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SCREENING, PRODUCTION, AND CHARACTERIZATION OF ANTIFUNGAL SECONDARY METABOLITES FROM *BACILLUS VELEZENSIS* ALO NA L4 OF *ALOE VERA*

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ABSTRACT

Bioprospecting of endophytes from understudied ecosystems is a promising source for extracting novel bioactive antifungal metabolites useful for pharmaceutical purposes.In present study twenty nine fungal endophytes and fifteen bacterial endophytes were isolated from the leaves of a well-known medicinal plant i.e. *Aloe vera* by optimizing suitable surface sterilization protocol. Cross streak and agar overlay methods were followed for preliminary screening for antifungal activity. Positive isolates were investigated for the efficiency of solvents for extraction of antifungal agents followed by secondary screening by agar well diffusion method. Out of all, aqueous extract of one bacterial isolate, Alo NA L 4 exhibited significant antifungal activity against *Candida albicans* (MCC 1154) and *Aspergillus species* (MCC 1074). Attempts were also made to partially characterize the antifungal metabolite(s) present in the broth culture using UV, FTIR, HPLC, and GC-MS. The characterization of the Alo NA L 4 extract by FTIR analysis showed the presence of possible functional groups. Active compounds were isolated by HPLC and GC-MS with major and minor peaks observed on the basis of retention time. The analysis of the derivatized extract by GC-MS led to the putative identification of three metabolites as 1,2 dichloro-1-ethoxy ethane ($C_4H_8Cl_2O$),S methyl methane thiosulphonate ($C_2H_6O_2S_2$),Disulphide dimethyl ($C_2H_6S_2$). The endophytic bacterial strain Alo NA L 4, was identified by 16S rRNA gene sequence and phylogenetic

analysis as *Bacillus velezensis* (Accession number MW356850). The bio-efficacy of Alo NA L 4 has warranted further studies to develop a baseline for the drug development.

Keywords: Endophyte *Bacillus velezensis, Aloe vera,* 16s rRNA antifungal activity, GC-MS

1. INTRODUCTION

In the last decade, increasingly invasive mycosis has become a public health problem. Because the vast majority of life-threatening fungal infections affect people with altered immune function, which include primary immune deficiency, cancer chemotherapy, hematologic and solid organ transplantation, prematurity, HIV/AIDS, and immunomodulatory medications **[1]**. The emergence of fungal resistance is also a prominent issue. Immunocompromised patients are mainly infected by *Candida*, *Aspergillus, Cryptococcus* and other opportunistic fungi. Moreover, the treatment of fungal infections has been limited due to toxicity, safety, spectrum of activity, pharmacokinetic properties **[2, 3]** low drug efficacy, increased incidence of multi-drug resistant fungal pathogens in regards to the existing antifungal drugs in the market, like amphotericin B, fluconazole and itraconazole. As compared to the development of new antibacterial, antifungal drug development faces a key fundamental challenge, in that fungal pathogens are more closely related to the host. It therefore seems fairly clear that the tempo of antifungal drug development has not kept pace with the clinical needs. These findings reinforce the growing need for bioprospection of new drugs with specific antifungal activity. Furthermore, increased demographic trends strongly suggest that the number of fungal infections are and will continue to increase, especially due to the aging of populations in developed countries **[4–6]**. Therefore there is an upsurge need to search novel antifungal compounds which will have potent activity, high specificity, less toxicity, eco-friendly and inexpensive nature and will have a novel mechanism of action. Hence, considerable research is being directed to screen novel antifungals or lead compounds with defined mechanisms of action that can serve as templates for further medicinal chemistry modifications. Whenever a novel niche of biodiversity is discovered and accessed, novel natural products including antifungals are discovered. Taking into consideration this fact, the best option is to use previously unexplored microbial niches or sources. Since the probability of obtaining a novel bioactive antifungal compound is higher in such cases we selected one such unexplored and less studied niche i.e. use of endophytes. Endophytes are the microbes existing in the inner tissues of the plants without causing any visible disease symptoms in the host'**[7]**. Bioprospection of such organisms and their products represents a promising source in the discovery of new antifungal drugs, which could be applied in the pharmaceutical industry **[8–10]**. The endophytes do survive in almost each plant species on earth **[10]**. Hence plants that are having an ethno-botanical history and are being used by the traditional healthcare practitioners for the treatment of a various diseases may be the most excellent choice. Further, the genetic origin of potent bioactive compound production have been predicted to be arises by a process called horizontal gene transfer from host medicinal plants to its associated endophytes **[11]**. The rationale behind selecting medicinal plants is that the therapeutic potential shown by host plant may also be observed in endophytes associated with such host plants. Medicinal plants have been used long been ago in treatment of various types of microbial infections. However owing to over exploitation of these genetic resources along with other biotic interferences, many plants which are used as medicines have now become critically endingered moreover they are on verge of extinction in near future. Furthermore, the extensive extraction procedures for valuable and potent bioactive compounds will have the risk of depletion of valuable medicinal plants diversity, hence the commercial collection of natural medicinal plant species are also legally regulated in many countries including India. Further plants, as compared to most drug producing microbes, exploited by the pharmaceutical industries, are often expensive and not easy to propagate on commercial scale because of seasonal specificity, restricted availability of land for their cultivation and environmental competence of plants etc. Moreover, national along with international regulations restrict the transport of many indigenous plant species **[12]**. However, if the endophytes can produce the similar rare and significant bioactive compounds like their host plant, this would not only reduce the need of harvesting slow growing and probably rare plants, but also will help to conserve the continually diminishing biodiversity of the globe. So in present study, we have focused endophytic population from well-known medicinal plant *Aloe vera.*

Aloe vera is a promising medicinal herb mentioned in ancient ayurvedic literature as having great therapeutic potential. The plant is indigenous to mostly native to sub-Saharan Africa, the Saudi Arabian Peninsula and many islands of the Western Indian Ocean. It grows in a variety of climates, including desert, grassland and coastal areas **[13]**. The leaf exudates of plant are used in traditional medicine and the cosmetic industry, due to its soothing, moisturizing and healing properties to the skin **[14]**. The plant also exhibits anticancer activity *in vitro* and *in vivo* **[15]**. Aloe is also used as an herbal medicine to treat wounds and burns, and to treat diabetes and elevated blood lipids in humans **[16, 17]**. It has also been documented to possess a number of antioxidant polyphenols, polysaccharides, phytochemicals and alkaloids. The pharmacological actions of *A. vera* include anti-inflammatory, antibacterial and hypoglycemic effects **[16]**. In addition to all these, it also has shown antifungal activity **[18–20]**. These various traditional uses created an interest to explore its endophytic flora for their antifungal compounds. Thus, an investigation was carried out wherein most active bacterial endophyte, Alo NA L 4 inhibited strongly *Aspergillus species* and *Candida albicans*. The characterization of antifungal compound from this isolate was carried out using UV spectroscopy, FTIR, HPLC and GC-MS. We detected the presence of antifungal 1,2 dichloro-1-ethoxy ethane, S methyl methane thiosulphonate, Disulphide dimethyl. The endophytic strain Alo NA L 4 was subjected to cultural and morphological characterization and was identified by homology of 16S rRNA gene sequence as *Bacillus velezensis* (Accession number MW356851).

2. MATERIALS AND METHODS

2.1 Site and collection of plant samples:

Apparently healthy, symptomless; disease free and mature plants were selected from relatively pollution free sites at medicinal plant garden of Satara College of Pharmacy, Satara in Maharashtra state, India. Leaves were cut off with an ethanol-disinfected sickle and placed into pre-sterilized zip lock plastic bags. The collected plant samples were brought to the laboratory and were immediately processed after sampling.

2.2 Isolation of endophytes:

The collected plant samples were first washed thoroughly by using running tap water for 7-10 mins so as to remove adhered debris, soil particles and then were rinsed with sterile distilled water before their surface sterilization. The plant samples were surface sterilized by using method of Petrini O. (1991) with some modification considering the fact that the time required in each sterilent usually varies depending upon the host plant, tissue thickness, texture and sensitivity. Washed leaves were initially soaked in 70% ethanol for 2 min followed by rinsing with sterile distilled water. These samples were then immersed in 5% sodium hypochlorite with

5 % available chlorine for 5 min and then rinsed thoroughly with sterilized distilled water. Then were immersed in 70% ethanol for 0.5 mins. Finally all were thoroughly rinsed by using sterile distilled water for three times, surplus water was allowed to surface-dry on sterilized filter paper under laminar airflow chamber **[21]**. Then, it was cut into smaller pieces of size approximately 1cm X 1cm and positioned on the sterile Nutrient Agar (NA Himedia) medium supplemented with Ketoconazole (100 µg /ml). Whereas for fungal endophytes sterile Potato Dextrose Agar medium (PDA Himedia) supplemented with chloramphenicol (50 µg/ml) was used. Plates were then sealed with parafilm so as to prevent desiccation of the culture media and subsequently incubated at 30 ±2°C for 3-5 days and at 27±2°C for 2 to 3 weeks for bacterial and fungal endophytes respectively. After incubation the individual endophytic colonies were used to obtain pure cultures. Pure cultures were preserved in refrigerator at 4°C by suitable coding. For confirmation of efficiency of surface sterilization process, sterility check was performed wherein 0.1 ml from final rinse was inoculated on respective agar media as control plate **[22, 23]**.

2.3 Preliminary screening of endophytes for antifungal activity:

Two fungal cultures *Aspergillus species (*MCC 1074) and *Candida albicans* (MCC 1154) used in the present investigation were procured from National Centre for Microbial Resource Centre of National Centre for Cell Science (NCCS) Pune. Preliminary anticandida activity was performed by using cross streak assay method. Wherein, sterilized nutrient agar plates were firstly inoculated with overnight grown cultures of endophytic bacteria as a single streak at the center of the plate and subsequently incubated at 35 ± 2^{o}C for 3-5 days. Then overnight grown culture of test organism *Candida albicans* (MCC1154) was streaked at right angle to the producer bacterial endophytes and monitored for its growth inhibition after 24-48 hours of incubation. The endophytic isolates with the maximum inhibition potential shown was selected for further studies. Whereas for antiaspergillus activity all isolated bacterial endophytic strains were spot inoculated separately at center of sterilized nutrient agar plates and incubated at 35 ± 2^{o}C for 72 hours. The full grown endophytic colonies were then exposed to chloroform for 20 mins for inactivation and then 100 microlitre of standard spore suspension of *Aspergillus species* (10^6-10^7spores / ml) mixed with sterilized 15 ml of semisolid PDA was aseptically layered on the surface of plate containing chloroform inactivated bacterial endophytic colonies. The plates were subsequently incubated at 27± 2°C for 3-4 days and were observed for the inhibition halos **[24]**. Anticandida activity of isolated endophytic fungi was tested by agar plug diffusion method **[25]** with some modifications and antiaspergillus activity of fungal endophytes was tested by dual culture assay method.

2.4 Production and extraction of antifungal metabolites:

Bacterial endophytes that showed promising antifungal activity in preliminary screening were selected and cultured in 50 ml presterilized nutrient broth at ambient temperature for 24 hours on orbital shaker with 150 rpm speed in order to obtain active culture. This active culture was inoculated and grown in 500 ml capacity Erlenmeyer flask containing 300 ml sterilized nutrient broth and further incubated at ambient temperature for 72 hours on orbital shaker with speed of 150 rpm. After incubation culture broth was subjected to centrifugation at 10,000 g for 20 mins at 4°C and the cell free supernatant was extracted with different types of organic solvents with increasing polarity such as hexane, chloroform, dichloromethane, ethyl acetate, methanol etc. All such extracts were then subjected to evaporation of organic solvent by rotary vaccum evaporator and the crude extract thus obtained was stored at 4° C until further analysis. Similarly a portion of cell free supernatant (CFS) was lyophilized, weighed and diluted in sterilized distilled water, to obtain crude aqueous extract at concentration of 10 mg/ml **[26]**. A method of acid precipitation in other reports was also tried **[27, 28]**, but it failed to produce any precipitate, even if the culture supernatant was adjusted to pH 2. This indicated that this product may be possibly different from lipopeptides reported previously.

2.5 Secondary antifungal assay:

The crude organic and aqueous extract obtained in above step was analyzed for activity against *Aspergillus species* (MCC 1074) and *Candida albicans* (MCC 1154), by agar well diffusion method. For antiaspergillus activity, the test fungus was initially grown on PDA until a sufficient spore formation has reached. 0.85 % sterile saline solution was poured onto fungal growth and spores were loosened by means of a sterile swab by gently scrapping surface of colonies. Density of spore suspension was adjusted to $1X10^5$ spores/ ml. For anticandida activity a freshly grown culture of *Candida albicans* (MCC 1154) was suspended into sterile saline to adjust the thickness of $1X10^6$ CFU / ml (0.5 Mc Farland scale). The above suspensions of test fungi were then aseptically mixed in a 15 ml sterilized PDA and YMA respectively,and was over layered on sterile PDA and YMA basal agar and allowed to solidify. By means of sterile cork borer (8 mm diameter), wells were prepared and filled with 100 microlitre of crude dried organic extract and crude aqueous extract dissolved in DMSO. To control wells, corresponding aliquot of pure DMSO (solvent control), sterile distilled water and standard antifungal drug ketoconazole were added to prepare negative and positive controls respectively. The plates were then kept in refrigerator at 4 ^{o}C for 4 hrs. For complete diffusion of antifungal compounds and subsequently incubated at 28±2^{0}C for 48 hrs. (for anticandida activity) and 3-4 days (for antiaspergillus activity). After incubation, zone of inhibition was measured around each well.

2.6 Detection and identification of potential antifungal endophyte:

2.6.1 Identification by cultural and morphological characteristics:

Endophyte showing promising antifungal activity was observed for colony characters, cell morphology and Gram reaction.

2.6.2 16S rRNA sequencing and phylogenetic analysis:

I6S rRNA gene sequencing was done at NCCS Pune. DNASTAR SeqMan Pro was used for sequence assembly. The assembled sequences were analysed for their closest relatives based on pairwise sequence similarity by using the EzBioCloud database. Phylogenetic tree was constructed from evolutionary distances using the neighbor joining method.The sequence has also been deposited in GenBank of NCBI.

2.7 Characterization of antifungal substances:

The crude aqueous extract obtained from endophytic bacterium which showed prominent antifungal activity, was characterized by employing UV, Fourier Transform Infra-Red (FTIR) spectroscopy, High Performance Liquid Chromatography (HPLC) and GC-MS techniques.

2.7.1 UV visible spectroscopy:

The UV-Visible spectrum of the sample compound was recorded using Shimadzu spectrophotometer. One mg of the sample was dissolved properly in 2.0 ml of water and filtered through a membrane filter of 0.22 µ pore size. The sample was taken into a cuvette and scanned at wavelengths between 200 - 800 nm with an increment of 100 nm using Shimadzu spectrophotometer (UV-2450).

2.7.2 FTIR:

1 mg crude extract was mixed well with 10 mg anhydrous potassium bromide and this mixture was made into a circular pellet and was placed in the IR holder. The transmission was recorded in the decreasing range of 4000 to 400 nm (JASCO, Model FT/IR-5300, Japan).

2.7.3 HPLC:

This analysis was performed by using the HPLC (Shimadzu HPLC, Japan LC 2010 CHT). The system consisted of the sample injector with C18 column and mobile phase i.e. acetonitrile and water having a flow rate of 1.5 ml per min. The quantity of sample injected in column was 20 µl and compounds present were detected using the UV-visible detector at 254 nm.

2.7.4 GC-MS:

The GC-MS analysis of crude aqueous extract was carried out using Shimadzu Japan GC 2010 MS-QP 2010 installed with silica capillarity column SHRxi5sil. MS having a length 30 meter and thickness 0.25 µm. Following temperature ramp was used: The oven temperature program was initially set at 50^{o}C, kept for 2 min, with ramp rate 10^{o}C min $^{-1}$until it reached 200^{o}C, and the temperature was kept at this level for 5 min. Then ramp rate was maintained as 10^{o}C min $^{-1}$upto 275^{o}C and held for 5 mins. The temperature of the injector was kept at 250^{o}Cand injected sample volume of 1 µlwith a split ratio of 1:70.The carrier gas was Helium at linear velocity with a flow rate of 1.0 ml/min. MS conditions employed were as follows: ion source temperature 200^{o}C and inlet temperature of 250^{o}C with detector voltage 70 eV.The identification and confirmation of compounds were performed by comparing and matching the obtained mass spectra, retention time and fragmentation pattern of compounds with an available database of the National Institute of Standards and Technology (NIST database/ chemstation data system) mass spectral library.

3. RESULTS

3.1 Isolation of endophytic bacteria and fungi:

Isolation of endophytes from *Aloe vera* leaves was done by optimizing the efficient method of surface sterilization as mentioned in method above and was found to be highly effective wherein sterility checks showed no growth, claiming no epiphytic contamination and the isolates obtained were true endophytes. After incubation, fungal growth was obtained on 9th day and bacterial growth on 3rd day. Total twenty nine fungal and fifteen bacterial endophytes were isolated. These were then subcultured on fresh sterile NA and PDA plates and coded alphanumerically.

3.2 Preliminary screening of endophytes for antifungal activity:

Among the 15 bacterial endophytes isolated, only 3 (20%) isolates designated as Alo NA L2, L 3 and L 4 showed anticandida as well as antiaspergillus activity. Among the most active endophyte, Alo NA L 4 inhibited strongly both test pathogens whereas L2, L3 inhibited relatively weaker. None of the rest isolates exhibited anticandida and antiaspergillus activity.

3.3 Production and extraction of antifungal metabolites:

Alo NA L2, L 3 and L 4 strainswere selected forfermentation and has shown maximum metabolite production at 30°C temperature, pH 7.0 for 03 days in nutrient broth. The crude secondary metabolites extracted by different solvents and cell free supernatant(aqueous extract) were tested for antifungal activity against the test pathogens, viz., *Aspergillus species (*MCC 1074) *Candida albicans (*MCC 1154). In this screening no organic solvent extracts has shown significant antifungal activity whereas the aqueous extract showed significant antifungal activity. Overall, it appears that the antifungal components may be mostly polar in nature.

3.4 Secondary antifungal assay:

After the mass production of antifungal metabolites by Alo NA L2, L 3, and L 4, confirmation of antifungal activity was done using crude extract against *Aspergillus species* (MCC 1074) and *Candida albicans* (MCC 1154) by using agar well diffusion method. The antifungal antibiotic, ketoconazole was used as a standard.The concentration of test compound and standard was 1000 µg/ml. Results were tabulated as diameters of zones of inhibition accordingly in **Table 1**. The crude aqueous extract of Alo NA L 4 exhibited the significant antiaspergillus activity as well as anticandida activity, whereas the crude aqueous extract of Alo NA L2, Alo NA L 3 has shown weaker antifungal activity against both test pathogens. The control i.e. pure DMSO had no inhibitory activity against selected fungal pathogens and a significant clear zone of inhibition was observed for the positive control in all the cases. Since Alo NA L 4 shows considerable antifungal activity this isolate was selected for characterization of antifungal metabolites **(Figure 1)**.

3.5 Detection and identification of potential antifungal endophytes:

3.5.1 Colony characteristics, Cell morphology and Gram reaction:

When grown on nutrient agar at 27^{o} C for 24 hrs., Alo NA L4 have shown a creamy white smooth, opaque colony with size of 1-3mm, round with irregular margin and flat elevation. Alo NA L 4 was Gram positive rod shaped occurring singly and in short chains.

3.5.2 16S rRNA sequencing and phylogenetic analysis of endophytic bacteria:

On the basis of 16S rRNA gene sequence, Alo NA L 4 was best pair-wise aligned with 16S rRNA gene sequence of *Bacillus velezensis-CR-502*(AY603658) with sequence similarity of 100%. Sequences obtained after molecular characterization were submitted to NCBI GenBank database and has GenBank Number -MW356850. The above isolate was also deposited in National Centre for Microbial Resource (NCMR) of NCCS with accession number *Bacillus velezensis* strain Alo NA L 4(MCC 3657) **(Figure 2)**.

Phylogenetic analysis:

With the help of sequence base pairs, phylogenetic analysis was done. *Bacillus licheniformis* ATCC 14580 CP000002 is the outgroup. Antifungal isolate forms divergent linkage from cluster of *Bacillus amyloliquifaciens subsp. plantarum, Bacillus siamensis*. As per the phylogenetic tree, strain Alo NA L 4 showed closest similarity with *Bacillus velezensis* which support the 16 S rRNA gene sequencing result **(Figure 3)**.

3.6 Characterization of antifungal metabolites:

The crude aqueous extract was subjected for chemical characterization by UV-visible spectroscopy, HPLC, FTIR spectroscopy and GC-MS.

3.6.1 UV visible spectral analysis:

The UV spectrum of crude aqueous extract was determined with a JASCO UV-Visible spectrophotometer at UV-vis range 200-800 nm and is represented in **Figure 4**. The sample shows maximum absorption (λmax) at wavelength 257nm. All the samples, which possess aromatic chromophores, absorb in 250 nm of UV spectra with pi-pi interactions.

3.6.2 FTIR:

IR spectrum of crude aqueous extract of Alo NA L 4 is shown in **Figure 5**. The mid-infrared, approximately 4000-400 cm^{-1} was used to study the fundamental vibrations and associated rotational-vibrational spectrum. The interpretation is given in **Table 2**.

From **Table 2** the presence of hydroxyl, primary amine, alkanes, alkenes carbonyl groups, aromatic hydrocarbons, carboxylic acids and esters in the sample can be inferred.

3.6.3 HPLC:

The HPLC chromatogram of this sample yielded two prominent peaks with retention times of *t*R=4.99 and 7.87 min indicating the presence of probably two compounds which represents bioactive fraction responsible for antifungal activity **(Figure 6)**.

3.6.4 GC-MS:

The chromatogram predicted the presence of three compounds and identified based on peak area, retention time, molecular weight and molecular formula. The name, molecular weight and structure of the components of the test sample were determined as 1,2 dichloro-1-ethoxy ethane ($C_4H_8Cl_2O$) with molecular weight of 142,S methyl methane thiosulphonate ($C_2H_6O_2S_2$)with molecular weight of 126,Disulphide dimethyl ($C_2H_6S_2$) with molecular weight 94 **(Figure 7a, b)**.

Table 1: Zones of inhibition in mm against selected fungal pathogens

Crude extract of endophyte/ Positive/Negative control	Zone of inhibition in mm	
	Against *Candida albicans*	Against *Aspergillus sp.*
Alo NA L 2	28	12
Alo NA L 3	30	20
Alo NA L 4	30	23
Pure DMSO	-	-
Ketoconazole (1000µg/ml)	34	32

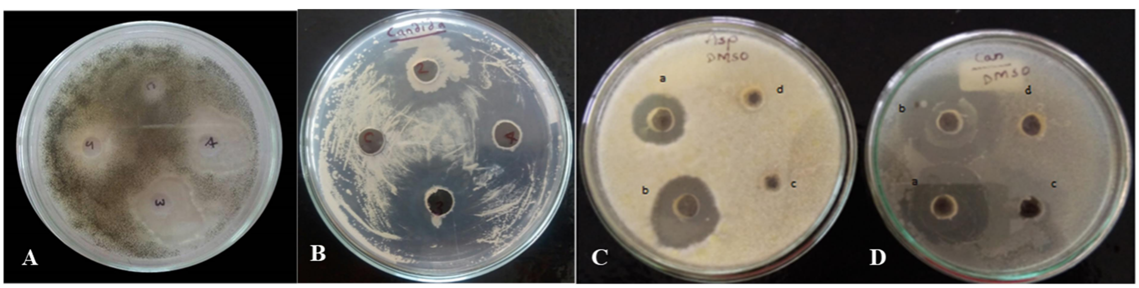


Figure 1: Antifungal activity of Alo NA L 2,3,4 against A) Aspergillus sp. B) Candida albicans C) Antiaspergillus and D) Anticandida activity of a) Crude extract of Alo NA L 4 b) Ketoconazole c) Pure DMSO d) distilled water

16S rRNA sequencing:

TGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTG
ATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGG
GGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATG
GACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
TGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGG
AAGAACAAGTGCCGTTCAAATAGGGCGGCACCTGACGTACTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTT
AAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAG
GAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCT
CTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGC
CTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCC
CTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCG
GAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCAG
AACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCA
ACTCGACTGCGTGAAGCTGGAATC

Figure 2: 16S rRNAsequence of Alo NA L 4

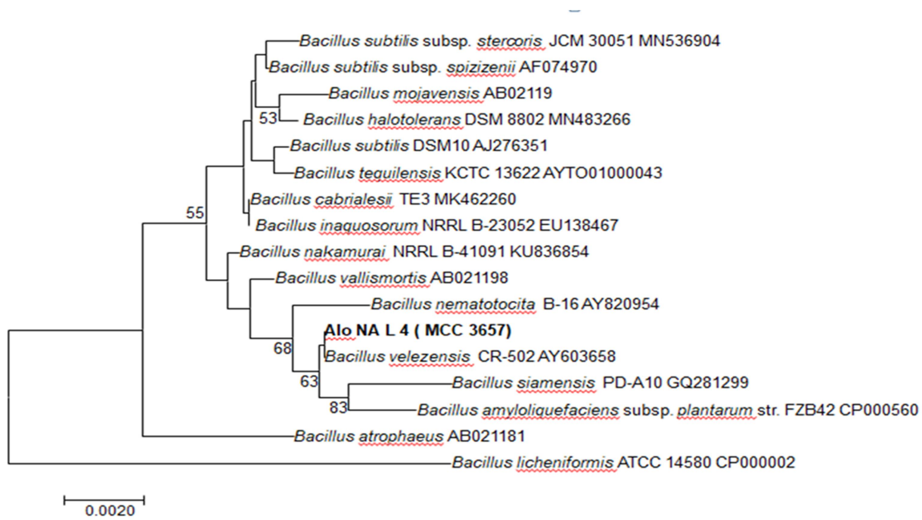


Figure 3: Phylogenetic tree showing the relationship among bacterial 16S rRNA gene sequences of potential fungal antagonistic endophyte with reference sequences obtained through BLAST analysis. The numbers on the tree indicates the percentages of bootstrap sampling derived from 1000 replications. Numbers in the bold are sequences obtained in the present study. Bar, inferred nucleotide substitutions per nucleotides

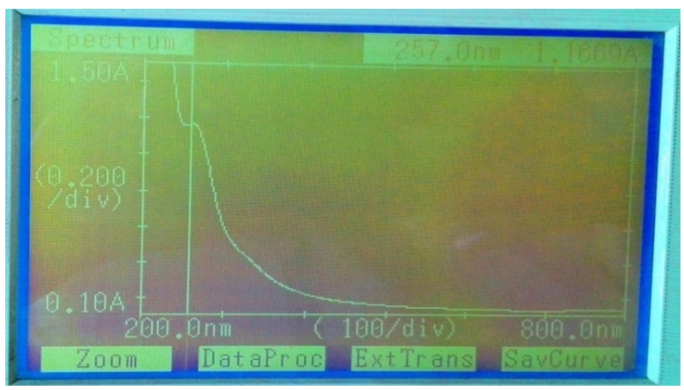


Figure 4: UV-Vis spectrum of crude aqueous extract of Alo NA L 4 showing the lambda maxima at 257 nm

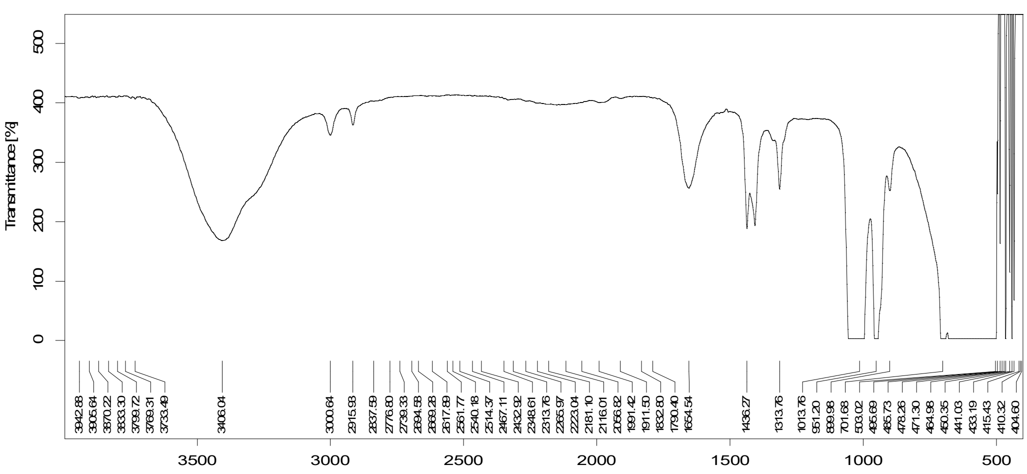
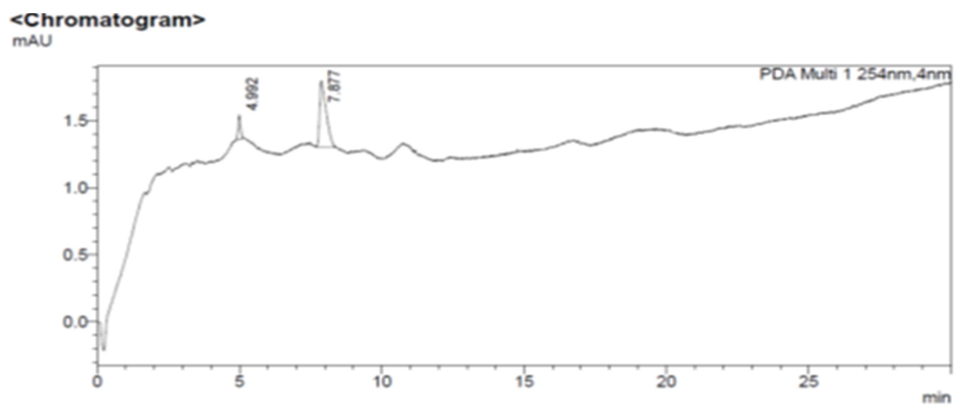


Figure 5: Infrared Spectrum of compound derived from *Bacillus velezensis* Alo NA L4 isolated from *Aloe vera* leaves

Table 2: Interpretation of IR spectrum

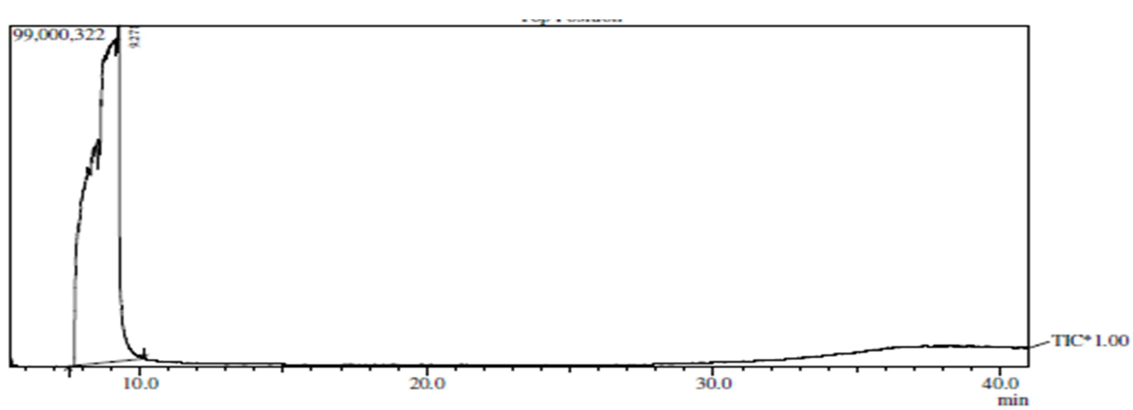
Sr. No.	Vibration	Obs. Frequency cm^{-1}	Part of molecule	Inference
1.	C-H stretching	2915.93	3000-2850 C-H of Sp^3 C	$-CH_3$
		3000.64	3000-3100 Alkyl gr. Of Sp^2 C	-C≡ C-H
2.	-O-H stretching	3406.04	3500-3200 -O-H stretching of phenol	-O-H
3	C=O stretching	1654.54	1630-1680 Sec. amide	-C-N-
4.	C=C stretching (Aromatic)	1436.27	1475 C=C(Aromatic)	C=C (Aromatic)
5	C-N	1313.76	1000-1350	C-N
6	C-O	1013.76	1000-1300	C-O
7	C-H out of plane bond	951.20	650-1000	C-H
8	C-H aromatic bond	899.98-701.68	690-900	C-H



<Peak Table>
PDA Ch1 254nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	4.992	1125	187	0.000			
2	7.877	7821	493	0.000			
Total		8946	680				

Figure 6: HPLC chromatogram of aqueous crude extract of Alo NA L 4



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Name
1	9.271	7.508	10.142	6632626377	100.00	
				6632626377	100.00	

Figure 7a: Gas chromatogram of Alo NA L 4 isolated from *Aloe vera*

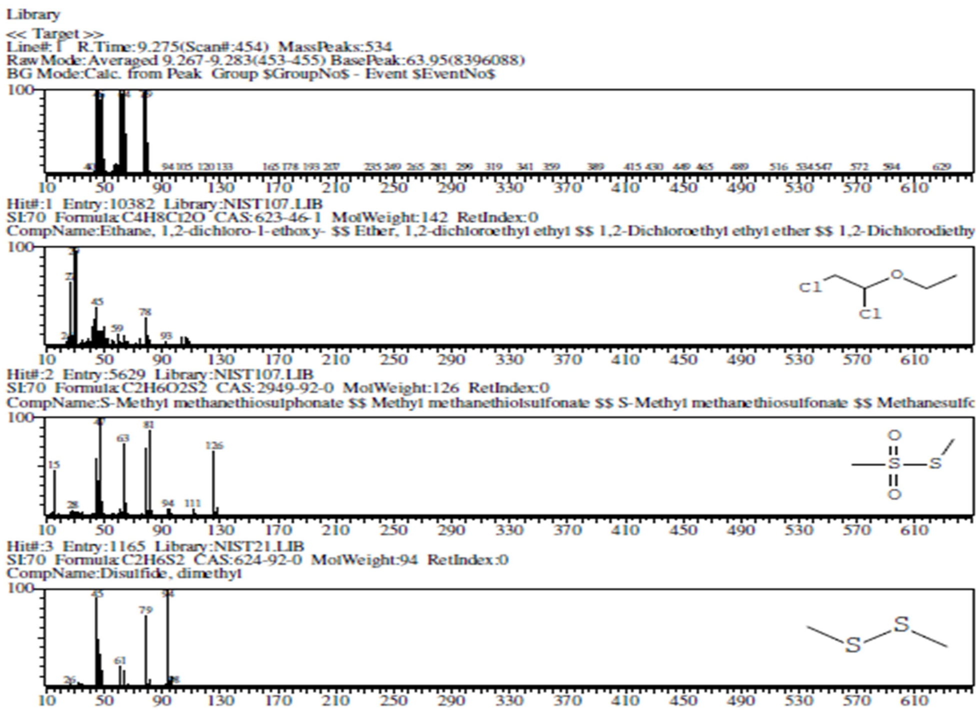
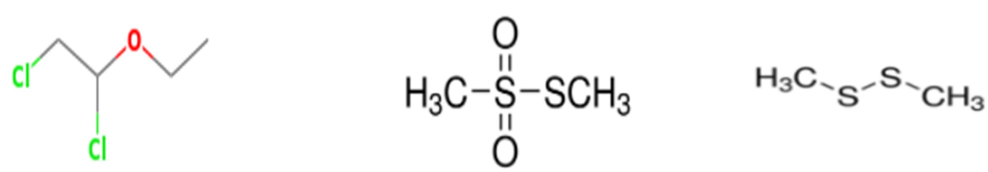


Figure 7 b: Mass spectra of the compounds from Alo NA L4



1,2 dichloro-1-ethoxy ethane   **S methyl methane thiosulphonate**   **Disulphide dimethyl**

Figure 8: Structures of antifungal compounds analysed by GC- MS

4. DISCUSSION

The increasing number of duplications and the urgent demand for few leading structures in pharmacology has enforced the search for metabolites in so far untouched habitats. Our interest focused on the endophytic group, the members of which have demonstrated interesting antimicrobial activity. In recent past, endophytes owing to their capacity to produce novel bioactive compounds have attracted the attention of the scientific community. However, it was observed that the endophytes in the traditional medicinal plants are relatively unstudied. Exploration of endophytes has potential to establish a scientific basis for traditional therapeutic uses of medicinal plants.

We have screened leaves of *Aloe vera*, from Satara region and were found to harbor varied types of bacterial and fungal endophytes. These observations are in accord of results stated by Suryanarayanan and Thennarasan **[29]**. Plants from the tropical rainforests were always known to be heavily manifested with endophytic fungi in their tissues. The endophytes isolated over the course of this work were obtained using a traditional method, in which only endophytes that can grow on microbiological media were accounted for which may create a negative bias towards endophytes that are slow growing or that cannot grow on biological media. To assess the full range of endophytes, future work should incorporate molecular techniques like DGGE or T-RFLP **[30, 31]**. However, the culturing of surface sterilized plant tissue segments on the media is the most accepted method for the isolation of the endophytes and widely used by the researchers **[32]**. When different methods of isolation of endophytes were studied it was observed that appearance of endophytes from sterilized tissues decreases with increase in treatment time of ethanol and sodium hypochlorite. It is obvious from study that type of surface sterilizing agent, its concentration as well as treatment time directly have an effect on qualitative and quantitative diversity of culturable endophytes. Therefore no single protocol of sterilization can be used as a comprehensive method appropriate for recovery of true endophytes from all plants. Various reports regarding the isolation of fungal endophytes have been reported by other workers similar to our studies. Kumar *et al*. **[33]** reported the isolation of eight fungal endophytes from different plant parts of *Menthe viridis* collected from Khamariya region of Jabalpur M.P. (India) and also studied their antibacterial effect against six different pathogens Dissanayake *et al*. **[34]** isolated twenty one morphologically distinct endophytic fungi from *Calamus thwaitesii* and also reported the antimicrobial activity of the compound mycoleptodiscin-B isolated from endophytic fungus *Mycoleptodiscus sp*. Qadri *et al*. **[35]** isolated fungal endophytes from selected plants of Western Himalaya, among which the conifers, *Pinus roxburgii, Abiespindrow*and *Cedrus deodara* harbored most diverse forms of endophytes. Gautam *et al*. **[36]** isolated two hundred and twelve fungal endophytes from *Cannabis sativa* and studied their antifungal potential.

Similarly, *Ho et al.* **[37]** isolated sixty seven endophytic fungi from the twigs of medicinal plants of Lauraceae family and eighty nine from Rutaceae family in central and northern Taiwan and studied their taxonomical features and antimicrobial activity against pathogens. Similarly, Jalgaonwala *et al*. **[38]** isolated seventy eight bacterial and one hundred and forty two fungal endophytes from aerial along with underground parts of various medicinal plants. One hundred and ninety four fungal endophytes were isolated from wild medicinal plants of Thailand by Theantana *et al*. **[39]**. Huang *et al*. **[40]** reported the isolation of one thousand, one hundred and sixty endophytic fungal strains from twenty nine traditional Chinese medicinal plants. Similarly, Nalini *et al*. **[41]** isolated ninety six fungal endophytes from eight hundred twig and bark segments of *Cratavea manga*. One hundred and twenty one fungal endophytes were isolated from leaves and stems of sixty two medicinal plants in Malaysia and were evaluated for their antimicrobial activity against various microorganisms by Radu S and Kqueen Cheah Yoke **[42]**. **Arnold *et al.*** **[43]** isolated four hundred and eighteen endophytic morphospecies from eighty three healthy leaves of *Ouratea lucens* and *Histeria concinna* in a low land tropical forest of central panama region, and proposed that tropical endophytes themselves might be hyperdiverse with host preference and spatial heterogeneity. Thus most of natural bioactive products have been reported to be obtained from endophytic fungi, however scanty information is available regarding bacterial endophytes from medicinal plants **[44, 45]**.

In preliminary screening by cross streak assay and agar overlay method, only 3 (20%) out of 15 bacterial endophytes showed anticandida as well as antiaspergillus activity. Among the most active endophyte, Alo NA L 4 inhibited strongly both test pathogens than rest two.None of the fungal endophytic isolates exhibited significant antifungal activity. Whereas most of the commercially used antibiotics are fungal products.However antimicrobial and antifungal activities of fungal endophytes from several other medicinal plants have been well documented **[46–52]**. Usually fungi and actinomycetes are used for bioprospecting purpose because most of the antibiotics and other therapeutic drugs of microbial origin produced today are by using members of these taxonomic groups. In contrast bacteria are mostly neglected from being studied as a producer of antimicrobial compounds. In our study we thus tried to make best use of endophytic bacterial isolate as producer of antifungal compounds. When the cell free supernatant of Alo NA L 4 was extracted with different organic solvents like ethyl acetate, chloroform, hexane, dichloromethane, petroleum ether, surprisingly no antifungal activity was observed. However, most of the antifungal antibiotics were extracted using ethyl acetate **[53]**. The antifungal compounds from Alo NA L 4 are soluble in water but the standard antifungal antibiotics, like itraconazole are insoluble in water. This may be indicative of the thing that the principle antifungal compound may not be a single and mixture of different compounds may be responsible for said activity and all these compounds may not have similar affinity towards organic solvents or they may be polar in nature.Snook M E **[54]** in his study have shown that culture filtrates from bacterial endophyte *Bacillus mojavensis* RRC 101 were antagonistic to the pathogenic and mycotoxic fungus *Fusarium verticillioides*. However, the inhibitory substance from extracts of this bacterium has not been identified. Further it has shown that direct analysis of culture filtrate by mass spectrometry established presence of Leu^7 surfactin as well as mixtures of several other isomers. In this study, we demonstrated that culture filtrates from the endophytic bacterium Alo NA L 4 grown aerobically in NB medium displayed antifungal activitiy against *Candida albicans* and *Aspergillus species*. These results indicate the presence of either excellent antimicrobial potency of the filtrates or else of a high concentration of some active principles in the filtrates of strains showing positive antifungal activities. Endophytic extracts which showed no antifungal activity in these assays probably may be active against other microbes which were not tested in present study. In earlier reports fungal endophytes of *Aloe vera* were shown to suppress mycelial growth of *F. oxysporum* **[55]** Whereas in another study the crude as well as ethyl acetate extracts of the metabolites of six isolates from *Aloe vera* , have shown broad spectrum antimicrobial activities against pathogenic *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus cereus, Proteus vulgaris, Salmonella typhimurium, Klebsiella pneumoniae, Streptococcus pyogenes Escherichia coli, and Candida albicans* **[56]**. But no any report uptill date is available on antiaspergillus activity of bacterial endophytes of *Aloe vera.* Lavermicocca *et al*. **[57]** has also shown that when the crude extract and fractions A, B, and C of bacterial culture filtrates were assayed by the antifungal disk assay using *E. fibuliger*IBT605 as the indicator only the crude extract and fraction C, containing more polar compounds, caused inhibition halo around the disks.When the same commercial compounds were used individually, only phenyllactic and *p*-hydroxyphenyllactic acids showed antifungal activities. A synergistic action has often been reported for antimicrobials produced by microorganisms, because in mixtures such compounds may interact with each other as well as with the test organisms **[58–60]**. A report by **Glienke *et al.* [26]** revealed that there is a difference between the antimicrobial activities of the crude extract of the plant with respect to their fractions, probably due to the existence of an interaction of compounds in the crude extract, that can enhance the activity against *C. albicans*. Therefore, when the extract was fractionated these compounds were put apart, reducing their potential to act. According to one more study about antimicrobial activity of peppertree it was confirmed that the aqueous extract when fractionated would lose inhibitory activity against *C. albicans* **[61]**, confirming the significance of synergism in this case. Apparently compound interactions that help crude extract activity with respect to fractions against yeast do not show the same effect to the other organisms tested.

In present study Alo NA L 4 strain shared 100 % sequence similarity in 16S rRNA gene with *Bacillus velezensis*-CR-502(AY603658).Based on 16S rRNA gene sequencing and phylogenetic analysis, Alo NA L 4 strain was identified as *Bacillus velezensis .Bacillus species.* is known to produce a wide variety of antifungal compounds, together with volatiles **[62]** lipopeptides **[63, 64]** in addition to several modified small peptides as well as proteins **[65, 66]**. *Bacillus velezensis* is a Gram-positive, rod-shaped bacterium that forms spores, and has long been recognized as one of the most important bacterium that is potentially promising to generate many antimicrobial compounds **[67, 68]** with potential applications in the various fields. The results of our study showed that the *Bacillus velezensis* Alo NA L 4 strain from *Aloe vera* exhibited strong antifungal activities. In present study, GC-MS technique was used to investigate the antifungal metabolites and we report for the first time, coproduction of different types of water soluble antifungal compounds like 1,2 dichloro-1-ethoxy ethane ($C_4H_8Cl_2O$), S methyl methane thiosulphonate ($C_2H_6O_2S_2$) and Disulphide dimethyl ($C_2H_6S_2$) by bacterial endophyte *Bacillus velezensis* from *Aloe vera*. Uptill date such work has not been reported in literature. Fernando *et al*. **[69]** has reported that sulfur-based compounds benzothiazole and dimethyl trisulfide possesses high fungicidal activity. Many commercially used fungicides and soil fumigants are also sulfur-based. Our results agree with the results obtained by Cheffi *et al*. **[70]** wherein the endophytic *Bacillus velezensis* was displaying significant antagonistic activity in vitro. The antimicrobial compounds that have been characterized previously from marine *Bacillus velezensis* include a group of bioemulsifiers known as lipopeptides with excellent biosurfactant activity and a broad spectrum of antimicrobial properties **[71]**.

Gao *et al*. **[72]** reported GC-MS analysis of volatile organic compounds from endophytic *Bacillus velezensis* ZSY-1 isolated from Chinese catalpa shows the major compounds as pyrazine (2,5-dimethyl), phenol (4-chloro-3-methyl), benzothiazole, and phenol-2,4-bis (1,1-dimethylethyl) have antifungal activity against *Alternariasolani, Botrytis cinerea, Monilinia fructicola, Valsamali, Fusarium oxysporum f. sp. capsicum,* as well as *Colletotrichum lindemuthianum*. However, the most common Volatile Organic Compound (VOC) types were ketones (seven unique compounds), alkanes (six), alcohols (six). Pyrazine (2,5-dimethyl), benzothiazole, 4-chloro-3- methyl, and phenol-2,4-bis (1,1-dimethylethyl) had considerable antifungal activity against *B. cinerea* and *A. solani*.Among the detected compounds in our GC-MS report, dimethyldisulfide which is produced by many microorganisms and found in garlic oil has been found to have antifungal activity **[73, 74]** and has shown that dimethyl disulfide has antifungal activity against *P. italicum*in vitro and inplanta through fumigant action **[74]**. Data produced by Lim *et al*. **[75]** indicate that strain G341 could inhibit mycelial growth of a variety of phytopathogenic fungi by producing dimethylsulfoxide, 1-butanol, and acetoin. Amongst them, acetoin was found to be a major volatile metabolite. Similarly Joller *et al*. **[76]** has also reported antifungal activity of S methyl methane thiosulfonate which also supports our results. Therefore, the *Bacillus velezensis* Alo NA L 4 strain and its antifungal compounds may be considered as potential antifungal agents in pharmaceutical and cosmetic formulations, in food preservation, as preservative, as an antifungal component in wall paints and as a biocontrol agent for crop diseases. Bacteria from *Bacillus* genus, such as those isolated and characterized in the present study, have a number of characteristics that are desirable in potential commercial applications such as: they can produce many biologically active substances and are persistent, forming endospores that are resistant to unfavorable environmental conditions.

5. CONCLUSION

In present study twenty nine fungal endophytes and fifteen bacterial endophytes were isolated from the leaves of *Aloe vera.* The study indicates that the endophytic bacterial strain *Bacillus velezensis* Alo NA L 4 exhibit antagonistic potential against *Aspergillus species* and *Candida albicans.* Antifungal compounds were characterized by FTIR, HPLC, and GC–MS and this study provide evidence for the secretion of antifungal metabolites 1,2 dichloro-1-ethoxy ethane ($C_4H_8Cl_2O$),S methyl methane thiosulphonate ($C_2H_6O_2S_2$), Disulphide dimethyl ($C_2H_6S_2$) by this endophyte. Our results suggest the potential use of this endophyte as a source of alternative antifungal compounds for use in pharmaceutical formulations, cosmetics and in wall paints, To the best of our knowledge, this is the first report demonstrating the mixture of antifungal compounds showing synergistic action produced byendophytic *Bacillus velezensis* from the medicinal plant *A. vera*. However, its antagonistic mechanism against pathogens need to be elucidated and detailed investigation on the characterization and their utilization in pharma formulation must be conducted. Hence we conclude that endophytic bacteria from medicinal plant like *Aloe vera* are a significant resource for drug discovery.

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