Journal of Applied Sciences

ISSN 1812-5654

science alert

ANSInet
an open access publisher
http://ansinet.com

OPEN ACCESS Journal of Applied Sciences

ISSN 1812-5654
DOI: 10.3923/jas.2019.140.147

Research Article
Identification of Antibacterial Compound from *Bacillus horikoshii*, Isolated from Rhizosphere Region of Alfalfa Plant

[1]Nisha M. Nair, [2]R. Kanthasamy, [3]R. Mahesh, [4]S. Iruthaya Kalai Selvam and [1]S. Ramalakshmi

[1]Department of Microbiology, Karpagam Academy of Higher Education, 641 021 Coimbatore, Tamil Nadu, India
[2]PG and Research Centre of Botany, Rani Anna Govt. College for Women, 627 008 Tirunelveli, Tamil Nadu, India
[3]PG and Research Centre of Botany, South Travancore Hindu College, 629 002 Nagercoil, Kanyakumari District, Tamil Nadu, India
[4]PG and Research Centre of Zoology, Jayaraj Annapackiam College for Women (Autonomous), Periyakulam, 625 601 Theni District, Tamil Nadu, India

Abstract

Background and Objective: With increase in multiple drug resistance pathogens, it is necessary to look for new drug study compounds of microbial origin. Thus study was aimed to identify the rhizosphere microflora of unexplored alfalfa plant for new antimicrobials. **Materials and Methods:** Based on screening done, the isolates were subjected to antibacterial activity against selected bacteria. The isolate was mass cultured and secondary metabolites were extracted using ethyl acetate. The crude extracts collected were subjected to FTIR and GC-MS analysis. **Results:** Based on functional diversity analysis, the isolate subjected to anti-bacterial activity revealed significant activity against *Klebsiella* and *Staphylococcus aureus* with zone of inhibition in the range of 17-18 mm. Based on GC-MS analysis reports, six compounds were identified and 11-Octadecanal responsible for bio-activity. FT-IR results showed that N-H stretching functional group dominantly present in the extract. Molecular identification of the isolate by 16S rRNA sequencing showed the isolate as *Bacillus horikoshii*. **Conclusion:** The study results showed that the isolate *Bacillus horikoshii*, Gram-positive spore forming bacteria had wide antibacterial activity due to 11-Octadecanal. Thus Alfalfa plant rhizosphere region harbors antibacterial potential microbes.

Key words: Alfalfa plant, antibacterial activity, *Bacillus horikoshii*, gas chromatography-mass spectrometry, rhizosphere

Citation: Nisha M. Nair, R. Kanthasamy, R. Mahesh, S. Iruthaya Kalai Selvam and S. Ramalakshmi, 2019. Identification of antibacterial compound from *Bacillus horikoshii*, isolated from rhizosphere region of alfalfa plant. J. Applied Sci., 19: 140-147.

Corresponding Author: Ramalakshmi Subbiah, Department of Microbiology, Karpagam Academy of Higher Education, 641 021 Coimbatore, Tamil Nadu, India Tel: 8608000835, 9600202880

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Alfalfa (*Medicago sativa*) means "father of all foods" belongs to Leguminosae family. It is a perennial herbaceous leguminous flowering plant known as Queen of Forage plant, lives upto 8 years. Due to its high protein content and fiber, they are cultivated worldwide and widely used as fodder for cows[1]. The crop is autorotated before reseeding due to its toxins having an allelopathic effect for other plants growth. The plant also has deep root system, thereby improving soil and water holding ability of plants. Alfalfa fixes high nitrogen than other plants due to its symbiotic association with microbes.

Medicago sativa, a therapeutic value plant has been reported for a number of phytopharmacological activities such as neuroprotective, hypocholesterolemic, antioxidant, antiulcer, antimicrobial, hypolipidemic, estrogenic and in the treatment of atherosclerosis, heart disease, stroke, cancer, diabetes[2] and menopausal symptoms in women[3-10].

The plant extract has been reported to possess bioactive compounds namely saponins, flavonoids, phytoestrogens, coumarins, alkaloids, amino acids, phytosterols, vitamins, digestive enzymes and terpenes[11-13].

Based on preliminary functional diversity studies carried (unpublished results) on the isolation of microbes from the rhizosphere of alfalfa plant. Out of 32 isolates, this isolate was selected for its amylase, cellulase, protease and phosphate solubilization activities. Thus the study was aimed at isolating the functionally diverse organism from rhizosphere soil region of Alfalfa plant (*Medicago sativa*) and identifying the bioactive compound responsible for antibacterial action.

MATERIALS AND METHODS

Sample collection: Soil sample was collected from the rhizosphere region of Alfalfa plant fields during June 2016 from Sulur, Coimbatore, Tamilnadu, India. Studies were carried out from June, 2016 to March, 2017 (Fig. 1).

Isolation and identification of micro-organism: One gram of the collected soil samples were used for serial dilution to isolate microbes by spread plate method. To the nutrient agar plates, 0.1 mL of serially diluted samples (10^{-1} to 10^{-7}) were plated, incubated at 37°C for 24-72 h. After incubation, bacterial isolates were checked for purity and preserved in glycerol stocks and as nutrient agar slants for further tests.

These colonies were observed for Gram's nature and morphological characters such as size, shape, color, texture, opacity, elevation, margin and mobility. They were further identified using biochemical methods as stated in Bergey's manual for characterization which includes Indole, Methyl Red, VogesPrauskaeur, citrate, urease and TSI slants etc.

Fig. 1: Alfalfa plant (*Medicago sativa*)

Antibacterial activity: The antimicrobial activities of crude extracts of all isolated bacteria were tested against bacterial pathogens by agar well diffusion method. Muller-Hinton agar (MHA) plates were prepared and the wells were made with sterile cork borer on the agar plates. The overnight grown nutrient broth cultures of all bacterial pathogens were uniformly swabbed on to the surface of MHA plates using sterile cotton swabs. Each 50 µL of cell free supernatants were aseptically incorporated into the well and the plates were incubated in an upright position at 37°C for 24 h. After incubation, the plates were observed for zone of inhibition.

Production and extraction of the bioactive compounds: For obtaining the large biomass, the active strain were inoculated into 1 L of nutrient agar medium and incubated in shaker at 30°C at 160 rpm for 36 h. After incubation, the media contents were centrifuged at 10,000 rpm for 10 min to obtain the cell free supernatant.

The cell free supernatant extracted with organic solvent-ethyl acetate and extraction carried out with 3 volume of solvent for 2 h by using rotary shaker supernatant fractions were flash evaporated at 45°C temperature to ensure complete removal of solvent and the extracts were evaporated to dryness. The resulting residues were dissolved in small amount of respective solvents and stored at -20°C until further purified.

Molecular identification and phylogenetic analysis of the bioactive compound

Genomic DNA isolation: DNA isolation from bacterial isolate performed according to the cold spring harbour lab protocol. Briefly, the isolates were grown in Nutrient Broth (Himedia, India) for 24 h days at 37°C, pelleted and washed in 1 mL Tris-EDTA (TE) buffer. The pellets were resuspended in 500 µL TE buffer containing 1 mg mL^{-1} lysozyme. After incubation at room temperature for 2 h, 75 µL of 10% Sodium Dodecyl Sulfate (SDS) and 125 µL of 5 M NaCl were added to this mixture. The samples were centrifuged (10,000 rpm for 10 min at room temperature) and incubated in ice cold ethanol (-70°C) for 3 min, later in a 65°C water bath for 3 min and on ice for 10 min. RNase (200 µg mL^{-1} of sample) added to the supernatant to remove RNA contamination and the mixture incubated at 37°C for 15 min.

Proteinase K (50 µg mL^{-1} of sample) added to content and the mixture was incubated at 37°C for 30 min. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) with upper aqueous phase recovered after centrifugation (10,000 rpm for 10 min at room temperature). To this an equal volume of chloroform/isoamyl alcohol (24:1) added and upper aqueous phase transferred to a new microfuge tubes after centrifugation (10,000 rpm for 5 min at room temperature). The DNA precipitated by mixing the aqueous phase with 50 µL of 3 M sodium acetate, 300 µL of ice-cold isopropyl alcohol and incubated at -20°C for 20 min. The DNA pelleted and washed twice with 70% ethanol. The pellet blot dried and resuspended in 40 µL of TE buffer and stored at -20°C.

Amplification of 16S rRNA gene: 16S rRNA genes were amplified from the extracted genomic DNA using the 8 F and 1541 R universal eubacterial primers designed to target the conserved regions in the genomic DNA of the isolates and amplify approximately 1.4 kb length gene. The forward primer 5`-AGAGTTTGATCCTGGCTCAG-3` and reverse primer 5`-AAGGAGGTGATCCAGCCGCA-3` were used for amplification.

The PCR mix contained 5 µL of 25×PCR buffer, 4 µL of 25 mM $MgCl_2$, 5 µL of 5 µM 518 Forward Primer and 5 µL of 5 µM 800 Revers Primer, 5 µL of 1 mM dNTP's, 0.5 µL of *Taq* DNA polymerase (Thermo Scientific, India) and 2 µL of genomic DNA.

The reaction volume adjusted and made up to a final volume of 50 µL with sterile double-distilled water and amplified in an automated thermal cycler (Vapo protect Pro S, Eppendorf). The PCR conditions were an initial denaturation stage at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 60 sec, extension at 72°C for 60 sec and a final extension step at 72°C for 10 min. Negative controls with no DNA template were included in all PCR experiments.

16S rRNA gene sequencing: The Polymerase Chain Reaction (PCR) products were purified with a Montage PCR Clean up kit (Millipore) as per the manufacturer's instructions. The purified PCR products were then sequenced. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Xcelris Laboratories, India).

Phylogenetic analysis: The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results performed followed by multiple sequence alignment. The program MUSCLE 3.7 used for multiple alignments of sequences. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise). Finally, the program PhyML 3.0 aLRT used for phylogeny analysis and HKY85 as Substitution model.

FTIR analysis: The purified bacterial extract was subjected to FTIR spectroscopic analysis (Perkin Elmer Lambda), equipped with KBr beam splitter with DTGS (Deuterated triglycine sulfate) detector. The technique work on the fact that bonds and groups of bonds vibrate at characteristic frequencies. A molecule that is exposed to infrared rays absorbs infrared energy at frequencies, which are characteristic to that molecule.

GC-MS analysis: The Thermo MS DSQ II used for the analysis packed DB 35-MS capillary standard non-polar column and the components were separated using Helium as carrier gas at a flow of 1 mL min^{-1}. The injector temperature set at 260°C during the chromatographic run. The volume of sample injected 1 µL at an oven temperature of 70°C (6 min). Interpretations on mass spectrums of GC-MS were done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectra of the unknown components will be compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials will be ascertained.

RESULTS AND DISCUSSION

Antibacterial activity: The rhizosphere region of soil carries a heterogeneous group of microbial population which can play a vital role in soil function. The antibacterial activity of the crude extract of the isolate studied against 7 clinical pathogens *(P. aeruginosa*, *Klebsiella* sp, *S. aureus*, *Proteus vulgaricus*, *S. pneumonia*, *E. coli* and *B. cereus*). The isolate showed significant zone of inhibition against *Klebsiella* sp. and *S. aureus* (18 and 17 mm) (Fig. 2).

Similar reports by researchers showed that the microbes isolated form rhizosphere region possessed significant anti-microbial activity. Ramakrishnan *et al.*[14] reported the wide range of antibacterial activity of *Streptomycetes* sp. isolated from the rhizosphere soil of medicinal plants at Kolli hills of Tamil Nadu. Ryandini *et al.*[15] isolated *Streptomyces* sp. from mangrove rhizosphere mud of rhizophora mucronata from east Segara Anakan mud and reported significant activity on multiple drug resistant bacteria.

Also Rajalakshmi and Mahesh[16] reported antimicrobial activity of *Aspergillus terrus* isolated from rhizosphere region of medicinal plants in and around Kuttralam, Tirunelveli. Upon GC-MS analysis, ten compounds were identified and tetracontane was reported to be bioactive potential compound.

Molecular characterization of the isolates: The genomic DNA of the isolate isolated and subjected to 16S rRNA gene amplification for the species identification. PCR product of the length 1,400 bp purified and sequenced in Yaazhxenomics lab, Coimbatore. The 16S rRNA sequences of the isolate subjected to BLAST analysis using mega blast tool of GenBank (http://www.ncbi.nlm.n ih.gov/). Among different species comprising of closet neighbouring strains in NCBI-BLAST analysis used in the phylogenetic analysis. The phylogenetic trees were constructed based on the neighbour joining method and percentage differences in the genetic relationships between the neighbouring strains of the two samples were analyzed.

Results revealed that the 16S rRNA partial gene sequence of the isolate showed 97% similarity with *B. horikoshii* (Fig. 3, 4). The 16S rRNA gene sequence submitted to the Gene bank (NCBI, USA) and Genebank ID accession number MK226527 received.

FTIR analysis: Figure 5 depicted the FTIR analysis of the *B. horikoshii* extract showing strong peaks at 3417, 1643.35, 1097.5, 1658.78, 2926 and 715 cm^{-1}, respectively. Major group was found to be N-H stretching at 3417 cm^{-1} (Table 1). The FTIR results elucidated an array of functional groups at a frequency ranges indicating the presence of functional groups

Table 1: FTIR analysis of *B. horikoshii* extract

Peak	Corr. intensity	Corr. area	Functional group	Type of vibration
3417.86	70.60	346.46	N-H	Stretch
1643.35	55.86	59.91	C=C	Stretch
1097.5	30.93	30.24	C-O	Stretch
1411.89	13.97	4.61	-CH-	Bending
2802.57	13.67	11.38	O-H	Stretch
2877.79	11.97	2.29	O-H	Stretch

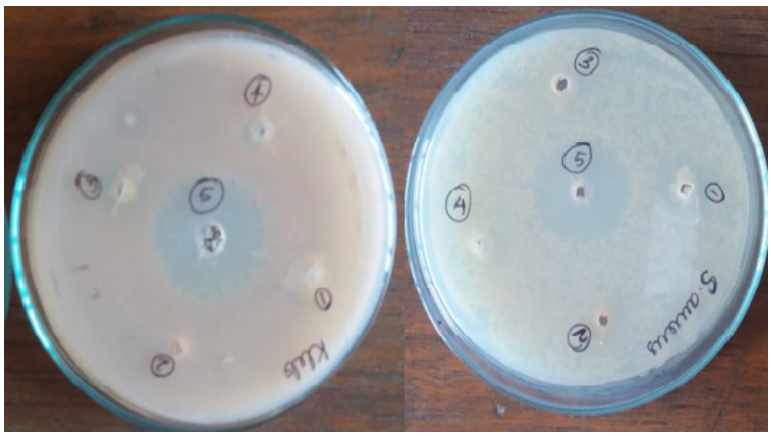

Fig. 2: Antibacterial activity of AL5 against *Klebsiella* and *S. aureus*

Table 2: GCMS analysis of compounds obtained from *B. horikoshii* extract

RT	Name of the compound	Molecular formula	MW	Peak area (%)
10.40	4-Cyano-2H-1-benzothiopyran	C10H7NS	173.00	8.12
15.49	Hexadecane	C16H34	226.00	4.89
19.59	1-Octadecene	C18H36	252.00	6.47
23.03	9,9-Dimethyl-8,10-dioxapentacyclo[5.3.0.0(2,5).0(3,5).0(3,6)]decane	C10H14O2	1663.75	
27.37	5-Octadecenal	C18H34O	226.00	3.73
36.19	11-Octadecenal	C18H34O	266.00	11.55

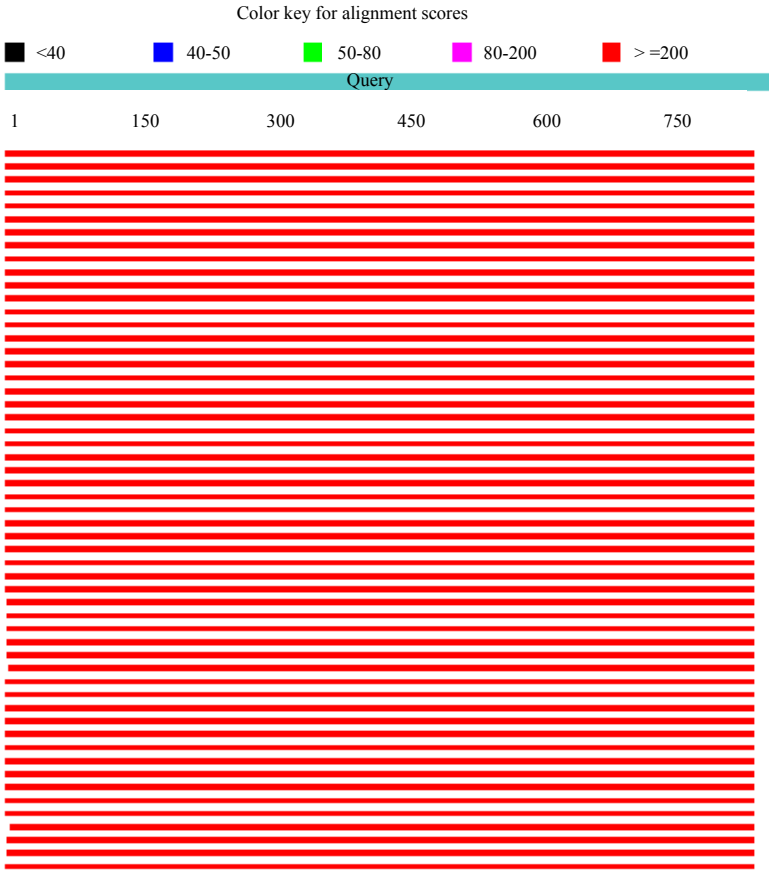

Fig. 3: Multiple alignment scores of *Bacillus horikoshii*

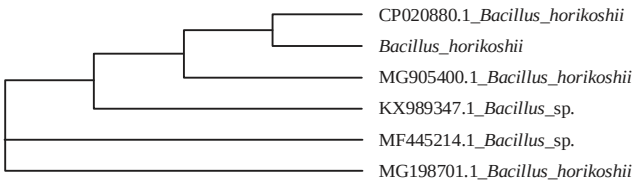

Fig. 4: Phylogenetic tree of *B. horikoshii* based on the 16S rRNA gene sequencing

corresponding to aromatic alkenes, aliphatic amines, compounds with aromatic rings, alkynes, amides, alcohols and phenols. The presence of such functional groups could be attributed to the bioactive nature of the partial fraction of *B. horikoshii* cell free supernatant.

GC-MS analysis: Figure 6 depicted the GC-MS analysis of *B. horikoshii* extract revealing presence of 31 peaks and 6 compounds were characterized and identified by comparison of the mass spectra of the constituents with the NIST library (Table 2, 3). The retention times (RT) are represented in minutes.

Stearyl alcohol, 11-octadecenal had highest intensity of 11.55% at retention time of 36.19 min. 11-Octadecenal has been reported to be present in essential oils from *Launaea resedifolia* L., possess antibacterial activity in range[17] of 11-37 mm. Similarly the presence of 9-octadecenal observed in marine red alga *Laurencia brandenii* showed various biological activities[18].

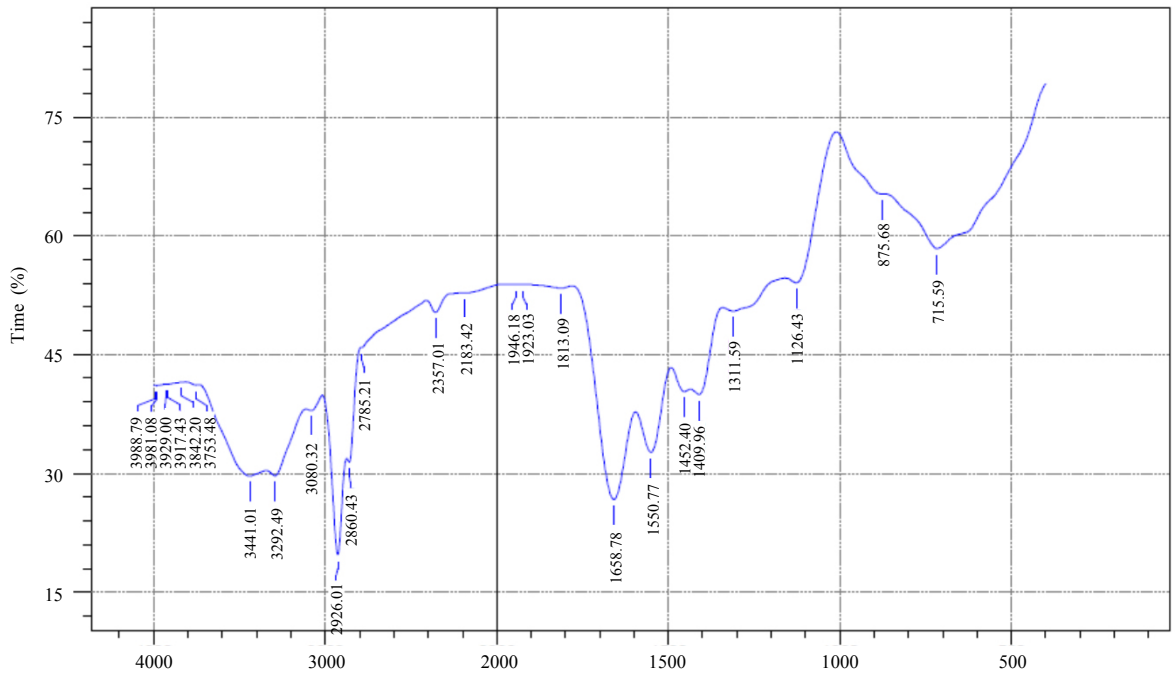

Fig. 5: FTIR spectrum of *B. horikoshii* extract

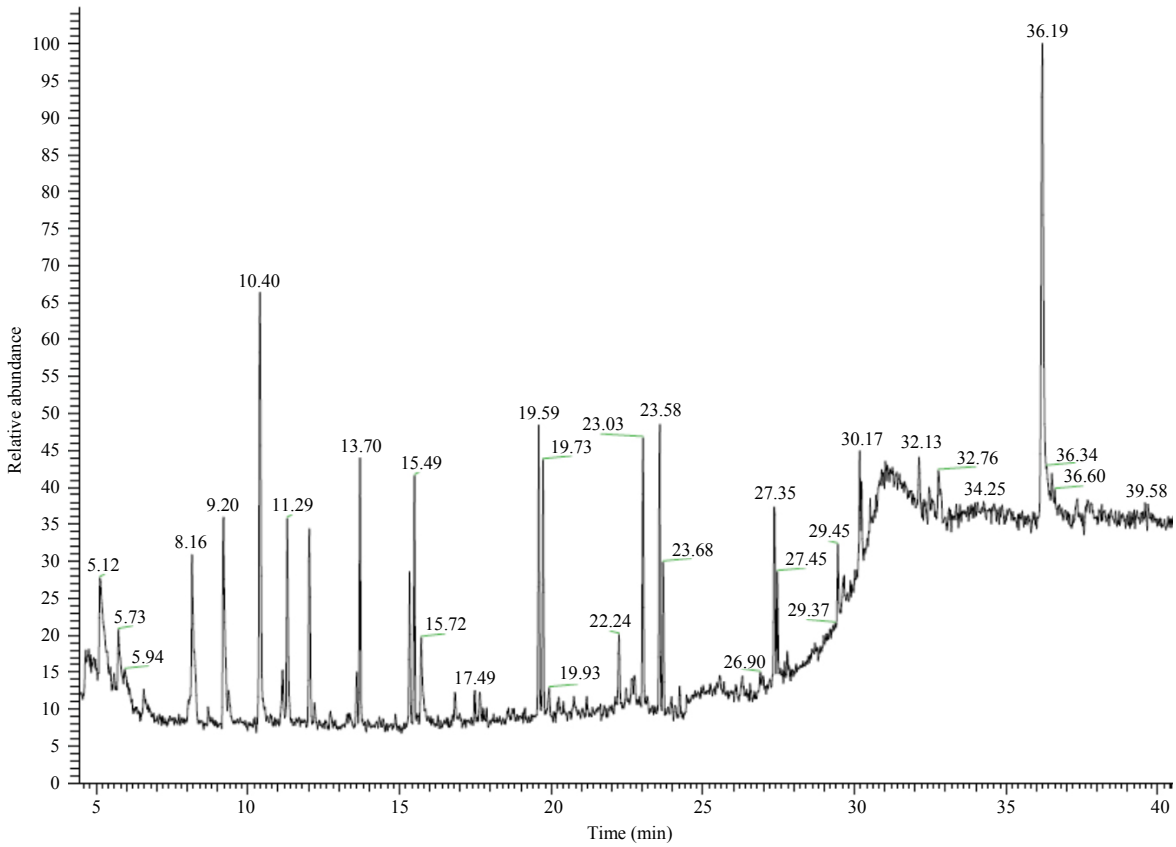

Fig. 6: GCMS spectrum analysis of *B. horikoshii* extract

Table 3: Activity of compounds identified in *B. horikoshii* extract

RT	Name of the compound	Compound nature	Activity
10.40	4-Cyano-2H-1-benzothiopyran	Bicyclic benzene	Antibacterial activity Antimalarial Anti-coagulant
15.49	Hexadecane	Fatty alcohol	Emulsifier
19.59	1-Octadecene	Alkane hydrocarbon	Not reported
23.03	9,9-Dimethyl-8,10-dioxapentacyclo[5.3.0.0(2,5).0(3,5).0(3,6)]decane	Alkane	Not reported
27.37	5-Octadecenal	Stearyl alcohol	Lubricant
36.19	11-Octadecenal	Stearyl alcohol	lubricant

The next major constituent for *B. horikoshii* extract was found to be 4-Cyano-2H-1-benzothiopyran at retention time 10.4 min and intensity of 8.12%. 4-Cyano-2H-1-benzothiopyran, a bicyclic benzene reported to have antibacterial action, antimalarial and anti-tumor activities especially against colon cancer[19,20]. Fatty alcohol hexadecane with retention time of 15.49 min. The alkane hydrocarbon octadecane was found at the retention time of 19.59 min.

Similarly *Bacillus* strain isolated from the groundnut rhizosphere soil reported by Bharose and Gajera *et al.*[21] had biocontrol activity against aflatoxin producing *Aspergillus* strain. GCMS analysis of *Bacillus subtilis* revealed presence of 55 compounds, out of which 2-hydroxy-4-phenyl-6-phenethyl pyrimidine (10%) identified as bioactive compound responsible for anti-fungal action.

Saranya Devi and Mohan[22] studied the rhizosphere region of *Casuarina equisetifolia* and identified that *Bacillus pumilus* showed high inhibition against *Fusarium oxysporum*. The ethyl acetate extract of *Bacillus pumilus* on GC-MS analysis revealed presence of 19 compounds and Cis-3-chloro all alcohol and 9-octadecenamide as predominant bioactive compounds responsible for salt tolerance and antifungal activity.

CONCLUSION

Through this study we were able to isolate new antimicrobials against potent pathogens. Based on molecular identification the active isolate of rhizosphere region of Alfaalfa plant was identified as *Bacillus horikoshii*. The studies on the rhizosphere region isolate *Bacillus horikoshii* extract lead to identification of 11 volatile compounds and study on the antimicrobial activity showed that action to be due to presence of 11-octadecenal. Thus further *in vitro* and *in vivo* biological studies are required for anticancer medical applications in various fields.

REFERENCES

1. Frame, J., 2005. *Medicago sativa* L. FAO Grassland Index: A Searchable Catalogue of Grass and Forage Legumes, Crop and Grassland Service, Agriculture Department, Food and Agriculture Organization, Rome, Italy.
2. Gray, A.M. and P.R. Flatt, 1997. Pancreatic and extra-pancreatic effects of the traditional anti-diabetic plant, *Medicago sativa* (lucerne). Br. J. Nutr., 78: 325-334.
3. Story, J.A., S.L. LePage, M.S. Petro, L.G. West, M.M. Cassidy, F.G. Lightfoot and G.V. Vahouny, 1984. Interactions of alfalfa plant and sprout saponins with cholesterol *in vitro* and in cholesterol-fed rats. Am. J. Clin. Nutr., 39: 917-929.
4. Avato, P., R. Bucci, A. Tava, C. Vitali, A. Rosato, Z. Bialy and M. Jurzysta, 2006. Antimicrobial activity of saponins from *Medicago* sp.: Structure-activity relationship. Phytother. Res., 20: 454-457.
5. Zhang, L., D. Zhang and K. Feng, 2006. Inhibition of refined components of *Medicago sativa* polysaccharides to the activities of reverse transcriptase of HIV and protease of HIV. J. Chin. Inst. Food Sci. Technol., 6: 59-62.
6. Huyghe, C., E. Bertin and N. Landry, 2007. Medicinal and Nutraceutical Uses of Alfalfa (*Medicago sativa* L.): A Review. In: Advances in Medicinal Plant Research, Acharya, S.N. and J.E. Thomas (Eds.). Research Signpost, Trivandrum, Kerala, India, pp: 147-172.
7. Bora, K.S. and A. Sharma, 2011. Phytochemical and pharmacological potential of *Medicago sativa*: A review. Pharmaceut. Biol., 49: 211-220.
8. Bora, K.S. and A. Sharma, 2011. Evaluation of antioxidant and cerebroprotective effect of *Medicago sativa* Linn. against ischemia and reperfusion insult. Evidence-Based Complement. Altern. Med., Vol. 2011. 10.1093/ecam/neq019.
9. Krakowska, A., K. Rafinska, J. Walczak, T. Kowalkowski and B. Buszewski, 2017. Comparison of various extraction techniques of *Medicago sativa*: Yield, antioxidant activity and content of phytochemical constituents. J. AOAC Int., 100: 1681-1693.
10. Argentieri, M.P., T. D'Addabbo, A. Tava, A. Agostinelli, M. Jurzysta and P. Avato, 2008. Evaluation of nematicidal properties of saponins from *Medicago* spp. Eur. J. Plant Pathol., 120: 189-197.

11. El-Khrisy, E.A.M., O.M. Abdel Hafez, A.A. Khattab and K.M. Ahmed, 1994. Chemical constituents of *Medicago sativa* L. Bull. Natl. Res. Centre (Egypt), 19: 117-122.
12. Biagioni, M., A. Alpi and P. Picciarelli, 1990. Aminopeptidases (EC. 3.4.11) in alfalfa (*Medicago sativa* L.) leaves. J. Plant Physiol., 135: 559-564.
13. Bialy, Z., M. Jurzysta, W. Oleszek, S. Piacente and C. Pizza, 1999. Saponins in alfalfa (*Medicago sativa* L.) root and their structural elucidation. J. Agric. Food Chem., 47: 3185-3192.
14. Ramakrishnan, J., M. Shunmugasundaram and M. Narayanan, 2009. *Streptomyces* sp. SCBT isolated from rhizosphere soil of medicinal plants is antagonistic to pathogenic bacteria. Iran. J. Biotechnol., 7: 75-81.
15. Ryandini, D., O.K. Radjasa and Oedjijono, 2018. Isolate actinomycetes SA32 origin of Segara Anakan mangrove rhizosphere and its capability in inhibiting multi-drugs resistant bacteria growth. J. Microb. Biochem. Technol., 10: 1-7.
16. Rajalakshmi, S. and N. Mahesh, 2014. Production and characterization of bioactive metabolites isolated from *Aspergillus terreus* in rhizosphere soil of medicinal plants. Int. J. Curr. Microbiol. Applied Sci., 3: 784-798.
17. Zellagui, A., N. Gherraf, S. Ladjel and S. Hameurlaine, 2012. Chemical composition and antibacterial activity of the essential oils from *Launaea resedifolia* L. Org. Med. Chem. Lett., Vol. 2. 10.1186/2191-2858-2-2.
18. Manilal, A., S. Sujith, B. Sabarathnam, G. Kiran, J. Selvin, C. Shakir and A. Lipton, 2011. Biological activity of the red alga *Laurencia brandenii*. Acta Bot. Croatica, 70: 81-90.
19. Nakazumi, H., T. Ueyama and T. Kitao, 1985. Antimicrobial activity of 3-(substituted methyl)-2-phenyl-4*H*-l-benzothiopyran-4-ones. J. Heterocycl. Chem., 22: 1593-1596.
20. Abbas, E.M.H., S.M. Gomha and T.A. Farghaly, 2014. Multicomponent reactions for synthesis of bioactive polyheterocyclic ring systems under controlled microwave irradiation. Arabian J. Chem., 7: 623-629.
21. Bharose, A.A. and H.P. Gajera, 2018. Antifungal activity and metabolites study of bacillus strain against aflatoxin producing *Aspergillus*. J. Applied Microbiol. Biochem., Vol. 2, No. 2. 10.21767/2576-1412.100024.
22. Saranya Devi, K. and V. Mohan, 2017. Screening and molecular characterization of salt tolerant bio-control bacterial isolates from *Casuarina equisetifolia* rhizosphere soil. Asian J. Plant Pathol., 11: 156-166.