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Exploration of endophytic microorganisms from selected medicinal plants and their control potential to multi drug resistant pathogens

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Abstract
Endophytes are microorganisms that live inside the host plant tissues which have novel metabolites exhibiting a variety of biological activities against different pathogens. This study was carried out to isolate endophytic bacteria and fungi from *Terminalia arjuna* (marutham), *Catharanthus roseus* (nithyakalyani) and *Azadirachta indica* (vembu) and to assess their antimicrobial activity against *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumonia, Enterococcus faecalis and Candida albicans*. Out of the several bacterial and fungal isolates the bacterial isolate NRL2exhibited a high antimicrobial activity against *Staphylococcus aureus*. This bacterial strain was investigated for the efficiency of solvents for extraction of antimicrobial agents. The extract of NRL2 isolates obtained from methanol and ethanol were effective. The bacterial crude extract was used against Methicillin resistant *S. aureus* by cup agar method. In GC MS analysis 24 compounds were obtained and four major compounds may responsible for antimicrobial activity in the inhibition of *S. aureus.*

Keywords: Endophytes, Methicillin resistant *Staphylococcus aureus*, Solvents, Antimicrobial.

1. Introduction
The World Health Organization estimates 25% of the total 57 million annual deaths that occur worldwide are caused by microbes and this proportion is significantly higher in the developing world. There are multiple factors that determine the impact of infectious diseases, but the most significant are probably health and housing conditions and access to medical care. Infections caused by multidrug-resistant microbe's present daily challenges to infectious diseases, physicians and their patients in the United States and throughout the world [22].
Antimicrobial resistance is a global quandary demanding urgent action to clearly understand the implications of resistance and to effectively manage patients infected with resistant pathogens, it is important to understand the epidemiology of resistant pathogens, the mechanisms of resistance and treatment options available. Because of the development and spread of drug-resistant pathogens, infectious diseases remain a global problem [19, 6]. Methicillin resistant *Staphylococcus aureus* (MRSA) strains cause a wide range of human diseases, from minor skin infections to life threatening deep infections such as pneumonia, endocarditis, meningitis, postoperative infections, septicemia and toxic shock syndrome. The high prevalence of MRSA strains around the world represents a serious public health problem, as this Gram-positive pathogen has become multidrug resistant [28, 13, 2, 11, 12].
Antimicrobial drugs generally act at three interfaces in the microbe viz. cell wall synthesis, protein synthesis and nucleic acids. β - lactams, which launched mankind journey into the golden era of antibiotics with drug like penicillin and methicillin, basically inhibit cell wall synthesis.
New antibiotics are continually required since there is an increase in the development of resistant pathogens, the evolution of new diseases, the existence of naturally resistant bacteria and the toxicity of some of the current compounds [25]. Natural products still remain the most important resource for the discovery of new and potential drug molecules [24]. New approaches need to be devised to efficiently access chemical diversity for the development of new medicines [21] to overcome the difficulties in the treatment of infections caused by resistant bacteria pathogens. Recent trends, however, show that the discovery rate of active novel chemical entities is declining [14].

Endophytes are microbes (fungi, bacteria and yeasts) that live within the plant tissue without causing any noticeable symptoms of disease. Endophytes have been found in all parts of plant, including xylem and phloem. The scientific community in searching for new therapeutic alternatives has studied and found variable bioactive metabolites in endophytes such as antiviral, anticancer, anti-diabetic and antibacterial compounds. During the long co-evolution of endophytes and their host plants, endophytes have adapted themselves to their special microenvironments by genetic variation, including uptake of some plant DNA into their own genomes [9]. This could have led to the ability of certain endophytes to biosynthesis some phytochemicals originally associated with the host plants [23]. Previously, the screening of 10000 natural products resulted in one commercial product. More than 22,000 biologically active compounds have been obtained from microbes by the end of 2002 [15].
Some of these endophytes may be producing bioactive substances that may be involved in a host-endophyte relationship. As a direct result of the role that these secondary metabolites may play in nature, they may ultimately be shown to have applicability in medicine. A worldwide scientific effort to isolate endophytes and study their natural products is now under way. Antibiotics, defined as low-molecular-weight organic, natural products made by microorganisms that are active at low concentration against other microorganisms, are the most bioactive natural products isolated from endophytes.
Strobel and Daisy [24] have summarized the discovery of antibiotics from the first discovery of penicillin to most of the novel antibiotics isolated from endophytes up to 2003. Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against pathogens and some of these compounds have been proven useful for novel drug discovery. Recent studies have reported hundreds of natural products including substance of alkaloids, terpenoids, flavonoids, steroids etc. Metabolites of endophytes have been reported to inhibit a number of microorganisms [8, 10]. Many important antifungal and antibacterial chemotherapeutic are either microbial metabolites or their semi-synthetic derivatives. It is estimated that there might be as many as one million different endophyte species, however, only a handful of them have been described [18], which means investigating the metabolites of endophytes can increase the chance of finding novel antimicrobial natural products. There are numerous new endophytic species may exist in plants, it follows that endophytic microorganisms are important components of microbial biodiversity [29].

2. Materials and Methods

2.1 Sample collection

The green, healthy mature plants of *Terminalia arjuna* (marutham), *Catharanthus roseus* (Nithyakalyani) and *Azadirachta indica* (vembu) leaves and stems were collected. These plants were selected based on its medicinal values such as antimicrobial, antitumor and antimutagenic properties.

2.2 Isolation of Endophytic Microorganism

The organism used in this study was isolated as endophyte from the leaves and stems of the medicinal plants of *Terminalia arjuna, Catharanthus roseus* and *Azadirachta indica*. The healthy plant samples were collected and cut into small pieces approximately 0.5 cm diameter using surgical blade and surface sterilized by the modified method of [5]. The leaves and stems were thoroughly washed in running tap water and immersed in 70% ethanol for 5 seconds, followed by 4% sodium hypochlorite for 90 seconds and then rinsed in sterile distilled water for 10seconds; the excess moisture was blotted with a sterile filter paper [4]. The surface sterilized leaf and stems segments were evenly spaced in petri dishes containing nutrient agar for isolation of endophytic bacteria and potato dextrose agar medium amended with chloramphenicol (30 mg/L) in order to prevent bacterial contamination for isolation of fungus. The petri dishes were incubated at room temperature for 3 days and monitored every day to check the growth of endophytic colonies from the leaf and stem segments, after the incubation, the colonies were sub cultured into the new respective medium and stored at 4 ºC.

2.3 Fungal and Bacterial Cultivation

The fungal endophytes were cultivated on potato dextrose broth (Himedia) by placing agar blocks of actively growing pure culture (3 mm in diameter) in 250 ml Erlenmeyer flasks containing 100ml of the medium. The flasks were incubated at 25±1 °C for 3 weeks with periodical shaking at 150 rpm. After the incubation period, the cultures were taken out and filtered through Whatman No.1 filter paper to remove the mycelial mats. The filtrate (80 µl) was used for preliminary testing of antimicrobial activity. For bacterial extraction the nutrient broths were prepared and inoculate the bacterial culture and incubated at 30 °C for 3 days then culture was centrifuged at 8000 rpm for 10 min and collects the supernatant for further analysis.

2.4 Screening for Antimicrobial Activity

The pure culture of endophytic bacterial and fungal cultures was used for antimicrobial activities by cross streak method. In this method the bacterial and fungal cultures were streaked in one edge of the nutrient agar plates and the human bacterial pathogens were horizontally streaked towards the pure endophytic microorganisms starts from other edges of the plates. The plates were kept for incubation at 37 ºC for 24 – 48 hours. The zone of inhibition was measured after the incubation period.

2.5 Extraction of Antimicrobial Compounds from the Bacterial Endophytes

Single pure efficient bacterial endophytes were inoculated into 200 ml of nutrient broth medium and kept for incubation in a rotary shaker at 30 ºC, 120 rpm for five days; after incubation, the culture was centrifuged at 8000 rpm for 10minutes. The supernatant was discarded and the pellet was collected in order to obtain the intracellular antimicrobial compounds. To this pellet 5 different polar and nonpolar solvents such as hexane, chloroform, ethyl acetate, ethanol and methanol were added and mixed thoroughly and kept this the mixture for 2 - 3 hours, after this the mixture was centrifuged. The supernatant containing antimicrobial compounds in the solvent was evaporated in order to get concentrated forms using rotary vaccum evaporator.

2.6 Evaluation of Antimicrobial Activity

The concentrated crude antibacterial compound was evaluated for antimicrobial activities using well or cup agar diffusion method. Cup agar was prepared by scooping out the media with a sterile cork borer (6mm in diameter), the multidrug resistant human pathogens *Staphylococcus aureus*, *Klebsiella pneumonia, Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Candida albicans* were swapped on nutrient agar and SDA

agar plates. All the clinical pathogens were obtained from Rhizosphere Biology Laboratory, Department of microbiology, Bharathidasan University, Trichy, Tamilnadu. The cups were filled with 80 µl of the crude extract and the plates were incubated at 35±1 ºC for 24 hours. After incubation the zone of inhibition was recorded and compared with control.

2.7 Genomic DNA Isolation

2 ml of overnight bacterial broth culture of was taken and centrifuged at 10000 rpm for 5 minutes and the supernatant was discarded. To the pellet, 570 µl of TE buffer, 30 µl of 10% SDS was added, mixed thoroughly by vortexing and incubated at 37 °C for one hour. After incubation, 5M NaCl was added and mixed thoroughly by vortexing. 80 µl of C-TAB/NaCl solution was added, mixed thoroughly and incubated at 65 °C for 10 minutes. Equal volume (0.7 – 0.8ml) of chloroform/isoamyl alcohol was added, mixed and centrifuged at 10000 rpm for 5 minutes.

The viscous supernatant was transferred to a fresh centrifuge tube (without disturbing the interface); equal volume of phenol/chloroform/isoamyl alcohol was added and spun at 10000 rpm for 5 minutes. The aqueous supernatant was transferred to a fresh centrifuge tube and 0.6 volumes of ice cold isopropanol was added and incubated for 20 minutes at -20 °C. Later, the tubes were centrifuged at 10000 rpm for 5 minutes. To the pellet, 70% of ethanol was added and centrifuged. The pellet was stored at -20 °C after adding TE buffer/ sterile milliQ water.

2.8 Agarose Gel Electrophoresis

For running the sample, 0.8% of agarose was prepared in TE buffer. To 50 ml of agarose 3 µl of ethidium bromide was added and cast into the boat which contains comb. After cooling, the comb was removed and the sample was loaded. To 5 µl of the sample 2 µl of the gel loading dye was added and loaded into the respective well.

2.9 Amplification of 16s rRNA gene

Amplification of bacterial 16S rRNA gene from the extracted genomic DNA was performed using the following universal 16S rRNA gene primers: 16S rRNA forward primer 5' AGAGTT TGATCMTGGCTCAG 3' (27f) and the reverse primer 5' TACGGYTACCTTGTTACGACTT 3' (1492r) (Nucleic Acids Research, Vol. 18, Supplement). Approximately 10 ng of DNA template in 50 µl reaction volume containing: 10 pmol of each primer, 200 µm dNTP, 1.5 mmMgCl2, 1X Taq buffer, and 1.0 U of Taq polymerase (Genei). The PCR profile comprised an initial denaturation of 5 min at 95 °C followed by 40 cycles of: 95 °C for 60 sec, 55 °C for 60 sec, 72 °C for 2 min; and final extension 72 °C for 10 min were carried out (Mastercycler Applied Biosystem - 9902). The amplification of 16S rRNA gene was confirmed by 1.5% agarose gel electrophoresis in 1x Tris-acetate-EDTA buffer. The amplified product was further resolved and amplicon size corresponding to 1400bp was purified from agarose gels using the QIA gel extraction kit following the manufacturer's protocol.

2.10 Sequencing of 16S rRNA gene

The amplified 16S rRNA gene after gel elution was sequenced using forward and reverse, about 1400 bp were carried out in Amnion sequencing.

2.11 Nucleotide Sequence Analysis

The sequences obtained were aligned with previously published sequences available in NCBI using BLAST [1]. Multiple sequence analysis was carried out using CLUSTALX [26], and further neighbor - joining plots were used to construct the phylogenetic tree.

2.12 Gas Chromatography-Mass Spectrum Analysis

GC-MS analysis of the ethanol extract of NRL2was performed using a Perkin–Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with an Elite-5MS (5% phenyl/95% dimethyl poly siloxane) fused a capillary column (30 × 0.25 µm ID × 0.25 µm df). For GC-MS detection, an electron ionization system was operated in the electron impact mode with an ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2 µl was employed (a split ratio of 10:1). The injector temperature was maintained at 290 °C, the ion-source temperature was 220 °C, the oven temperature was programmed from 50 °C (isothermal for 1 min), with an increase of 7 °C/min to 220°C, then 8 °C/ min to 290 °C, ending with a 9 min isothermal at 290 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 600 Da. The solvent delay was 0 to 2 min, and the total GC/MS running time was 30 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas.

3. Results

3.1 Isolation Endophytes

In this work, a total of 14 fungus and 7 bacteria was isolated from three different medicinal plants *Azadirachta indica*, *Terminalia arjuna,* and *Catharanthus roseus* the details of the isolates given the table 1 and Figure 1.

Table 1: Endophytic Microbes from Three Different Medicinal Plants.

S. No.	Plant Name	Bacteria	Fungi
1	Azadirachta indica	NRL1-NRL2	NRL1-NRL6
2	Terminalia arjuna	TRL1-TRL5	TRL1-TRL2
3	Catharanthus roseus	-	CRL1-CRL6

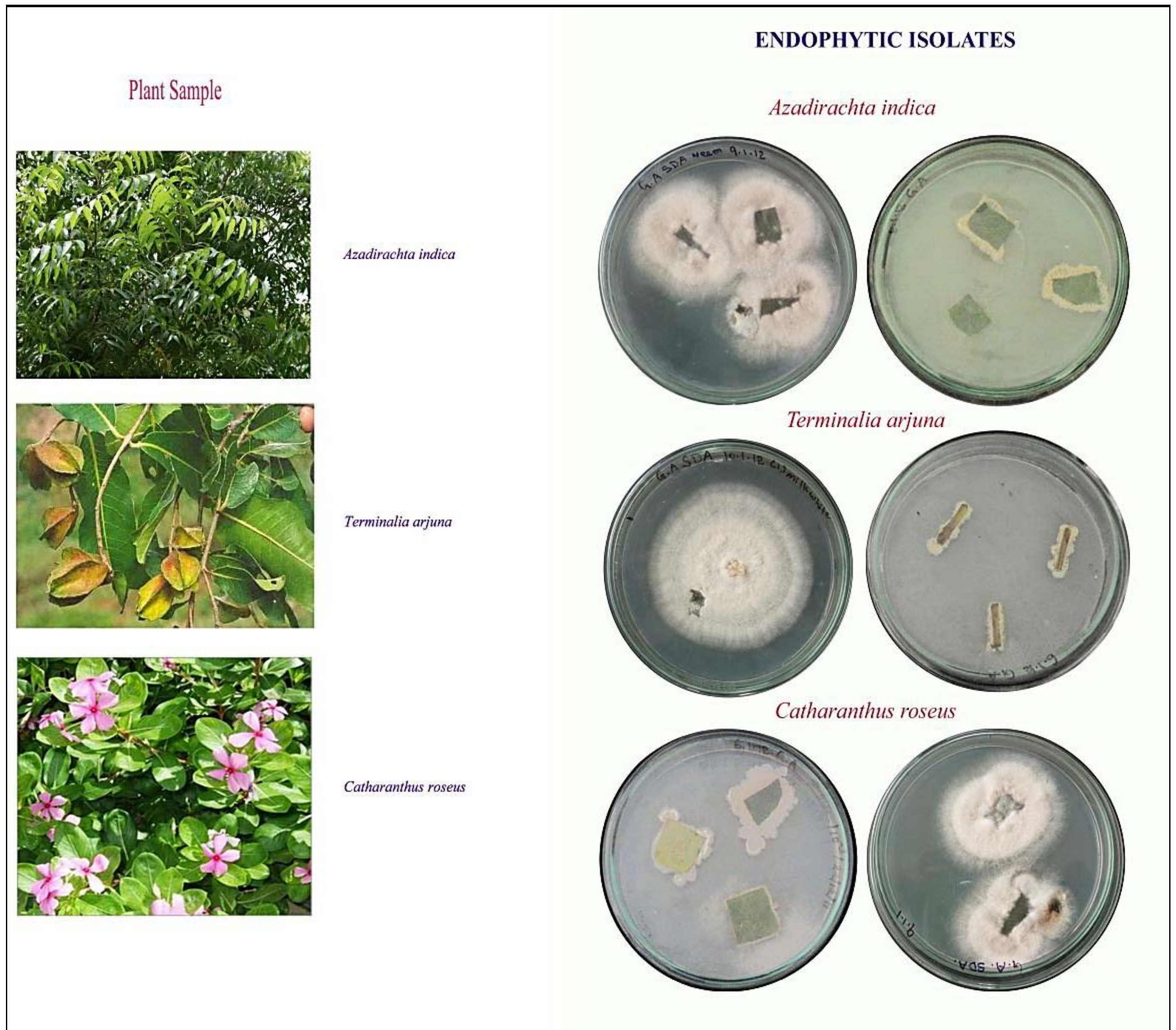

Fig 1: Selected medicinal plants and the isolated microbes

Screening for Antimicrobial Activity

The pure culture of endophytic bacteria was used for antimicrobial activities against the multidrug resistant human pathogens *staphylococcus aureus*, *Klebsiella pneumonia*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and yeast (*Candida albicans*) by the cross streak method. Out of 7 isolates 1 showed inhibition against Methicillin resistant *S. aureus* (Table.2) other 6 isolates doesn't control the pathogens. The active resulted bacterial crude extract was collected and used for cup agar method. The zone of inhibition was obtained in methicillin resistance *S. aureus* plate. The zone of inhibition range was 24 mm.

Table 2: Antimicrobial Activity of Endophytic Microbes

Bacterial code	*staphylococcus aureus*	*Klebsiella pneumonia*	*Enterococcus faecalis*	*Pseudomonas aeruginosa*	*Candida albicans*
NRL1	-	-	-	-	-
NRL2	+	-	-	-	-
TRL1	-	-	-	-	-
TRL2	-	-	-	-	-
TRL3	-	-	-	-	-
TRL4	-	-	-	-	-
TRL5	-	-	-	-	-

3.2 Extraction of Antimicrobial Compounds

The resulted NRL2 bacterial crude extract was used solvent extraction. Five different polar and non-polar solvents such as hexane, chloroform, ethyl acetate, ethanol and methanol were used. After extraction the solvents are performed by cup agar method to check the antimicrobial activity no result was obtained. Then try to intracellular metabolites here the bacterial (NRL2) pellet was taken and perform the solvent extraction. The results showed that methanol extract had an effective antimicrobial activity against the tested

microorganisms the methanol extract produced a maximum inhibition zone of 33 mm against *S. aureus*. The ethanol extract exhibited moderate antimicrobial activity against *S. aureus* the zone of inhibition range is 27 to 29 mm. The methanol extracts making analysis for GC-MS to identify the dissolved compounds (Figure 2).

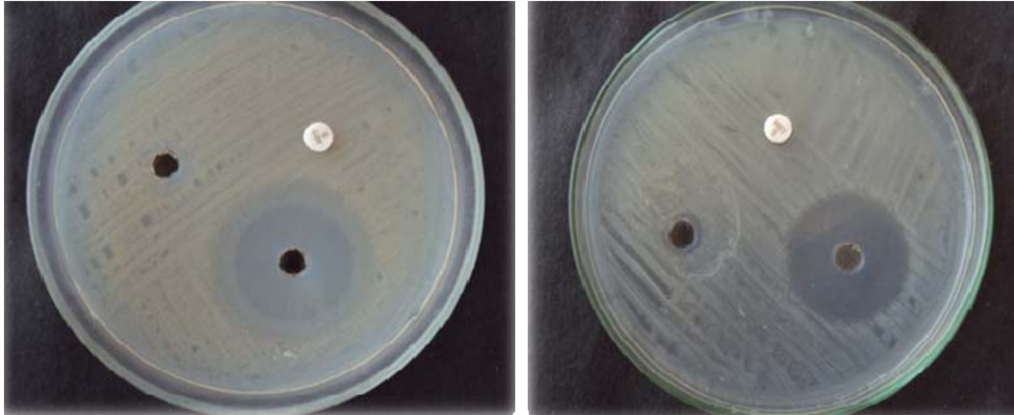

Fig 2: Methanol Extract to Control the *S. aureus* Ethanol Extract to Control the *S. aureus*

3.3 Amplification of 16S rRNA

The amplification of 16S rRNA gene was confirmed by 1.5% agarose gel electrophoresis in 1x Tris-acetate-EDTA buffer. The amplified product was further resolved and amplicon size corresponding to 1400bp was purified from agarose gels using the QIA gel extraction kit following the manufacturer's protocol

3.4 Phylogenetic Analysis

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 41.07001828 is shown. The percentage of replicating trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 829 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

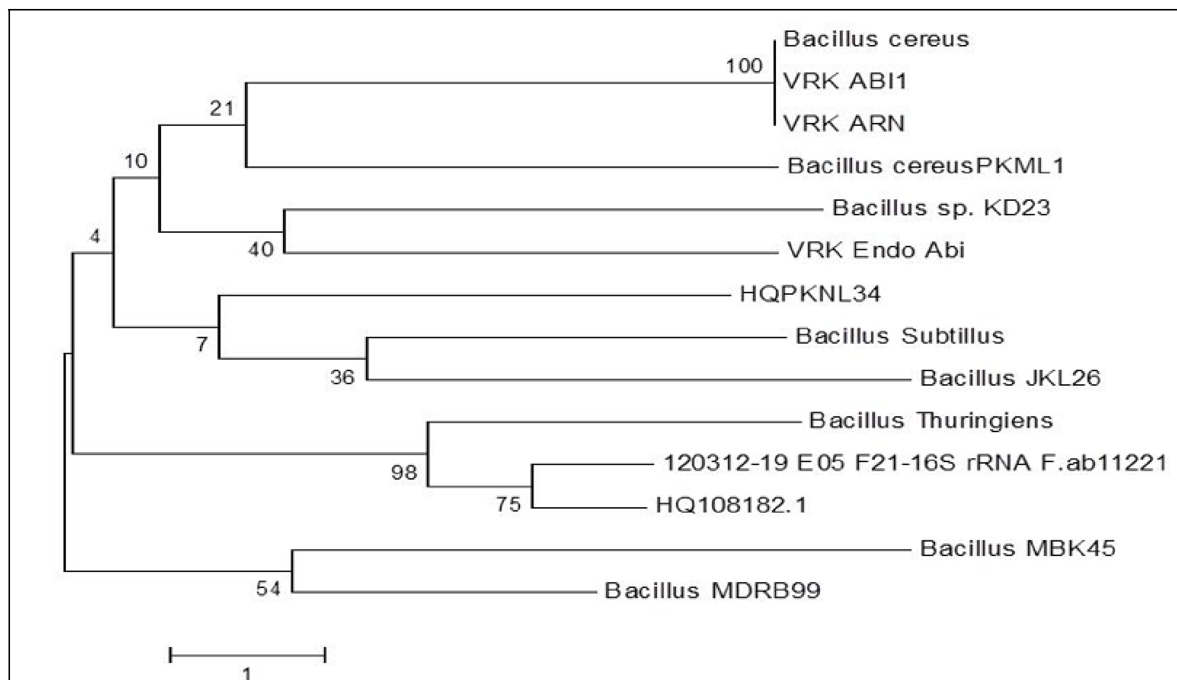

Fig 3: Neighbour-joining phylogenetic tree from analysis of 16S rRNA gene sequence

The strains were identified by 16S rRNA gene sequencing. Preliminary Sequence analysis by NCBI BLASTn algorithm of the strains revealed 100% homology to *Bacillus cereus*, with an average identity of 100% to 1400 bp of the 16S rRNA gene sequenced. Our Isolates 16S rRNA sequences were analyzed with other closely related Bacillus cereus previously described. The Phylogenetic analysis of bacteria associated with the endophytic isolate showed that *Bacillus cereus* was the predominant bacterial member (Figure3), In addition to the BLASTn analysis.

3.5 GC-MS Analysis

The methanol extract exhibits a good antimicrobial activity against Methicillin Resistant *Staphylococcus aureus.* This study shows that the *Bacillus cereus* extract exhibit high antibacterial activity. The methanol extract given to GC MS

analysis. The sample was then fed into the instrument and based on the difference of the retention time of different compounds the samples were analyzed. Some amount of purified and distilled methanol was also fed into the instrument to check the purity of the solvent. Different peaks were obtained which correspond to different compounds which were subsequently mass analyzed. The mass spectrum of different compounds was analyzed based on the peaks and the Mass/Charge ratio.

The studies on the active principles content in *Bacillus cereus* by GC/MS clearly show the presence of 24 components (Figure 4; Table.3). Four major peaks was obtained Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (Retention time 25.17), Methyl-2-O-methylál-arabinopyranoside (Retention time 28.42), : Propionylfilicinic acid (Retention time 33.08), The most identified compound to have antimicrobial property were Benzene carboxylic acid (Retention time 14.18), this compound is used as preserves food by killing bacteria, Topical medications, such as Whitfield's Ointment and Bensal HP are combined with benzoic acid and salicylic acid to treat bacterial infections on skin.

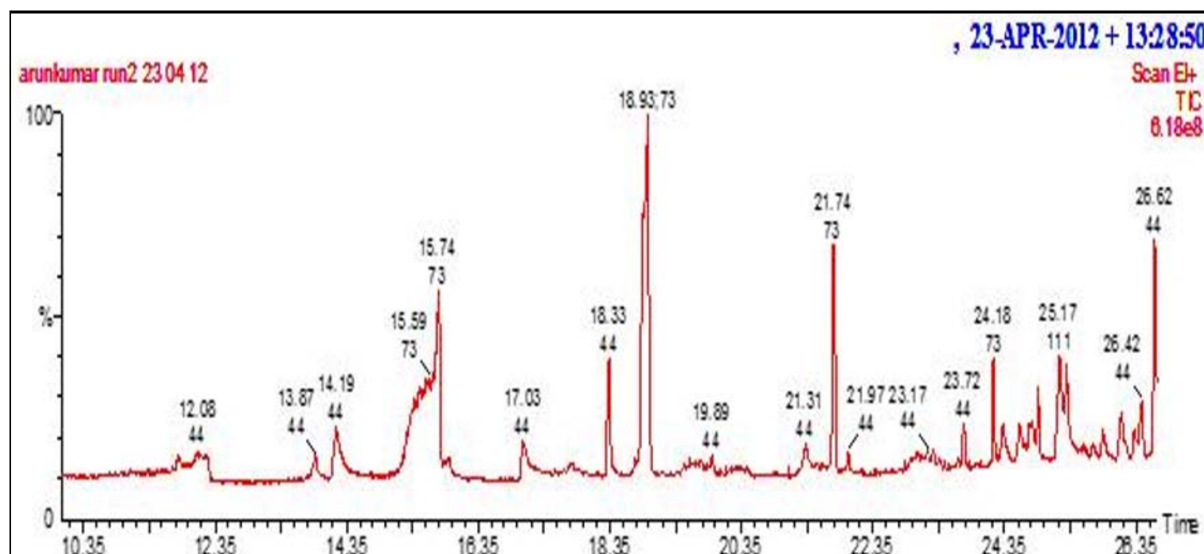

Fig 4: Chromatogram (x-axis = Retention time; y-axis = % intensity/% abundance/ concentration)

Table 3: Activity of Antimicrobial Compounds identified by GC-MS Analysis.

S.NO	Peak Name	Retention time	Peak area	% Peak area
1	Name: (3R)-(+)-3-Acetamidopyrrolidine Formula: $C_6H_{12}N_2O$ MW: 128	3.84	3987954	3.2876
2	Name: Heptanoic acid Formula: $C_7H_{14}O_2$ MW: 130	11.80	5250297	4.3283
3	Name: 1-Butanamine Formula: $C_4H_{11}N$ MW: 73	12.08	285357	0.2352
4	Name: Octanoic Acid Formula: $C_8H_{16}O_2$ MW: 144	13.87	3551558	2.9279
5	Name: Benzenecarboxylic acid Formula: $C_7H_6O_2$ MW: 122	14.18	11554576	9.5255
6	Name: 2-Propenoic acid, tridecyl ester Formula: $C_{16}H_{30}O_2$ MW: 254	15.90	1241223	1.0233
7	Name: Phthalic anhydride Formula: $C_8H_4O_3$ MW: 148	17.03	7877652	6.4943
8	Name: Propanoic acid, 2-(aminooxy)- Formula: $C_3H_7NO_3$ MW: 105	17.77	2920066	2.4073
9	Name: Dodecanal Formula: $C_{12}H_{24}O$ MW: 184	18.33	8725502	7.1932
10	Name: Amidephrine Formula: $C_{10}H_{16}N_2O_3S$ MW: 244	18.73	88947	0.0733
11	Name: Propanedioic acid, (hydroxyimino)-, diethyl ester Formula: $C_7H_{11}NO_5$	18.93	8477873	6.9891

	MW: 189			
12	Name: 2-Decen-1-ol, (E)- Formula: C10H20O MW: 156 CAS	21.97	1611991	1.3289
13	Name: Tetradecanal Formula: C14H28O MW: 212	23.64	753556	0.6212
14	Name: Methyl tetradecanoate Formula: C15H30O2 MW: 242	23.72	2759548	2.2749
15	Name: 7(4H)-Benzothiazolone, 2-amino-5,6-dihydro- Formula: C7H8N2OS MW: 168	24.32	4297972	3.5432
16	Name: Tetradecanoic acid Formula: C14H28O2 MW: 228	24.57	4731153	3.9003
17	Name: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- Formula: C7H10N2O2; MW: 154 CAS	25.17	12404951	10.2265
18	Name: Heptanoic acid, 3,5,5-triethyl- Formula: C13H26O2 MW: 214	25.67	953888	0.7864
19	Name: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- Formula: C11H18N2O2 MW: 210	25.84	2488475	2.0515
20	Name: 10-Nonadecanone Formula: C19H38O MW: 282	26.10	5513344	4.5451
21	Name: Methyl-2-O-methylál-arabinopyranoside Formula: C7H14O5 MW: 178	28.42	12988904	10.7079
22	Name: 10-Nonadecanone Formula: C19H38O MW: 282	30.03	2570787	2.1193
23	Name: E,E,Z-1,3,12-Nonadecatriene-5,14-diol Formula: C19H34O2 MW: 294	32.03	3842277	3.1675
24	Name: Propionylfilicinic acid Formula: C11H14O4 MW: 210	33.08	12423929	10.2422

4. Discussion
In our study three plant samples were *Azadirachta indica, Terminalia arjuna, Catharanthus roseus* used for isolation of endophytic organism. From these plants totally 14 fungi and 7 bacteria were isolated and the seven bacteria were screened antimicrobial activity against multi drug resistance pathogens. Other investigators have also described the isolation of bacteria and fungi from medicinal plants. A total of 77 endophytic fungal isolates belonging to 15 genera were isolated from the inner bark of *Acalypha indica* [16]. The dominant endophytic fungi isolated were *Phomopsis oblonga*, *Cladosporium cladosporioides, Pestalotiopsis* sp., *Trichoderma* sp, and *Aspergillus* sp. Genera such as *Periconia*, *Stenella,* and *Drechslera* are reported here for the first time as endophytes from this host plant of *A. indica* [27]. There were 24 different types of bacterial species, namely, *Bacillus amyloliquefaciens*, *B. cereus*, *B. marisflavi*, *B. methylotrophicus*, *B. mycoides*, *B. pumilus*, *B. safensis*, *B. subtilis*, *Cronobacter sakazakii*, *Enterobacter cloacae*, *E. cowanii*, *E. hormaechei*, *E. pulveris*, *Lysinibacillus sphaericus*, *Pantoea agglomerans*, *P. ananatis*, *P. dispersa*, *P. stewartii*, *Pseudomonas argentinensis*, *P. flavescens*, *P. fulva*, *P. oleovorans*, *P. oryzihabitans*, and *Terribacillus saccharophilus*. Thirty-two (32) % of the isolates represented *Bacillus* genus (Jagadesan Preveena and Subhash J. Bhore,)

Among the seven isolates one isolate crude extract control the Methicillin resistance *S. aureus* by cup agar method. Eteghad *et al.* had isolated new *Streptomyces* sp. strainABRIINW111 that produces highly active secondary metabolites that have a broad spectrum of activity and are particularly active against clinical isolates of MR-CNS 9 (Methicillin resistant coagulase negative *S. aureus*). The effect of culture conditions on the production of bioactive secondary metabolites by the endophytic fungus *Arthrinium state* of *Apiospora montagnei* was investigated. Antibacterial activity, especially against *E. coli* (MIC 110 µg/ml) was detected in the extract from fungus cultivated for 9 days [20]. The 43 isolates were screened for their antimicrobial activity against six test organisms spread on specific medium plates. The result showed that 88.4% of the epi- and endophytes possessed inhibitory activities against one or more test organisms. 79.1% inhibited Gram positive bacteria (*S. epidermidis* and/or *B. subtilis*), 62.8% inhibited Gram negative bacteria (*Pseudomonas fluorescens*, *P. aeruginosa*, and/or *Escherichia coli*), and 48.9% inhibited both Gram positive and Gram negative bacteria [30].

The methanol extract had an effective antimicrobial activity against *S. aureus* the maximum inhibition zone is 33mm. The ethanol extract exhibited moderate antimicrobial activity against *S. aureus* the zone range is 29mm. Similarly Arivudainambi *et al.* [3] reported *Colletotrichum gloeosporioides*, was isolated from the medicinal plant *Vitex negundo (L)*. Extracts of *C. gloeosporioides* were obtained using hexane, ethyl acetate and methanol solvents. The methanol extract produced a maximum inhibition zone of 21.6 mm against *S. aureus.*19.6 mm against *B. subtilis*, 18.3 mm against *E. coli*, 18.6 mm against *P. aeruginosa* and 17.6 mm against *C. albicans*. The ethyl acetate extract exhibited moderate antimicrobial activity against all the tested microorganisms.

The methanol extract was given to GC–MS analysis for identifying the responsible compounds 24 compounds was listed majorly four compounds may responsible for antimicrobial activity's (Table 3). Ezra *et al.* [7] was reported that the original *M. albus* isolate (CZ-620) does not make naphthalene or naphthalene, 1,19-oxybis- but does produce two different nonanones, ethanol and acetic acid, 2-phenylethyl ester and many esters which were not detected in the new *M. albus* isolates. The bioactive compounds from *Aspergillus ochraceus (A. ochraceus)* MP2fungi were characterized the first eluting was identified as α- Campholene aldehyde with chemical formula $C_{10}H_{16}O$ and molecular weight 152 Da. The second Lucenin-2 and chemical formula $C_{27}H_{30}O_{16}$ and molecular weight 610 Da. The third eluting was identified as 6-Ethyloct-3-yl-ethylexyl ester with chemical formula $C_{26}H_{42}$ o4 with molecular weight 418 Da. The isolated compounds showed significant antimicrobial activity against potential human pathogens [17].

The Endophytic bacterial isolate was determined by 16s rRNA gene sequencing. Preliminary Sequence analysis by NCBI BLASTn algorithm of the strains revealed 100% homology to *Bacillus cereus*, with an average identity of 100% to 1400 bp of the 16S rRNA gene sequenced. Li *et al*. an endophytic bacterial strain ZZ120 that was isolated from healthy stems of *Prunus mume* (family: *Rosaceae*) was identified as *Bacillus subtilis* based on biochemical and physiological assays and 16S rRNA, rpoB and tetB-yyaO / yyaR genes analysis. The entire rpoB sequence (3574 bp) of strain ZZ120 which was amplified with 3 pairs of primers and analyzed phylogenetically showed that the strain ZZ120 was likely to be *B. subtilis* 168 with 99.88% similarity.

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