet' in the Mt. Elgon region in Kenya. Traditionally, the plant is used for management of medically diverse ailments such as burns and as a decoction against roundworms, applied to wounds, sores and snake bites (Ugwah-Oguejiofor et al., 2015). Plants of the genus Leucas are important sources of natural antibiotics, oils for aromatherapy, perfumes and cosmetics for moisturizing purposes (Regina et al., 2015). Apparently all plant species harbor endophytes in their inter-cellular tissues (Santoyo et al., 2016). In this study, we report isolation, identification and antibacterial activity of fungal endophytes isolated from *L. martinicensis* leaves collected from Mt Forest in Kenya.

MATERIALS AND METHODS

Plant material

Fresh leaves from 20 *L. martinicensis* plants were collected from Mt. Elgon National Forest in Kenya prior to the onset of long rains in March 2018. The forest stretches from N 01°01.995' and E 034°46.815' at an altitude of 2080 m. Plants were selected based on ethno botanical information and sampling was undertaken randomly within the forest in sites that appeared to have less interference by human activities. Herbarium specimens were collected and identification undertaken with the help of a taxonomist prior to depositing at Biological Science Department, Egerton University. The young leaf samples collected for isolation of endophytes were transported to the laboratory and processed within 24 h.

Isolation of fungal endophytes

Fungal endophytes were isolated following the procedure described by Marcellano et al. (2017) with slight modifications. Briefly, leaves were washed under running tap water to remove soil debris, then surface sterilized with 1% sodium hypochlorite for 3 minutes, 70% ethanol for 3 min and rinsed in three changes of sterile distilled water. Mid region of the sterilized leaves were cut into sections of 1 × 4 mm and inoculated on Sabourand Dextrose Agar (SDA) media amended with 2 mg/ml streptomycin sulphate. The inoculated plates were placed in an incubator at 28±2 °C in the dark until fungal growth from inoculated leaves was observed. Hyphal tips of the endophytes were sub-cultured onto fresh SDA media amended with streptomycin sulphate to generate axenic cultures. To test the efficacy of sterilization protocol 100 µl of final rinse water was plated on SDA media and incubated for 5 days.

DNA extraction and PCR amplification

Genomic DNA was extracted from approximately 60 mg of mycelia obtained from 3 to 5 days old endophyte cultures using BIO BASIC EZ-10 Spin column miniprep kit (BIO BASIC INC.) according to the manufactures' instructions. The ribosomal DNA region, Internal Transcribed Spacer (ITS) was amplified using ITS1F (CTTGGTCATTTAGAGGAAGTAA) forward and ITS4 (TCCTCCGCTTATTGATATGC) reverse primers. Amplification was conducted using 25 µl reaction mix consisting of 2 µl (0.5 µg) genomic DNA, 12.5 µl JumpStart Taq Ready Mix (Sigma-Aldrich), 9.5 µl dH_2O, 0.5 µl ITS1F and ITS4 primers. The amplification was performed using Eppendorf® Mastercycler® nexus thermocycler under the following conditions; initial denaturation of 5 min at 94°C, followed by 34 cycles of denaturation for 30 s at 94°C, annealing at 52°C for 30 s and elongation for 1 min at 72°C. The final elongation was conducted for 10 min at 72°C. To confirm the presence of PCR amplicons, 3 µl of PCR products were mixed with 5 µl of Midori green loading dye and resolved on 1.8% agarose gel. The gel electrophoresis was conducted for 40 min at 100 V using 1×TAE buffer and the gel was viewed under Nippon Genetics trans-illuminator.

Amplicon DNA sequencing and phylogenetic analysis

The polymerase chain reaction (PCR) products were purified using EZ-10 Spin Column PCR purification kit (Bio Basic INC.) following the manufacturers' instructions. The amplified ITS fragments were then bidirectional sequenced using Sanger technique and consensus sequences assembled in Geneious software version 11.0. 4 (Grada and Weinbrecht, 2013). The sequences were then subjected to NCBI BLASTN-Targeted loci search on NCBI GenBank database (http://www.ncbi.nlm.nih.gov/blst) to determine the identity of the axenic endophytes. Multiple sequence alignment of ITS ribosomal DNA fragments was done using Clustal Omega to compare ITS fragments. The evolutionary relationship of the endophytic fungi was determined by construction of a phylogenetic tree based on Maximum likelihood method (Neighbor Joining approach) with 1000 bootstrap replications using Molecular Genetics Analysis MEGA-X software (Kumar et al., 2018) and UNITE fungal identification database updated on 2018-12-08 (https://unite.ut.ee/) (Nilsson et al., 2018).

Prescreening endophytes for antimicrobial activities

Determination of antimicrobial activity of the endophytes isolated was conducted using dual culture assay as described by Wanga et al. (2018). The test pathogens used were *E. coli* DSM498*, S. typhi* ERS223417, *K. pneumoniae* ATCC13883*, S. aureus* ATCC25922 and *P. vulgaris* ATCC49132. Briefly, 100 µl of overnight culture at 1.5×10^8 CFU/ml of the test pathogens were spread on petri dishes containing Mueller Hinton agar and allowed to air dry under sterile conditions. Using a 7 mm cork borer, plugs of each axenic endophyte isolate were prepared from 7-day old cultures and placed onto the MHA media containing inoculated respective test bacterial pathogen. For control treatment, standard chloramphenicol discs (30 µg/disc) were used as positive controls. The plates were then incubated at 28°C for 24 h followed by determination of the inhibition zones. Three independent replicate experiments were carried out to determine antimicrobial activities of the endophytes isolated.

Endophyte fermentation and secondary metabolites extraction

Endophytes possessing antimicrobial activities were subjected to solid state fermentation. This was achieved by inoculating 7 mm agar plugs for each endophyte obtained from 7-day old axenic cultures into twenty separate 500 ml Erlenmeyer flasks containing 90 g of parboiled rice media. One flask without the inoculum was kept as a control. Solid state fermentation was conducted at 28°C for 21 days and flasks were periodically checked for any contaminants. The fermented endophyte cultures were cut into pieces using a spatula, then 150 ml of methanol was added and left to stand overnight in an ultrasonic cleaner (SB-120 DTN) to allow complete extraction of secondary metabolites. The mixture was filtered using a Whatman No. 1 filter paper followed by repeated extraction with methanol till exhaustion. The filtrate was evaporated in a rotary evaporator (BUCHI rotavapor R-205) under reduced pressure to yield methanol extract that was subjected to solvent partitioning with ethyl acetate and hexane. The ethyl acetate extract was then purified by Thin Layer Chromatography using the solvent system 6:4 (ethyl acetate: hexane) and column chromatography on silica gel.

Screening ethyl acetate fractions for antimicrobial activity

Antimicrobial activity for the endophytic fractions was conducted following Balouiri et al. (2016) protocol with slight modifications. The media used was MHA (65 g/L) and Nutrient broth (38 g/L). Briefly, overnight bacterial pathogen cultures containing 1.5×10^8 CFU/ml was spread on MHA media plates and left to air dry under sterile conditions. The endophytic extracts were dissolved in 0.1% Dimethyl Sulfoxide (DMSO) to achieve 5.0 mg/ml concentration. For each extract, 20 µl was used to impregnate 6 mm diameter sterile Whatman filter paper No. 1 discs and placed on petri dishes containing test pathogen. A standard chloramphenicol disc (30 µg/disc) was used as a positive control, while the negative control consisted of blank sterile disc soaked in 0.1% DMSO. The plates were incubated at 32±2°C for 24 h and followed by determination of zones of inhibition.

Minimum Inhibitory Concentration (MIC) Assay for *Nigrospora osmanthi* fractions was quantified using agar disc diffusion and their inhibitory effects assessed based on two-fold dilution following Hengameh and Rajkumar (2017) protocol with slight modification. Briefly, a stock solution of 5.0 mg/ml was prepared by dissolving 50 mg of the fractions in 10 ml of 0.1% DMSO. The stock solutions were further diluted to obtain the following concentrations (2.5, 1.25 and 0.625 mg/ml). Approximately 100 µl of freshly grown test bacterial cultures (24 h) containing 1.5×10^8 CFU/ml was spread on MHA media using a sterile swab and allowed to dry. Thereafter Whatman No. 1 filter paper discs (6 mm) were soaked in 50 µl of different concentrations of the extract fractions. Using sterile forceps, four discs containing different concentrations of the extracts were placed on MHA petri dishes. The positive control was standard chloramphenicol discs (30 µg/disc) while the negative control was sterile Whatman No.1 filter paper discs soaked in 0.1% DMSO. The petri dishes were incubated at 28°C for 24 h and the zones of inhibition determined.

Preparation of pure compound and analysis

Following fractionation, 150 µg of 1 mg/ml ethyl acetate extracts of LM-L(1) and AD-L(1) were prepared for all fractions and analyzed in a preparative High Performance Liquid Chromatography (HPLC) (Shimadzu CTO-20 AC) to establish the purity of the metabolites present in the fractions. The stationary phase consisted of silica gel (Germini® 10 µm C18 110Á (LC column 250 x 10 mm) packed column. The chromatogram obtained from the fraction analysis was used to determine the gradient for the preparative HPLC run. The mobile phase used for the gradients was methanol (solvent B) and distilled water (solvent A) with an injection volume of 150 µl. The detection was carried out at 254 nm wavelength. The fractions obtained were evaporated under reduced pressure in a Buchi rotary evaporator (RE 100-Pro). The pure compounds were analyzed by a combination of 1D and 2D NMR and mass spectroscopy and pure compounds subjected to antibacterial assay.

Data analysis

The mean inhibition zones were calculated and data generated for each endophyte were analyzed separately using two-way analysis of variance (ANOVA). The post-hoc analysis was conducted using Turkeys test (Honestly Significant Difference) at $p<0.05$ to determine significant differences between the means. Data analysis was conducted using SPSS programme version 20.

RESULTS

Isolation of endophytes

A total of three endophytes were isolated from leaves of *L. martinicensis* plants on SDA media. Fully grown axenic

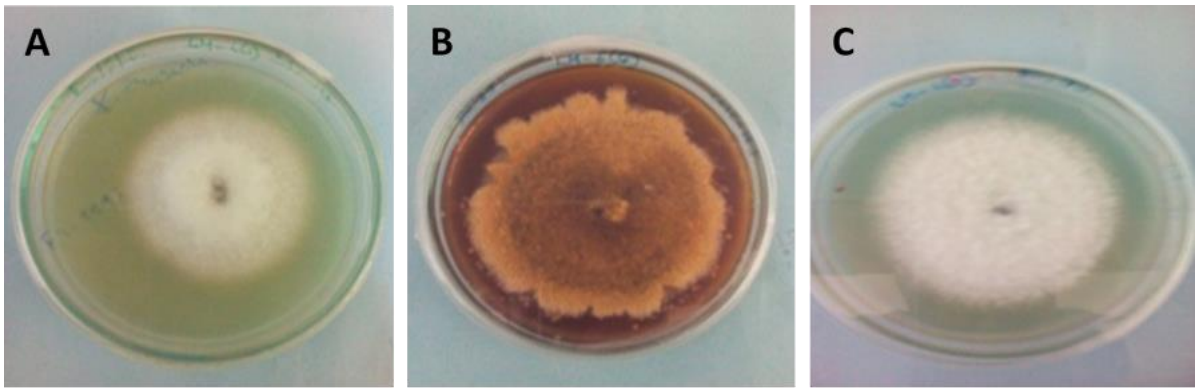

Figure 1. Morphological characteristics of fungal endophytes isolated from *L. martinicensis* leaves collected from Mt Elgon in Kenya. The colony form and colour ranged from (A) white colony with circular form; (B) red colony with irregular form; (C) white colony with filamentous form.

cultures displayed varied forms namely circular, irregular and filamentous, while colony colour was either red or white (Figure 1A-C).

Molecular identification of the endophytes

The ITS amplicons sequenced were 564bp, 533bp and 528bp for isolate LM-L (1), AD-L (1) and LM-S (6), respectively. Alignment of the three sequences using Clustal Omega also revealed variation between the ITS sequence nucleotide residues (Supplementary Figure 1). NCBI BLASTN-Targeted loci search using ITS sequences revealed that the three endophytes belong to phylum Ascomycota. In addition, determination of genus and species based on sequence identity of 100%, e-values of 0 and query coverage ≥ 90%ee, BLASTN-Targeted loci search using ITS revealed that isolate LM-L(1), AD-L(1) and LM-S(6) share sequence similarity with *Nigrospora osmanthi* (NR_153474.1), *Diaporthe novem* (NR_111855.1) and *Epicoccum italicum* (NR_158264.1), respectively. In contrast to BLASTN-Targeted loci hits, search against UNITE fungi identification databases revealed that ITS sequences for LM-L (1) AD-L(1) and LM-S(6) isolates share sequence similarity with *Nigrospora sphaerica* (GenBank: MH645137), *Diaporthe pseudolongicolla* (GenBank: KU672724) and *Epicoccum nigrum* (GenBank: MG719634), respectively. Alignment of *Nigrospora* and *Epicoccum* sequences revealed truncation of nucleotides in the 5'and 3' end for sequence obtained from BLASTN-Targeted loci database compared to the UNITE database, whereas for *Diaporthe* the sequences from the two database were identical except for a single nucleotide substitution at position 111 (Supplementary Figure 2).

Evolutionary relationship of the three fungal endophytes with the top 10 BLASTN-Targeted loci hits and UNITE sequences inferred using Maximum likelihood method (Neighbor Joining approach), identified AD-L(1) isolate up to genus *Diaporthe* level. Whereas LM-l(1) shares identity with two species from GenBank (*N. osmanthi* and *N. lactocolonia*) and one from UNITE (*N. sphaerica*) databases. On the other hand, LM-S(6) shares identity with *E. italicum* and *E. nigrum* from GenBank and UNITE databases, respectively (Figure 2).

Proliferation of endophytes is antagonistic to growth of test bacteria

Agar plugs from all the endophyte isolates inhibited growth of tested bacterial strains. Irrespective of the test bacteria, overall highest and lowest activity was obtained with isolate LM-L(1) and LM-S(6), respectively, although activity of LM-S(6) was not significantly *($p \geq 0.05$)* different from AD-L (1) isolate (Table 1). There were no significant *($p \geq 0.05$)* differences in the zones of inhibition produced by the three isolates against *E. coli* and *P. vulgaris*. On the other hand, LM-L(1) produced significantly ($p<0.05$) higher inhibition zone against *S. aureus*, *K. pneumoniae* and *S. typhi* compared to LM-S(6) isolate (Table 1). Though the endophytes inhibited growth of test bacteria, the positive control (chloramphenicol at 30 µg/disc) displayed a significant (*$p<0.05$*) activity of 2-3 fold higher than the endophytes (Table 1).

Endophytes ethyl acetate extracts display antibacterial activity

All ethyl acetate fractions obtained from *Diaporthe* sp. isolate displayed antibacterial activity against test bacteria except fraction F2, F3 and F4 when tested against *K. pneumoniae*. Generally, antibacterial activities of AD-L(1) fractions were not significantly (*$p \geq 0.05$*) different when tested against *E. coli*, *P. vulgaris* and *S. typhi*. On the other hand, when tested against *K. pneumoniae* and *S. aureus* significant ($p<0.05$) activity

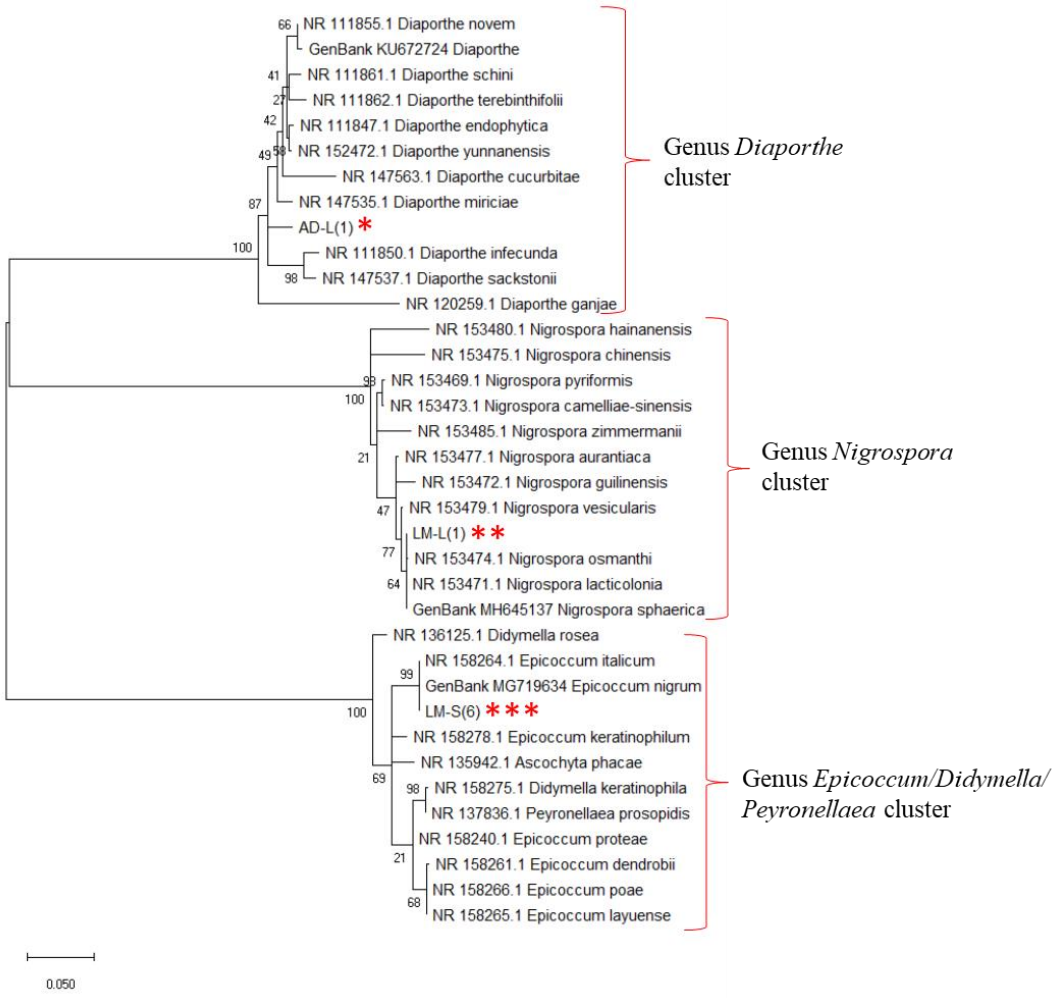

Figure 2. Evolutionary phylogenetic tree obtained from analysis of rDNA ITS sequences of fungal endophytes LM-L (1) AD-L (1) and LM-S (6) isolated from *L. martinicensis* leaves and their closest relatives obtained from GenBank using BLASTN-Targeted loci and UNITE fungal identification database. The tree was constructed with MEGA-X using Maximum likelihood method and 1000 bootstraps. The isolates LM-L (1) AD-L (1) and LM-S (6) clustered with fungi of the genus *Nigrospora*, *Diaporthe* and *Epicoccum*, respectively.

Table 1. Antibacterial activity of endophytic fungi isolated from *L. martinicensis* assessed through dual culture assay.

Isolate	Endophytic fungi	Inhibition diameter zones (mm)				
		Escherichia coli	***Proteus vulgaris***	***Staphylococcus aureus***	***Klebsiella pneumoniae***	***Salmonella typhi***
AD-L(1)	*Diaporthe*	8*±0.00$^{b\#}$	8.3±0.58^{b}	9.7±1.15^{bc}	8.3±0.58^{bc}	9±1.00^{b}
LM-L(1)	*Nigrospora*	10±1.73^{b}	10±1.00^{b}	10.7±0.58^{b}	9±1.00^{b}	9.3±0.58^{b}
LM-S(6)	*Epicoccum*	8±0.00^{b}	7±0.00^{b}	7±0.00^{c}	7±0.00^{c}	7±0.00^{c}
Chloramphenicol		22±2.00^{a}	20±2.00^{a}	32±2.00^{a}	32±1.00^{a}	22±1.00^{a}

*The values are the mean of the three experiments ± S.E of the mean. $^{\#}$Values in a column with same superscript are not significantly ($p \geq 0.05$) different based on Turkey HSD test.

was obtained with fraction F1 and F4, respectively (Table 2). Although ethyl acetate fractions showed activity against test bacterial strains, the activity of chloramphenicol was 2-3 fold higher (Table 2).

For endophyte LM-L(1), antibacterial activity of fractions F1 to F4 against *P. vulgaris*, *E.* coli and

Table 2. The activity of ethyl acetate secondary metabolite fractions extracted from AD-L(1) (*Diaporthe* sp.) endophyte isolate against test bacteria strains.

Fraction	Inhibition diameter zones (mm)				
	Escherichia coli	***Proteus vulgaris***	***Klebsiella pneumoniae***	***Salmonella typhi***	***Staphylococcus aureus***
F1	8*±0.58$^{b\#}$	9±0.58^{b}	10±0.58^{b}	9.7±0.88^{b}	8±0.58^{c}
F2	9±0.58^{b}	10.3±0.88^{b}	0±0.00^{c}	8±0.58^{b}	8±0.58^{c}
F3	9±0.58^{b}	8.7±1.20^{b}	0±0.00^{c}	11±0.58^{b}	9.3±0.33^{c}
F4	9.3±0.88^{b}	8±0.58^{b}	0±0.00^{c}	10.3±0.88^{b}	13.7±0.88^{b}
Chloramphenicol	21±2.00^{a}	21±1.76^{a}	32±0.58^{a}	22±0.58^{a}	32±1.15^{a}
DMSO	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}	0±0.00^{d}

*Values are the mean of the three replicate experiments ± S.E of the mean. #Values in a column with same superscript are not significantly (p≥0.05) different based on Turkey HSD test.

Table 3. The activity of ethyl acetate secondary metabolite fractions obtained from LM-L(1) (*Nigrospora* sp.) against test bacteria.

Fraction	Inhibition diameter zones (mm)				
	Escherichia coli	***Proteus vulgaris***	***Klebsiella pneumoniae***	***Salmonella typhi***	***Staphylococcus aureus***
F1	8*±1.00$^{b\#}$	10±1.15^{b}	8.3±0.67^{b}	9.7±0.33^{b}	8.7±0.67^{c}
F2	8.7±1.20^{b}	11.3±2.03^{b}	8.7±0.88^{b}	0±0.00^{c}	10.7±0.67^{c}
F3	8±0.58^{b}	10.7±2.19^{b}	7±0.00^{b}	0±0.00^{c}	15±0.58^{b}
F4	8.3±1.33^{b}	12±2.08^{b}	8±0.58^{b}	0±0.00^{c}	15±1.53^{b}
Chloramphenicol	21±1.15^{a}	20±1.15^{a}	33±0.58^{a}	22±0.58^{a}	32±1.15^{a}
DMSO	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}	0±0.00^{c}	0±0.00^{d}

*Values are the mean diameter (mm) of three replicate experiments ± S.E of the mean. #Values in a column with same superscript are not significantly (*p≥0.05*) different based on Turkey HSD test.

K. pneumonia bacteria were not significantly (p≥0.05) different, while only discs impregnated with fraction F1 showed activity against *S. typhi*. The activity of fraction F3 and F4 compared to F1 and F2 were significantly (p<0.05) different when tested against *S. aureus* (Table 3). However, activity of chloramphenicol was significantly (p<0.05) higher than those obtained on discs impregnated with LM-L(1) fractions which was approximately 2-3 fold higher (Table 3).

Antibacterial activity of increasing concentrations of ethyl acetate fractions

The MIC assay showed that increasing the concentration of AD-L(1) fractions from 0.625 to 5.0 mg/ml led to increased antibacterial activity for fractions F2 and F3, with highest activity obtained at 5.0 mg/ml. Antibacterial activity at 5.0 mg/ml for fraction F2 and F3 against *E. coli* and *S. typhi* was significantly (p<0.05) higher compared to *P. vulgaris*, *S. aureus* and *K. pneumoniae* where 5.0 mg/ml concentration was only significantly different from the activity of 0.625 mg/ml (Table 4).

For LM-L(1) MIC assay, all the fractions concentrations tested, showed activity against all test bacteria. Similar to AD-1(L) increasing fractions concentration from 0.625 mg/ml to 5.0 mg/ml resulted to increased antibacterial activity against test bacteria (Table 5). Furthermore, in all fractions the discs impregnated with 5.0 mg/ml generally had significantly (*p<0.05*) higher activity compared to those impregnated with 0.625 and 1.25 mg/ml. This trend was mainly observed against *E. coli*, *P. vulgaris* and *K. pneumoniae*, while for *S. aureus* and *S. typhi* the trend was observed for fractions F3 and F4, and fraction F4, respectively. Unlike in the disc diffusion assay data presented in Table 5, fraction F2-F4 produced activity against *S. typhi* (Table 5). Highest activity was obtained at a concentration of 5.0 mg/ml against *P. vulgaris* produced by discs impregnated with all fraction (F2, F3 and F4). However, only discs impregnated with F3 at 5.0 mg/ml produced activity that was not significantly (*p≥0.05*) different from the positive control chloramphenicol antibiotic (Table 5). LM-L(1) and AD-L(1) ethyl acetate fractions subjected to column chromatography and HPLC produced two pure compounds. For LM-L(1), a compound 4, 7- dihydroxy-9-methoxy-1-methylchromen-6-one was obtained from fraction F2, as a white powder with a mass of 4.3 mg

Table 4. Minimum inhibitory concentrations (mg/ml) of ethyl acetate fraction of secondary metabolites obtained from AD-L(1) isolate (*Diaporthe* sp.) against test bacteria.

Fraction: Conc. (mg/ml)	Inhibition diameter zones (mm)				
	Escherichia coli	***Proteus vulgaris***	***Staphylococcus aureus***	***Salmonella typhi***	***Klebsiella pneumoniae***
F2 5.0	14.7±0.67^{b}	14±1.15^{b}	14.3±0.88^{b}	13.3±0.67^{bc}	11.3±0.67^{cd}
F2 2.5	12.7±0.67^{bc}	12±1.15^{bc}	12.7±0.67^{bc}	10.7±0.67^{cd}	9.7±0.33^{de}
F2 1.25	10.7±0.67^{cd}	10.3±0.88^{bc}	10.7±0.67^{bcd}	10±0.58^{d}	8.3±0.33^{de}
F2 0.625	8.3±0.33^{de}	8±0.58^{c}	8±0.58^{d}	8±0.58^{d}	7.3±0.33^{e}
F3 5.0	13.3±0.67^{bc}	12±1.15^{bc}	14±1.15^{b}	15±0.58^{b}	16±1.15^{b}
F3 2.5	10.7±0.67^{cd}	10.3±0.88^{bc}	12±1.15^{bc}	13±0.58^{bc}	14±1.15^{bc}
F3 1.25	9±0.58^{de}	9±0.58^{c}	10.3±0.88^{bcd}	10.7±0.67^{cd}	12±1.15^{cd}
F3 0.625	7.3±0.33e	7.7±0.33^{c}	8.3±0.88^{cd}	8.3±0.33^{d}	9±0.58^{de}
Chloramphenicol	22±1.15^{a}	20±1.15^{a}	32±1.15^{a}	23±0.58^{a}	33±0.58^{a}
DMSO	0±0.00^{f}	0±0.00^{d}	0±0.00^{e}	0±0.00^{e}	0±0.00^{f}

*The values are the mean diameter (mm) of three replicate experiments ± S.E of the mean. #Values in a column with same superscript are not significantly (*p≥0.05*) different based on Turkey HSD test.

While for AD-L(1) a compound 4, 7, 9- trihydroxy-1-methylchromen-6-one was obtained from F3 fraction. Unfortunately, none of the two pure compounds obtained failed to display activity against all test bacterial strains.

DISCUSSION

Isolation of fungal endophytes

Fungi are known to produce bioactive compounds which can be useful in agricultural, industrial and pharmaceutical applications. However, isolation and characterization of fungi producing these useful compounds is crucial for large-scale production. In our study, isolation of endophytic fungi from medicinal plant *L. martinicensis* focused on leaf tissues. This choice was informed by reports indicating that distribution of endophytic fungi is organ specific with highest numbers and diversity obtained from leaf tissue (Suryanarayanan, 2013; Banhos et al., 2014). Despite leaves being a rich source of endophytes, from leaves of 20 different *L. martinicensis* plants sampled in our study we isolated a total of three different endophytes. This number of isolates is relatively low, especially when compared to other studies. For example, Pádua et al. (2018) isolated 187 fungi endophytes from *Myracrodruon urundeuva* leaves. Although plant tissue has influence on endophyte population and diversity, however other factors such as host species, host developmental stage, density of inoculum and environmental conditions has also great influence on population and diversity too (Wanga et al., 2018; Dudeja and Giri, 2014). While the number of endophytes was low, the isolates represented three fungi genera obtained from *L. martinicensis* leaves collected from undisturbed section of tropical rain forest. Since only leaf tissue was considered in our study, sampling of different plant part of *L. martinicensis* may yield more diverse endophytes.

Identification of fungal endophytes

Cultural and morphological characteristic distinguished the endophytes isolated, however, it was difficult to characterize them up to genus level, since all the isolates failed to sporulate on PDA media. The efficiency and consistency of the rDNA ITS in identification of Ascomycetes has been demonstrated in various studies (Raja et al., 2017; Hibbett et al., 2016). Similarly, we were able identify the three endophytes through ITS-approach unlike morphological, furthermore phylogenetic analysis confirmed that indeed the three endophytic fungi belonged to phylum Ascomycota and but from different genera. On the other hand, inconsistency in identification of the endophyte isolates at species level using ITS sequences in GenBank and UNITE databases suggests variation between ITS sequences deposited in the two databases. There are propositions that relying on ITS-based identification of fungal isolates based on GenBank search alone could lead to misidentification, because it may contain erroneous names associated with ITS sequences, unlike for UNITE database, which is often curated by mycology experts (Raja et al., 2017).

Antibacterial assay

All endophytes inhibited growth of *E. coli*, *P. vulgaris*, *K. pneumoniae*, *S. typhi* and *S. aureus* bacteria. This clearly demonstrates that these endophytes possess antibacterial compounds. This is in line with reports indicating that

Table 5. Minimum inhibitory concentrations (mg/ml) for fractions obtained from LM-L(1) isolate (*Nigrospora* sp.) against test bacteria.

Fraction: conc. (mg/ml)	Inhibition diameter zones (mm)				
	Escherichia coli	***Proteus vulgaris***	***Staphylococcus aureus***	***Salmonella typhi***	***Klebsiella pneumoniae***
F2: 5.0	13.3*±0.67$^{b\#}$	15.3±0.67^{cd}	11±0.58^{cde}	12±1.15^{cd}	11.7±0.33^{cd}
F2: 2.5	11±0.58^{bcd}	13±0.58^{cde}	9.7±0.33^{defg}	10.3±0.88^{cde}	10.3±0.33^{cde}
F2: 1.25	9.3±0.67^{cd}	9.7±0.33^{efg}	8.7±0.33^{efg}	8.7±0.33^{de}	8.7±0.33^{ef}
F2: 0.625	8±0.58^{d}	7.7±0.33^{g}	7.3±0.33^{g}	7±0.00^{e}	7±0.00^{f}
F3: 5.0	13±0.58^{b}	19±0.58^{ab}	14±0.58^{b}	11.3±0.67^{cd}	12.7±0.67^{bc}
F3: 2.5	11±0.58^{bcd}	15.7±0.33^{bc}	12.7±0.67^{bc}	9.7±0.33^{de}	10.3±0.33^{cde}
F3: 1.25	10±0.58^{bcd}	13±0.58^{cde}	10.7±0.67^{cdef}	8.7±0.33^{de}	9.3±0.33^{def}
F3: 0.625	8.3±0.33^{cd}	9.3±0.33^{efg}	9±0.58^{efg}	7.3±0.33^{e}	7.7±0.33^{f}
F4: 5.0	11.7±0.88^{bc}	16.3±1.33^{bc}	12±0.58^{bcd}	16±1.15^{b}	14.7±0.67^{b}
F4: 2.5	10.3±0.88^{bcd}	14.3±0.88^{cd}	10.7±0.67^{cdef}	14±1.15^{bc}	12.7±0.67^{bc}
F4: 1.25	9±0.58^{cd}	12±1.15^{def}	9.3±0.33^{defg}	10.7±0.67^{cde}	11±0.58^{cd}
F4: 0.625	7.7±0.67^{d}	8.7±0.33^{fg}	8±0.58^{fg}	8.7±0.33^{de}	9±0.58^{ef}
Chloramphenicol	22±1.15^{a}	20±1.15^{a}	32±0.88^{a}	23±1.15^{a}	33±0.58^{a}
DMSO	0±0.00^{e}	0±0.00^{h}	0±0.00^{h}	0±0.00^{f}	0±0.00^{g}

*Values are the mean diameter (mm) of three replicate experiments ± S.E of the mean. #Values in a column with same superscript are not significantly (*p≥0.05*) different based on Turkey HSD test.

fungi of *Nigrospora* sp., *Diaporthe* sp. and *Epicoccum* sp. are known to produce different metabolites with antimicrobial activity against both Gram positive and negative bacteria (Meepagala *et al*., 2015; Wu et al., 2019). Species of the genus *Diaporthe* are known to produce a huge number of exceptional high and low molecular weight metabolites and polyketides with different antibacterial activities as well as biocontrol of fungal pathogens (Gomes et al., 2013).

Fractions obtained from *Nigrospora* isolate exhibited varied level of activity depending on the concentration of the extracts. For instance, the most notable activity was obtained with fraction F3 at 5.0 mg/ml against *P. vulgaris*, which was comparable to chloramphenicol (30 µg/disc). The response obtained with *Nigrospora* fraction F3 demonstrated that the semi pure compound had anti *P. vulgaris* activity similar to chloramphenicol. This result obtained with *Nigrospora* isolate fraction is in line with reports in literature and according to Chen et al. (2016), members of *Nigrospora* genus are thought-provoking sources of natural products for pharmaceutical uses. Secondary metabolites possessing antimicrobial activities such as 6-phenylhexanoic acid derivative, Uridine, Phomalactone among others also possess antimicrobial activity have been extracted from *Nigrospora* species (Chen et al., 2012). Antibacterial activity of metabolites extracted from *Epicoccum* showed relatively low activity. In contrast to our study, species of genus *Epicoccum* have been reported to produce active metabolites against Gram-positive and Gram-negative bacteria (Dzoyem et al., 2017; Perveen et al., 2017). Despite fractions of the endophyte secondary metabolites showing activity against all the test bacterial strains, there was no activity observed for pure compounds prepared from the fractions obtained. Lack of antibacterial activity for the pure compounds suggests that the antibacterial activity observed in the fractions could be due to the interaction of two or more compounds.

Conclusions

To the best of our knowledge the study reported herein is the first with regard to isolation and evaluation of antibacterial activities of endophytes associated with *L. martinicensis* leaves. Despite lack of antibacterial activities for pure compounds prepared, overall the results indicate that isolated *L. martinicensis* endophytes possess antimicrobial secondary metabolites which could be exploited further or be lead compound for developing drugs for treatments of microbial infections.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY MATERIAL

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CLUSTAL O(1.2.4) multiple sequence alignment


LM-S(6)      TCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTTGTGGACTTCGGTCTG-----   55
ADL(1).      TCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCTTCGGCGCACCCAGAAACC   60
LML(1).      TCCGTTGGTGAACCAGCGGAGGGATCATTACAGAGTTATCCAACTCCCAAA--------C   52
             ***** ******** ***** ******** *

LM-S(6)      ----------------------CTACCTCTTACCCATGTCTTTTGAGTACCTTC---GTT   90
ADL(1).      CTTTGTGAACTTATACCTACTGTTGCCTCGGCGCAGGCCGGCTTCCTCACCGAAGCCCCC   120
LML(1).      CCATGTGAACATATCTC-TTTGTTGCCTCGGCGCAAGCTACCCGGG-----------ACC   100
                              * ****    *

LM-S(6)      TCCTCGGCGGGTCCGCCCGCCGGTTGGACAACATTCAAACCCTTTGCAGTTGCAATCAGC   150
ADL(1).      TGGAAACAGGGAGCAGCCCGCCGGCGGCCAACTAAACTCTGTTTCTATAGTGAATCTCTG   180
LML(1).      TCGCGCCCCGGGCGGCCCGCCGGCGGACAAACCAAACTCTGTTATCTTCGTTGATTATCT   160
             *        **     **  * *  *   ***          *       *  *

LM-S(6)      GTCTGAAAAAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT   210
ADL(1).      AGTAAAAAACATAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT   240
LML(1).      GAGTGTCTTATTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT   220
                      *  * * * ************************************

LM-S(6)      GAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAAT   270
ADL(1).      GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT   300
LML(1).      GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT   280
             *************************** ********************************

LM-S(6)      CTTTGAACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTT   330
ADL(1).      CTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCATTT   360
LML(1).      CTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTT   340
             ********************  * ******    *************************

LM-S(6)      GTACCTTCAAGCTCTGCTTGGTGTTGGGTGTTTTGTCTCGCCTCCGCGCGCAGACTCGCC   390
ADL(1).      CAACCCTCAAGCCTGGCTTGGTGATGGGGCAGTGCCTTGGAGACAAGGCACGCCCTGAAA   420
LML(1).      CAACCCCTAAGCACAGCTTATTGTTGGGCGTCTACGTCTGTAGTGCCTCAAAGACATTG-   399
               ***   ****   ****  ** ****    *      *        *     *

LM-S(6)      TTAAAACAATTGGCAGCCGGCGTAT-TGATTTCGGAGCGCAGTACATCT-CGCGCTTTGC   448
ADL(1).      TCCAGTGGCGAGCTCGCCAGGACCCCGAGCGTAGTAGTTATATCTCGCTCTGGAAGGCCC   480
LML(1).      -----GCGGAGCGGCAGCAGTCCTCTGAGCGTAGTAATTCTTTATCTCGCTTCTGTTAGG   454
                        * *           * * *      *    *

LM-S(6)      ACTCATAACGACGACGTCCAAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGG   508
ADL(1).      TGGCGGTGCCCTGCCGTTAAACCCCCAACTTCTGAAAATTTGACCTCGGATCAGGTAGGA   540
LML(1).      CGCTGCCCCCCCGGCCGTAAAACCCCCAATTTTTTCTGGTTGACCTCGGATCAGGTAGGA   514
                     *   * *    **      * ** *      ********************

LM-S(6)      ATACCCGCTGAACTTAAGCA----      528
ADL(1).      ATACCCGCTGAACTTAAGCATATC      564
LML(1).      ATACCCGCTGAACTTAAGC-----      533
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Figure 1. CLUSTAL Omega alignment of the Three *Leucas martinicensis* endophyte rDNA ITS sequences

(a) Isolate LM-S(6)

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LM-S(6)                -------TCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTTGTGGACTTCGGTC   53
ENUnite                CAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTTGTGGACTTCGGTC   60
Epicoccumitalicum      ------------------------------ATCATTACCTAGAGTTTGTGGACTTCGGTC   30
                                                      ****************************

LM-S(6)                TGCTACCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTCCGCCCGCCGG   113
ENUnite                TGCTACCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTCCGCCCGCCGG   120
Epicoccumitalicum      TGCTACCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTCCGCCCGCCGG   90
                       ************************************************************

LM-S(6)                TTGGACAACATTCAAACCCTTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTAC   173
ENUnite                TTGGACAACATTCAAACCCTTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTAC   180
Epicoccumitalicum      TTGGACAACATTCAAACCCTTTGCAGTTGCAATCAGCGTCTGAAAAAACTTAATAGTTAC   150
                       ************************************************************

LM-S(6)                AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA   233
ENUnite                AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA   240
Epicoccumitalicum      AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA   210
                       ************************************************************

LM-S(6)                AGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCT   293
ENUnite                AGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCT   300
Epicoccumitalicum      AGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCT   270
                       ************************************************************

LM-S(6)                TGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTG   353
ENUnite                TGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTG   360
Epicoccumitalicum      TGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTGCTTGGTG   330
                       ************************************************************

LM-S(6)                TTGGGTGTTTTGTCTCGCCTCCGCGCGCAGACTCGCCTTAAAACAATTGGCAGCCGGCGT   413
ENUnite                TTGGGTGTTTTGTCTCGCCTCCGCGCGCAGACTCGCCTTAAAACAATTGGCAGCCGGCGT   420
Epicoccum italicum      TTGGGTGTTTTGTCTCGCCTCCGCGCGCAGACTCGCCTTAAAACAATTGGCAGCCGGCGT  390
                       ************************************************************

LM-S(6)                ATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGT   473
ENUnite                ATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGT   480
Epicoccum italicum      ATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACGTCCAAAAGT  450
                       ************************************************************

LM-S(6)                ACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCA--      528
ENUnite                ACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATA      537
Epicoccum italicum      ACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACC-----------------     490
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(b)Isolate AD-L(1)

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ADL(1)                 -----------TCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCTTCGGCGC      49
DPUnite                GTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCTTCGGCGC      60
Diaporthe novem         --AACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCTTCGGCGC     58
                              ***********************************************

ADL(1)                 ACCCAGAAACCCTTTGTGAACTTATACCTACTGTTGCCTCGGCGCAGGCCGGCTTCCTCA      109
DPUnite                ACCCAGAAACCCTTTGTGAACTTATACCCACTGTTGCCTCGGCGCAGGCCGGCTTCTTCA      120
Diaporthe novem         ACCCAGAAACCCTTTGTGAACTTATACCCACTGTTGCCTCGGCGCAGGCCGGCCTCTTCA     118
                       *************************** ************************ ** ***

ADL(1)                 CCGAAGCCCCCTGGAAACAGGGAGCAGCCCGCCGGCGGCCAACTAAACTCTGTTTCTATA      169
DPUnite                CTGAGGCCCCCTGGAAACAGGGAGCAGCCCGCCGGTGGCCAACTAAACTCTGTTTCTATA      180
Diaporthe novem         CTGAGGCCCCCTGGAAACAGGGAGCAGCCCGCCGGCGGCCAACTAAACTCTGTTTCTATA     178
                       * ** ****************************** ************************

ADL(1)                 GTGAATCTCTGAGTAAAAAACATAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTT      229
DPUnite                GTGAATCTCTGAGTAAAAAACATAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTT      240
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Figure 2. Clustal Omega alignment of the Three *Leucas martinicensis* endophyte rDNA ITS sequences with Hits from Unite and best hits from NCBI-Targeted loci BLAST.