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In vitro Anti-staphylococcal Potential of Endophytic Fungi from *Aegle marmelos*

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***Staphylococcus aureus* is the third most dreaded human pathogen which is getting refractory to current armamentarium of antimicrobial drugs. Endophytic fungi have been recognized as a fountainhead of novel bioactives for human therapeutic intervention and drug development. In the present investigation we report isolation and screening of endophytic fungi isolated from *Aegle marmelos* inhabiting conserved forest area in Western Ghats, India. A total of 25 endophytic fungi representing 10 genera were isolated by adopting a standardized protocol. *Fusarium* species exhibited maximum colonization (28%), followed by *Aureobasidium* (12%) and *Lasiodiplodia* species (8%). *In vitro* screening of culture broth of these fungi using a standardized anti-microbial assay exhibited activity only in one isolate #1005AMLBRT. Subsequently ethyl acetate extracts of the culture broths revealed that 15 isolates did not possess any anti-staphylococcal potential. However the best anti-staphylococcal potential was found in ethyl acetate extract of #1005AMLBRT. #1005AMLBRT was found to be a new species of *Alternaria* named as *Alternaria marmelos* based on phylogenetic and morphological studies. Further studies on *Alternaria marmelos* are warranted for production, isolation and characterization of the anti-staphylococcal principle.**

Keywords: Endophytic fungi, Anti- Staphylococcal activity, Fermentation broth, *Alternaria, Aegle marmelos*.

Staphylococcus aureus has been recently identified as the third most dreaded pathogen responsible for mortality and morbidity in hospitals and community due to its refractory behaviour[1]. *S. aureus* is an opportunistic pathogen which causes soft tissue and skin infections, respiratory infections, osteomyelitis, endocarditis and pneumonia[2]. Patients with prosthetic devices are also found to be prone to resistant Staphylococcal infections apart from those who have undergone invasive surgical procedures[3]. Methicillin resistant *Staphylococcus aureus* (MRSA) and its resistant clones are responsible for nosocomial infections as well as community epidemics[4-6]. Currently MRSA and its variants are posing a critical situation for clinicians since they are frequently getting refractory to current armamentarium of antibiotics[1,7]. Accordingly it is imperative to explore for new anti-MRSA agents to treat chronic staphylococcal infections.

Endophytic fungi largely inhabit as biotopes namely in higher plants and therefore have been considered as wellspring of compounds exhibiting diverse activities like anti-microbial, anti-fungal, anti-tumor, anti-oxidant etc. Endophytic fungi are ubiquitous in healthy tissues amongst all plants and have been accepted a rich source of bioactive compounds[8,-10]. Metabolites produced by fungal endophytes could be used as an arsenal for pathogenic bacteria as they possess better biosynthetic abilities presumably as a result of their

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gene recombination with the host genes while residing within the healthy plant tissue[11, 12]. There are several reports on the broad spectrum antimicrobial activity of fungal endophytes from medicinal and higher plants[12-16] but limited experimental information exists on emphasis of single pathogenic bacteria vis. *Helicobacter pylori*[5], *Mycobacterium tuberculosis*[17], *Staphylococcus aureus* etc.

Aegle marmelos or Bael (Family, Rutaceae) is a traditional medicinal plant possessing a moderate size and aromatic nature. Bael has enormous traditional uses against various diseases and many bioactive compounds have been identified and isolated from this plant[18]. Traditionally the plant has been used for treating intermittent fever, intestinal ailments, fertility control and treatment after childbirth and fish poison[19]. The fruit of *Aegle marmelos* possess anti-diarrhoel and anti-dysentery properties leading to its inclusion in the British Pharmacopeia[20]. Further studies have indicated that the roots of *Aegle marmelos* also exhibit anti-diarrheal properties, methanolic extract of fruit possess a very strong activity against multidrug resistant *Salmonella typhi*[21, 22].

In the present paper we report the evaluation of fermentation extracts of endophytic fungi of the medicinal plant *Aegle marmelos* for its anti-staphylococcal potential and identification of the isolate exhibiting potential anti-staphylococcal activity.

MATERIALS AND METHODS

Plant Sample collection

The plant samples were collected from the heart of conserved rain forest area geographically located at 11°362 183 N 76°042 593 E and 11°592 383 N 77°82 263 E in the Western Ghats region of India during rainy season after seeking due permission from Ministry of Environment and Forests, Govt. of India. The samples were properly sealed in sterilized sample pouches and stored at 4°C till further use.

Isolation of endophytic fungi

For isolation of the endophytic fungi, adult and healthy leaves and stem of *Aegle marmelos* were surface sterilized by aseptically immersing in 1 % sodium hypochlorite solution for 2-3 minutes followed by dipping in 70 % ethanol for 1-2 min. and finally by washing with 30% ethanol. The surface sterilized samples were then cut into 1 mm pieces with the help of a sterile blade. These 1mm pieces were inoculated on PDA plates containing 50 µg/mL of chloramphenicol with the ventral side facing media surface. Maximum of eight pieces were inoculated in single plate. The plates were incubated at 26 ± 2°C for maximum of 25 days[13]. The cultures which appeared on the plate were subsequently transferred to PDA plates containing no antibiotic to obtain pure culture. Endophytes were numbered and stored on PDA slants at 4°C.

Fermentation

Mycelial plugs of 5mm diameter of 7-days old fungal culture was inoculated in 50 ml pre-sterilized Potato Dextrose Broth (PDB), pH 5.1 (Hi Media) in Erlenmeyer flasks (Schott Duran). The flasks were incubated at shaker at 26°± 2°C, 120 rpm for 12 days for production of secondary metabolites[23]. After 12 days broth was separated from mycelia by filtration. Filtration was carried out aseptically using a muslin cloth and subsequently through Whatman paper 4 [23]. Solvent fractionation of the cell free filtrates was carried out using Ethyl acetate. The solvent fraction of different fungal fermentation broths so obtained were freeze dried to obtain residues for testing their antimicrobial activity.

Test Microorganisms

Standard and clinical isolates of *Staphylococus aureus* were used as test organism. S. aureus NCTC 6571 was used a standard isolate while *S. aureus* G3 (Pus Culture, MRSA and VRSA) *S.aureus* G26 (Burn culture) were the clinical isolates tested. Clinical isolates were collected from Govt. Rajindra Medical College, Patiala.

In vitro antimicrobial activity

Agar Well Diffusion Assay, Wells of 5 mm were scooped with the help of pre sterilized cork borer on MH agar (Hi Media) plates to provide a depth of 4 to 5 mm. 30 µl (Stock of 6.4 mg/ml of each residue) of the cell free filtrate / solvent extract in DMSO was dispensed in the wells. Subsequently solvent along with DMSO used for the dissolution of the residues was also loaded as the control and allowed to diffuse for 15 min. Thereafter wells were sealed with molten MH agar. Finally after, 15 min. the plate was swabbed with 18 24 hrs old 0.5 McFarland adjusted culture of the

test isolate. Antibacterial activity was determined by measuring halo formation. All the tests were performed in triplicates[24-25].

DNA extraction and PCR amplification potential anti-staphylococcal exhibiting fungal endophyte

The fungal DNA was extracted from 3-4 day old culture grown on PDA using the Wizard® Genomic DNA purification kit (Promega, USA). 2-3 discs (0.5 mm) of cultured mycelia were cut from 3-4 day old culture and crushed in liquid nitrogen to a fine powder. Further DNA extraction was carried out according to the instructions of kit manufacturer.

The ITS1, 5.8S, ITS2 rDNA sequence was amplified using a Thermocycler (My Cycler, Bio-Rad Laboratories, Inc.) PCR reaction was carried out by using the primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') AND ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3'). The PCR reaction was performed in a 25µl reaction mixture containing 1µl of extracted fungal DNA, 10 µM of each primer (ITS1 and ITS4), 2.5mM of dNTP (Bangalore GeNei), 25 mM $MgCl_2$ (Bangalore GeNei), 1.5 U of Taq DNA Polymerase (Bangalore GeNei) in 10 X Taq buffer (Bangalore GeNei). The PCR cycling conditions consisted of initial denaturation at 96ºC for 5 min followed by 39 cycles of 95ºC for 45 sec, 60ºC for 45 sec, 72ºC for 45 sec followed by final extension at 72ºC for 5 mins. The PCR products were examined using gel electrophoresis in a 1.5 % agarose gel dissolved in 1X TAE buffer at 40V for 1.30 hr. Gel imaging was performed under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software. An approximate 500 bp PCR product was purified by using the Wizard® SV Gel and PCR clean up system kit (Promega, USA). Purified PCR products were sequenced (**Xcelris Genomics,** Xcelris Labs Ltd. Ahemadabad, Gujarat, India) by using 96 capillary high through put sequencer (ABI 3730 XL).

Phylogenetic analysis

The ITS/5.8 rRNA sequences obtained from the respective primers were aligned and the consensus sequence was deposited in GenBank NCBI database under the accession numbers JN400741. Further the consensus sequence was subjected to a NCBI-BLAST search to verify the identity of #1005AMLBRT. The sequences were edited with BioEdit 5.0.6 and aligned using MAFFT v 6.240 with other sequences obtained from GenBank. The alignment acquired from MAFFT was submitted to TreeBASE (http,//www.treebase.org) and obtained the submission ID of 11798. GARLI 2.0 (26) was used to perform the maximum likelihood analysis with default parameters except that the number of searches was brought to 5. The resultant best tree having the lowest likelihood ratio was selected and edited in MEGA 4.0. Branch support was estimated by performing 100 bootstrap replicates (27) in GARLI. The resulting trees were fed into PAUP version 4b10 [28] to obtain a majority rule consensus tree. Bayesian posterior probabilities (PP) for each internodes were calculated with a Metropolis-coupled Markov Chain Monte Carlo (MCMC) sampling method as implemented in MrBayes version 3.1 [29]. Six simultaneous Markov chains were run for one million generations (resulting 10K total trees). The first 2,000 trees were discarded and the remaining 8,000 were used for calculating PP in the majority rule consensus rule tree. These analyses were repeated five times starting from different random trees to ensure trees from the same space were being sampled during each analysis.

RESULTS AND DISCUSSION

25 fungal isolates were recovered from the leaves, stem and internal stem tissue of *Aegle marmelos* representing 10 endophytic taxa. The distribution of the endophytic fungus varied, maximum isolates were recovered from the internal tissue of the stem. In all six *Fusarium* species were isolated, two each from leaves, stems and internal stem tissue. One *Alternaria* species was reported from the leaves of *Aegle marmelos.* Three endophytic *Aureobasidium* were also isolated from internal tissue of the stem of *Aegle marmelos*. (Table 1). 2 isolates of *Lasiodiploida* sp. were also isolated from internal tissue of the stem of *Aegle marmelos*. Other isolates included one each of *Sphaeropsis sp., Barriopsis* sp., *Cunninghamella* sp. and *Penicillium* sp. All the cultures were identified on the basis of morphological and cultural characteristics[30].

***In vitro* Anti-Staphylococcal assay of spent broth**

Spent PDB of all these fungal isolates were subjected to screening for their anti-staphylococcal potential against a panel of three organisms comprising of control and clinical

isolates of *Staphylococci* exhibiting Methicillin and Vancomycin resistance. Only one spent broth of #1005 AMLBRT exhibited antimicrobial activity in the in vitro agar well diffusion assay with zone sizes of 13 mm, 14mm and 15mm respectively against *S. aureus* NCTC6571, Sau G3 and SauG26 respectively (Fig.1).

***In vitro* assay of ethyl acetate fraction of fungal spent broth for Anti-Staphylococcal assay**

Highest broad spectrum anti-staphylococcal activity was exhibited by ethyl acetate fraction of spent broth of #1005 AMLBRT (Fig 2). It was followed by #6 AMLWLS> #1079AMSTITYEL > # 9AMLBRT (Fig.3). The diameter of inhibition zone against the test isolates ranged between 18.0-26.7 mm, 17.0-21.7 mm, 14.7-19.7 mm and 12.3-18 mm respectively for #1005 AMLBRT, #6AMLWLS, #1079 AMSTITYEL and #9AMLBRT. 15 endophytic fungal isolates did not exhibit any inhibitory activity in the ethyl acetate fraction of their spent broth against any of the test Staphylococcal isolates. Ethyl acetate extracts of fermentation broth of fungal endophytes from *Mallus haliana* were found to possess broad spectrum anti-bacterial activity. However *Alternaria brassicicola* ML-P08 was selected from them as it possessed the strongest activity against all the isolates in the test panel[31].

Phylogenetic identification of #1005 AMLBRT

As #1005 AMLBRT exhibited potential anti-staphylococcal activity, rDNA analysis was carried out using consensus sequence (JN400741) from genomic DNA after amplification of ITS1 and ITS4 primers followed by BLASTN. The BLASTN report revealed close similarity of #1005 AMLBRT to the genus *Alternaria* (Table 2). Subsequently a data matrix was obtained after aligning 24 BLAST sequences with the consensus sequence of endophyte #1005AMLBRT (JN400741). Maximum likelihood analysis using GARLI 2.0 was conducted using the aligned data matrix comprising of a total of 1023 characters of which 907 (88.7%) were constant, 80 (7.8%) un-informative variable and 36 (3.5%) parsimony informative. The best tree obtained with lowest likelihood ratio of -2155.2073 has been represented in Fig.4. Bootstrap values (≥90%) are based on 100 replicates while PP values are presented in brackets. The branch support is

Table 1. Endophytic fungi isolated from leaves and Stem of *Aegle marmelos* from western Ghats

S. No.	Endophytic fungi	Cultures isolated from different part plants			Total No.
		Leaf	Stem	Internal tissue of Stem	
1	*Fusarium sp.*	# 9AMLBRT, #6 AMLWLS	#9(b)AMSTYEL, #7 AMSTYEL	#1070 AMSTITYEL, #1022 AMSTITYEL, #1007 AMLBRT	07
2	*Alternaria sp.*	#1005 AMLBRT	-	-	01
3	*Penicillium sp.*	-	-	#1011 AMSTITYEL	01
4	*Aureobasidium sp.*	-	-	#23(b) AMSTYEL, #11 AMBAWLS, #23 AMSTYEL	03
5	*Lasiodiplodia sp.*			#1079 AMSTITYEL, #1104 AMSTITYEL	02
6	*Sphaeropsis sp.*			#1003 AMSTITYEL	01
7	*Barriopsis sp.*			#1111 AMSTITWLS	01
8	*Cunninghamella sp.*			#1032 AMSTITYEL	01
9	Mycelia-Sterilia			#16 AMLWLS, #1088 AMSTITWLS, #20 AMSTYEL,	03
10	Unidentified			#1103 AMSTITYEL, #1095 AMSTITWLS, #1082 AMSTITWLS, #32 AMSTYEL, #18 AMSTYEL,	05

considered wherein the PP values are higher than 0.95 in the MCMC analysis. The phylogenetic tree reveals the clustering of the characters of #1005 AMLBRT with *Alternaria palandui* (DQ 323687) exhibiting 98% bootstrap values and a posterior probability of 0.98 in one clade.

Morphological studies

Morphological identification #1005 AMLBRT culture was based on the colony or hyphal morphology of the culture on potato dextrose agar, characteristics of spores, discernible reproductive structures. #1005 AMLBRT culture on potato dextrose agar (PDA) exhibited a colony 6 cm diameter after 5-7 days. Colony development on PDA is concentric with obvious rings of sporulation influenced by light/dark cycle. The colony on PDA is so dense as to hide the agar surface. Sporulation is very abundant. Culture medium gradually turns grayish to olivaceous and the substratum turned orange (Fig 5a). The size of a mature conidium body ranges to approximately 35-80 µm x 12-19 µm. It has a 5-6 transverse septa and one longiseptum in 3or 4th transverse segment and is dull brown in colour (Fig 5b).

Molecular tools indicate that #1005 AMLBT is related to *Alternaria palandui* but morphological studies (macroscopic as well as microscopic) do not confer to the molecular studies. The branch length of #1005 AMLBRT is significantly longer than *Alternaria palandui* indicating molecular difference between the two species. *Alternaria palandui* does not have growth rings of sporulation influenced by light / dark cycle; the conidial colour is moderate to dark brown with a size of 35-55 x 7-9 µm. Further *Alternaria palandui* is epiphytic and pathogenic to *Allium* species. Previously endophytic existence of *Alternaria alternata* in leaves of *Aegle marmelos* has been reported purely on morphological and classical taxonomical studies[32]

Table 2. Similarity pattern of ITS sequence of #1005AMLBRT with genus *Alternaria* based on BLASTN

S. No	Accession	Description	Max Score	Query coverage	E value	Max. Identity
1	DQ156344	*Alternaria brassicae strain B*	897	100%	0	100%
2	AY762949	*Alternaria triticimaculans strain EGS 40-150*	863	99%	0	97%
3	FJ755199	*Alternaria tenuissima strain CZ075A*	856	37%	0	96%
4	DQ323687	*Alternaria palandui isolate Alt14*	879	95%	0	96%
5	AB369424	*Alternaria longipes isolate, GL1YS4*	875	97%	0.0	96%
6	FR846400	*Alternaria compacta strain 15*	897	98%	0	95%
7	EU520097	*Marssonina mali isolate NW195*	863	96%	0	95%
8	AF314583	*Alternaria pomicola strain PL1*	897	100%	0	94%
9	AF314579	*Alternaria citri strain AC2*	897	100%	0	94%
10	AY154683	*Alternaria mali*	901	38%	0	94%
11	AY154712	*Alternaria tenuissima strain IA287*	901	38%	0	94%
12	AY751457	*Alternaria longipes strain EGS30-033*	901	99%	0	94%
13	FJ869872	*Alternaria brassicae*	901	99%	0	94%
14	GQ121322	*Alternaria alternata isolate IEIHBT*	901	66%	0	94%
15	AY923859	*Alternaria aff. dianthicola RHR2*	897	100%	0	94%
16	AB470908	*Bionectria ochroleuca isolate, X12*	899	98%	0.0	94%
17	EF432256	*Phomopsis sp. G26*	901	89%	0	94%
18	EU 520078	*Alternaria gaisen isolate NW576*	899	98%	0.0	94%
19	AF455448	*Alternaria alternata wb398isolate*	901	96%	0.0	94%
20	AF314578	*Alternaria abutilonis strain QMT*	897	100%	0.0	94%
21	AY154683	*Alternaria mali*	901	38%	0.0	94%
22	Y17066	*Alternaria infectoria isolate 4A*	666	88%	0.0	90%
23	Y17069	*Alternaria solani isolate ICMP 6519-79*	697	99%	0.0	89%
24	Y17086	*Alternaria linicola*	699	99%	0.0	89%
25	AF229464	*Alternaria crassa strain DGG Acr1*	702	99%	0.0	89%

(a) *S. aureus* NCTC6571 (b) *S. aureus* G3 (c) *S. aureus* G26

Fig.1. *In vitro* anti- staphylococcal assay of whole spent broth of endophytic fungal isolates from *Aegle marmelos*

(a) *S. aureus* NCTC6571 (b) *S. aureus* G3 (c) *S. aureus* G26

Fig.2. *In vitro* anti- staphylococcal assay of ethyl acetate fraction from spent broth of #1005 AMLBRT an endophytic fungus from *Aegle marmelos*

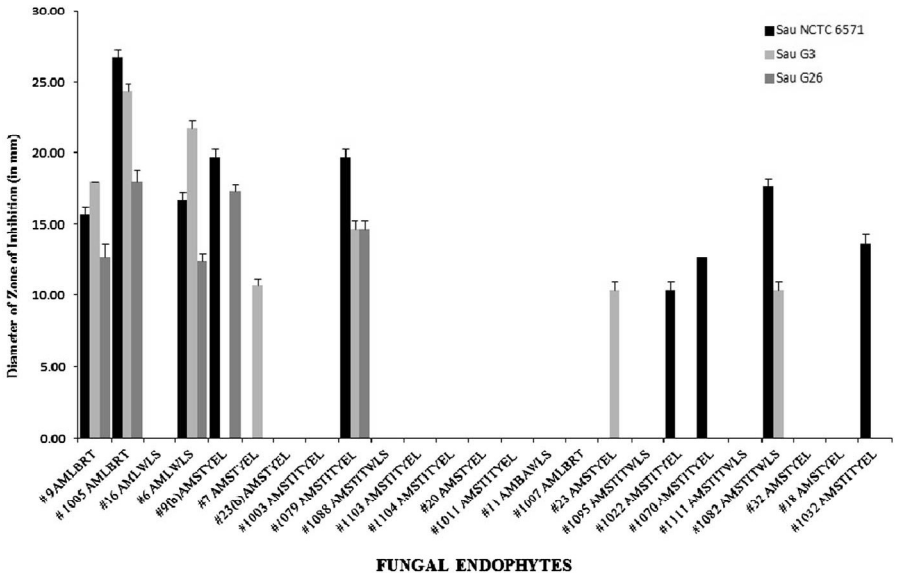

Fig. 3. *In vitro* anti- staphylococcal activity of ethyl acetate fractions of spent broth of endophytic fungi

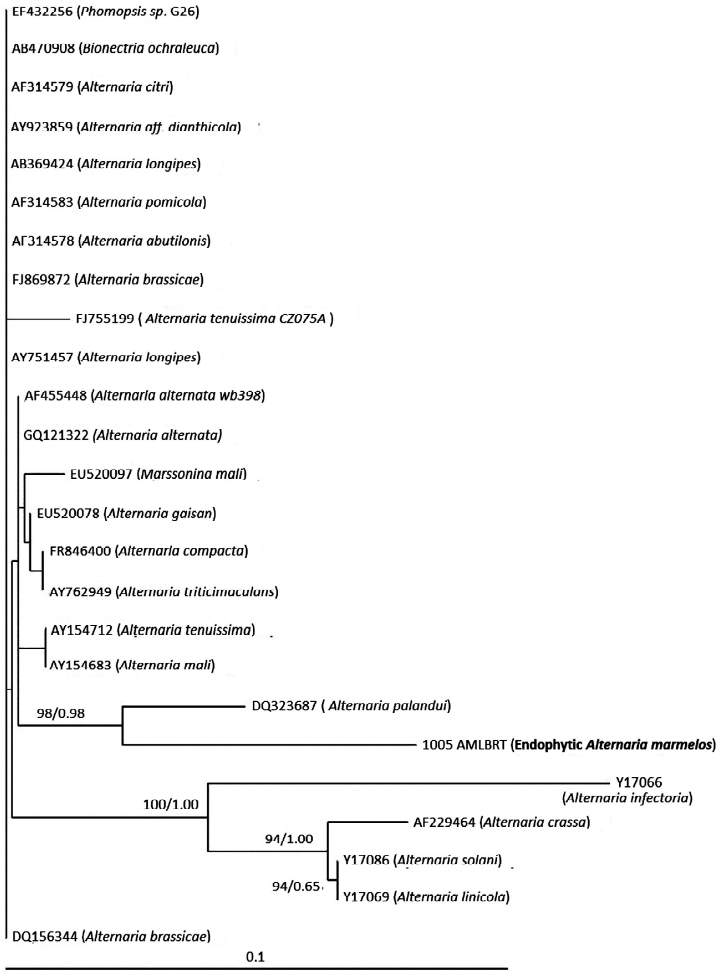

Fig.4. A maximum likelihood (ML) tree generated based ITS and 5.8 S rDNA sequence data (-lnL = 2155.2073). Bootstrap values (equal to or above 90%) based on 100 replicates are shown on the upper branches followed by Posterior probability values from MCMC analyses hyphen

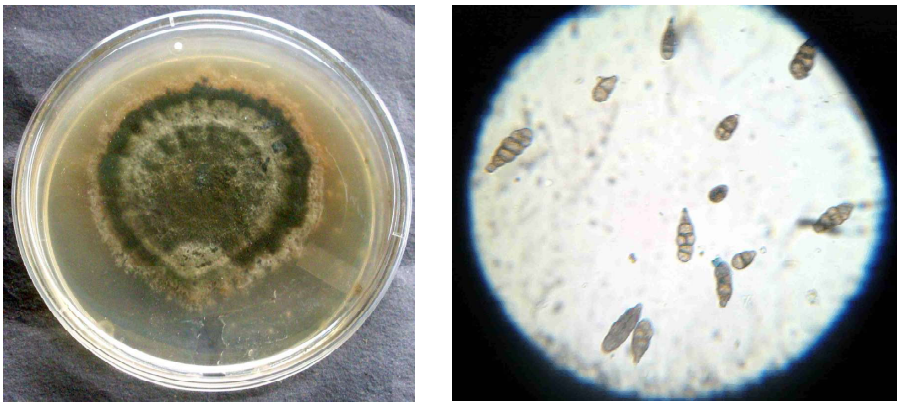

Fig. 5. Morphological features of #1005 AMLBRT
(a) 10 day old culture on PDA plate (b) Conidia of #1005AMLBRT at 400x magnification

however phylogenetic studies are necessary to further substantiate identification of endophytic microflora more conclusively.

The present *Alternaria* isolate #1005AMLBRT from leaves of *Aegle marmelos* is a new species based on morphological as well as molecular data indicating its non- similarity to *Alternaria palandui* (Fig 4). #1005AMLBRT is thus named as *Alternaria marmelos*. Further the production of anti-staphylococcal principles elaborated by *Alternaria marmelos* (#1005AMLBRT) substantiate the fact that it has overcome the antifungal phytochemicals present in the host plant *Aegle marmelos* leaves[33]. Xanalteric acids I and II have been isolated from an endophytic *Alternaria* isolate from mangrove plant *Sonneratia alba* and have been found to possess a weak anti-staphylococcal activity (34). Anti-staphylococcal activity has also been reported from *Alternaria alternata* existing as an endophyte in *Coffea arabica*[35]. Endophytic Alternaria has also been a potential source of anti-fungal agents to overcome the fungi *Plasmopara viticola* responsible for downy mildew in grapevine (36). The present study highlights the immense need explore endophytic fungi from medicinal plants and their screen them for their antibiotic potential in developing new drugs. *Alternaria* species has proved to be resources of newer compounds when it exists as an endophyte. Only one endophytic fungi from bark of *Aegle marmelos* namely *Bartalinia robillardoides* Tassi has been found to produce taxol in the free fermentation medium[37] till date.

CONCLUSION

Thus the endophyte *Alternaria marmelos* (#1005AMLBRT) from *Aegle marmelos* exhibits potential for further investigations to isolate and characterize the secondary metabolites/bioactive compounds from ethyl acetate extract to overcome multidrug resistant *Staphylococcus aureus*.

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