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Evaluation of Bioactive chemical constituents by Gas chromatography-Mass spectrometry analysis isolated from *Bacillus* species

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A B S T R A C T

The aim of the research study concerns on screening, production and partial purification of Secondary metabolites isolated from soil sample collected from Western ghats, India. The sample was been serially diluted and plated on Nutrient Agar and bacteria were selectively isolated. The broth culture of the nutrient medium was preferred for fermentation process and extracellular metabolites were been extracted using the solvent ethyl acetate. The crude compound (ABTRI 3 strain) was been primarily screened for antimicrobial activity and based on the activity potent strain was been selected for secondary metabolites production. Secondary metabolites showed distinct bands by thin layer chromatography and maximum inhibition was observed at final concentration for antibacterial activity. The potential strain was characterized by GCMS and identified as *Bacillus sp.* by 16s rRNA sequencing. The findings of the present study suggested that the selected isolate could be a potent natural source of bioactive compounds.

Introduction

The word antibiotic is derived from Greek term antibiosis, which literally means against life. It can be purified from microbial fermentation and modified chemically or enzymatically for either chemical use or for fundamental studies(Robbers *et al*., 1996; De Mondena *et al*.,1993). The antibiotics are widely distributed in the nature, where they play an important role in regulating the microbial population of soil, water, sewage and compost. Of the several hundred naturally produced antibiotics that have been purified, only a few have been sufficiently non-toxic to be of use in medical practice.

Throughout many years, natural products have been playing a major role in the search for novel drugs or drug candidates. They are an ongoing and inspiring source for

researchers due their enormous structural diversity and complexity. Today, more than 50% of the drugs prescribed in the USA are natural products or semisynthetic derivatives (Schneider *et al.*,2008*).*The most prominent producers of natural products can be found within three groups of organisms: plants, bacteria (actimycetales, mycobacteria and cyanobacteria) and fungi.

Bacteria constitute a large domain of prokaryotic microorganisms. Typically a few micrometers in length, bacteria have a number of shapes, ranging from spheres to rods and spirals (Usha.,2011) There are typically 40 million bacteria cells in a gram of soil and a million bacterial cells in a millilitre of fresh water. There are approximately $5x10^{30}$ bacteria on Earth, forming a biomass which exceeds that of all plants and animals.(Mire *et al.*, 2014).

While plants and plant extracts have been playing an important role in traditional medicine for thousands of years, the discovery and investigation of bacterial and fungal natural products had only been made possible with the discovery penicillin in 1929, metabolites of microbial origin are known for valuable supply of new compounds and lead structures in the quest for drug candidates against infectious diseases, cancer and many other illnesses (Koehn and Carter, 2005; Newman and Cragg, 2007).

Among them are therapeutically used alkaloids like ergotamine, the immunosuppressive peptide cyclosporine A, other peptidic compounds like penicillin and cephalosporin, the polyketide lovastatin and the antibacterial terpenoid fusidic acid (Carmichael *et al.*, 1992 and Demain *et al.*, 1999).

Most of the bio active compounds are secondary metabolites. Secondary metabolites are typically organic compounds produced through the modification of primary metabolite synthesis (Beardy *et al*., 2005). Secondary metabolites do not play a role in growth, development, and reproduction like primary metabolites do, and are typically formed during the end or near the stationary phase of growth (Challis and Hopwood, 2003). In many of the identified secondary metabolites have a role in ecological function, including defence mechanism(s), by serving as antibiotics and by producing pigments (Blats *et al*., 2008).

Secondary metabolites are also called as anti-infective metabolites, such as bacteriocins. Based on their ability to produce a wide range of secondary metabolites are have biological actions such as anti bacterial and fungal activity. The anti-infective metabolism of the genera is dominated compounds derived from the polyketide pathway and from elaboration of amino acid metabolism. (Ajijur Rahman *et al*., 2011).

Natural products are pharmacologically or biologically potent chemical compounds produced by living organisms. Bacterial aromatic polyketides include the antibiotic tetracycline and the compound doxorubicin, used in the treatment of breast and other cancers. Because many of these natural products are synthesized by organisms that are difficult to collect, grow and maintain, researchers have sought to produce them using simpler organisms like *E. coli*., resulting in the production of groups of drugs that include antibiotics like erythromycin and vancomycin, as well as terpenes and alkaloids, attempts to synthesize bacterial aromatic polyketides had previously been hindered by the compounds' complicated assembly process, most for engineering and mass producing these compounds.

Materials and Methods

Sample Collection and Isolation of Bacteria

The samples were collected from Tamilnadu kerala border, Madurai and Sadhuragiri hills (Western Ghats) Tamilnadu using sterile polythene bags and transported to laboratory.The sample was been serially diluted (10^{-1} to 10^{-9}) ,followed by spread plate method. The plates were incubated at 28°C for 3-5 days (Srividhya *et al*., 2009). The same procedure was followed for the other types of the soil. Morphologically different isolates were selected, subcultured and maintained on nutrient agar slants and preserved at 4°C.

Screening for Bioactivity against Various Pathogens

Primary screening against human bacterial pathogens

Nutrient agar was prepared, sterilized and poured into sterile Petriplates and the media was allowed to solidify. The isolated bacteria was been perpendicularly streaked (5cm measurement) on each plate. The plates were incubated for 24 hours. 24 hrs old human bacterial pathogens such as *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Klebsiella pneumoniae* were streaked (3cm measurement) by perpendicular streaking method, incubated overnight and observed for inhibition.

Primary Screening against Human Fungal Pathogen

Nutrient agar and potato dextrose agar was prepared separately in equal volume, sterilized and both the media were mixed together and poured into sterile petriplates and the media was allowed to solidify. A disc(8mm) of fungi *Aspergillus niger* was placed in one side of the petriplate and after the partial growth of the fungi, the isolated bacteria was perpendicularly streaked (5cm measurement) on each plate. The plates were incubated for 24-48hours and observed for inhibition.

Extraction of Secondary Metabolites from Selected Isolate

The effective strain resulted from the screening process was been processed for extraction of crude compounds by ethyl-acetate extraction method. The strain was inoculated in Nutrient broth and the broth was kept in an orbital shaker for incubation for 7days in order to reach maximum production. After incubation culture broth was centrifuged at 8000 rpm for 15 minutes and the supernatant was collected and mixed with an equal volume of ethyl acetate. The extracted crude compounds were dried at 40°C. The crude compound was been processed for secondary screening by agar well diffusion method to confirm the presence of bioactive metabolites.

Partial purification of secondary metabolites by thin layer chromatography

The technique for separation of bioactive compounds extracted from bacteria was achieved through the method of Hao *et al*., 2004.

The ethyl acetate extract of crude compound was further subjected to TLC to study its compound profile. The extract was spotted on pre-coated silica plates and developed with Ethyl acetate : hexane mixture in varying ratio. The run TLC plates were visualized under UV illumination and iodine vapours. The ratio in which distinct bands appeared was optimized and Rf values of the bands was calculated.

Calculation of Rf value:

Rf value = Distance travelled by solute / Distance travelled by solvent.

Bioactivity of the crude compound by Well diffusion assay (Eloff, 1998)

24 h growing bacterial cultures *Staphylococcus aureus Bacillus subtilis*, *Escherichia coli* and *Klebsiella pneumoniae* were swabbed on sterile Nutrient agar plates. The stock solution was prepared *i.e*, 10mg/ml (crude compound/DMSO). Then, 5 wells (8mm diameter) were made by using a sterile cork borer. The 4 different concentrations (250µg, 500µg, 750µg and 1000µg) of the test sample were loaded in the wells. Tetracycline was used as control. The plates were then incubated at 37ºC for 24h. After incubation the inhibition diameter was measured.

Identification of Bioactive Metabolites

The active eluent compounds from TLC plates were identified using gas chromatography and mass spectrometry (GC-MS) method, (TSQ QUANTUM XLS).

Strain Identification by 16S rRNA Sequencing

The DNA was isolated by QIAGEN DNA isolation kit (Qiagen) and amplified by PCR as per user manual. The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/blast) against the 16S ribosomal RNA sequence database.

Results and Discussion

Sample Collection and Isolation of Bacteria

Morphologically different isolates were selected, sub-cultured and maintained on nutrient agar slants and preserved for further usage. All the plates were observed with bacterial colonies by serial dilution after an incubation of 24hours. The distinct colonies were well observed in 10^{-9} dilution (sadhuragiri soil) and so it was sub-cultured onto NA plates and incubated and so the plates were observed with a well grown adherence. The cultures were named as ABTRI 1, ABTRI 2, ABTRI 3, ABTRI 4, ABTRI 5, ABTRI 6, ABTRI 7, ABTRI 8 and ABTRI 9 and these cultures were used for identification process.

Screening for bioactivity against various pathogens

Primary screening against human bacterial pathogens

The ABTRI 1-9 were tested for their antibacterial activity by perpendicular streaking. From the 9 strains, ABTRI 3 was selected as an effective strain and ABTRI 3 was selected based on inhibition. The zone of inhibition for ABTRI 3 against *Bacillus subtilis* was recorded as 1.2cm. The ABTRI 3 was effective and so it was selected to check the antifungal property.

Primary screening against human fungal pathogen

The ABTRI 3 was tested for their antifungal activity by perpendicular streaking. The ABTRI 3 was screened for antifungal activity against *Aspergillus niger*. The zone of inhibition for ABTRI 3 against *Aspergillus niger* was recorded as 2.6cm.The ABTRI 3 was screened for antibacterial and antifungal activity. From the results, it is evident that ABTRI 3 exhibited both antibacterial and antifungal activity. Thus, ABTRI 3 was selected for compound production.

Extraction of Secondary Metabolites from Selected Isolate

After 24 hours of the ethyl acetate extraction method, the solvent layer was collected and condensed to obtain the crude extract. This resulted in yellow coloured, thick viscous extract.

Partial purification of secondary metabolites by thin layer chromatography

Partial purification of secondary metabolites was done by TLC method. Active compounds were identified based on the optimized solvent system.

The TLC profile of the crude extract of ABTRI 3 showed distinct band with R_f value 1.4, under UV illumination as given in fig.1. These compounds might be responsible for antimicrobial activity of crude extract.

Bioactivity of the crude compound by Well diffusion method

The crude compound showed antibacterial activity against the organisms. The ABTRI 3 showed maximum activity against *Bacillus subtilis* as depicted in table. 1.

The extracted bioactive compound was subjected to well diffusion assay to study its antibacterial property against *S.aureus*, *K.pneumoniae*, *B.subtilis* and *E.coli*. The compound showed maximum inhibition against *Bacillus subtilis* with ZOI of 14mm at a concentration of 1000µg/ml.

Identification of Bioactive Metabolites

Interpretation of mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST-011 library. The names of the components of the test materials were ascertained. The active constituents with their retention time (RT), molecular weight (MW) Molecular formula (MF), concentration (peak area %) were determined in Table.2 and fig. A. The GCMS analysis of crude compound showed 20 bioactive compounds with high concentration of 2,4-bis(1,1-dimethylethyl)Phenol having retention time 21.26 (RT) and peak area 5.90% , followed by some constituents such as Hydroxylamine having retention time 21.37 (RT) and peak area 1.87% , 3-Heptafluorobutyroxy tetradecane having retention time 25.21 (RT) and peak area 1.91%, Nonanedioic acid having retention time 23.32 (RT) and peak area 0.69%, Allylmethallyl ether having retention time 18.94 (RT) and peak area 0.61%. Other constituents ranged with varying retention time and peak area.

Strain Identification by 16S rRNA sequencing

The selected bacterial strain (ABTRI 3) was sequenced and identified as *Bacillus amyloliquefaciens.* Using similarity search program, BLAST (Basic Local Alignment Search Tool), the bacterial isolate as depicted in fig.2 was identified as *Bacillus amyloliquefaciens*.

For the preparation of inoculum, the potential strain was inoculated on the nutrient broth and incubated at 28° C for 18hours. About 10% of bacterial inoculum adjusted to 0.5 McFarland standard was transferred into each 100 ml of fermentation medium was incubated in rotary shaker for 120 hours at 28°C (Hasan *et al.*, 2009).

Table.1 Antibacterial activity for the Bioactive Compounds against Human Bacterial Pathogens

Organism	Zone of inhibition (mm)				
	Control	250µg/ml	500µg/ml	750µg/ml	1000µg/ml
S.aureus	23mm	-	-	-	11mm
K.pneumoniae	23mm	-	-	-	11mm
B.subtilis	23mm	11mm	14mm	14mm	14mm
E.coli	23mm	-	-	-	10mm

Table.2 Bioactive Constituents of Crude Compound (ABTRI 3) by GCMS Analysis

S.No	RT	Name of the compound	MF	MW	Peak Area%
1	12.16	Toluene	C_7H_8	92	71.63
2	16.58	5-Hexenoic acid	$C_6H_{12}O_2$	116	0.26
3	17.04	1-Heptene	C_7H_{14}	98	0.12
4	17.53	Z-1, 9-Hexadecadiene	$C_{16}H_{30}$	222	0.11
5	18.22	Trifluoroacetic acid	$C_2HF_3O_2$	114	0.16
6	18.85	Benzaldehyde	C_7H_60	106	0.46
7	18.94	Allylmethallyl ether	$C_7H_{12}0$	112	0.61
8	19.84	Butanoyl chloride	$C_4H_7C_{10}$	106	4.31
9	20.00	1-Propene	C_3H_6	42	2.40
10	20.24	2-Undecanethiol	$C_{11}H_{24}S$	188	1.64
11	20.35	d-glycero-d-ido-heptose	$C_7H_{14}O_7$	210	1.83
12	20.58	1-Butanol	$C_4H_{10}O$	74	1.00
13	20.97	6-Nonynoic acid	$C_9H_{14}O_2$	154	1.59
14	21.26	2,4-bis(1,1-dimethylethyl)Phenol	$C_{14}H_{22}O$	206	5.90
15	21.37	Hydroxylamine	C_1H_4NO	69	1.87
16	21.90	Cyclopentaneundecanoic acid	$C_{16}H_{30}O_2$	254	1.83
17	23.32	Nonanedioic acid	$C_9H_{16}O_4$	188	0.69
18	23.43	Diethyl phthalate	$C_{12}H_{14}O_4$	222	1.33
19	25.21	3-Heptafluorobutyroxy tetradecane	$C_{10}H_{16}O_4$	200	1.91
20	28.50	Amylnitrite	$C_7H_{13}NO_2$	143	0.34

MF-Molecular Formula., MW-Molecular weight

Fig.1 Visualization of Bands by TLC (Under UV) for ABTRI 3

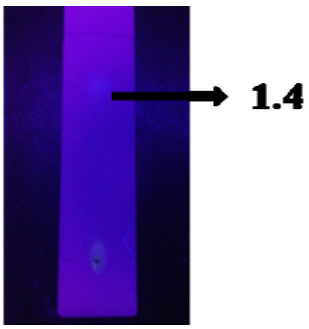

Fig.2 16s rRNA Sequence of *Bacillus* Species

Bacillus amyloliquefaciens strain KAVK1 16S ribosomal RNA gene, partial sequence
Sequence ID: gb|KP792772.1| Length: 1415 Number of Matches: 1

Range 1: 36 to 792 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1399 bits(757)	0.0	757/757(100%)	0/757(0%)	Plus/Plus

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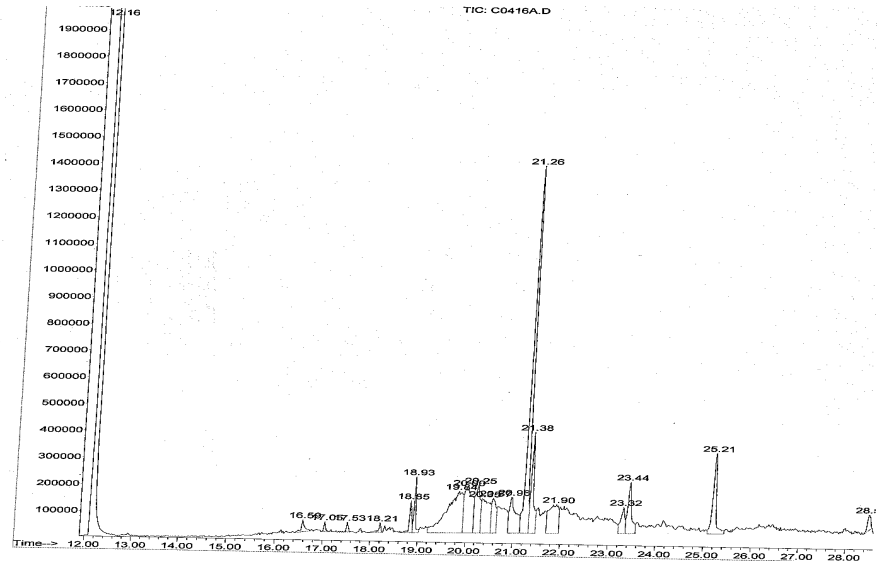
Query  1    GTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACG  60
Sbjct  36   GTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACG  95
Query  61   TGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCT  120
Sbjct  96   TGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCT  155
Query  121  TGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACC  180
Sbjct  156  TGTTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACC  215
Query  181  CGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGAC  240
Sbjct  216  CGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGAC  275
Query  241  CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG  300
Sbjct  276  CTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG  335
Query  301  CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGA  360
Sbjct  336  CAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGA  395
Query  361  AGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCG  420
Sbjct  396  AGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCG  455
Query  421  GCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA  480
Sbjct  456  GCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA  515
Query  481  TACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCT  540
Sbjct  516  TACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCT  575
Query  541  TAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTT  600
Sbjct  576  TAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTT  635
Query  601  GAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAG  660
Sbjct  636  GAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAG  695
Query  661  GAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTG  720
Sbjct  696  GAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTG  755
Query  721  GGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC  757
Sbjct  756  GGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC  792
```

Fig.A GCMS Chromatogram for Bioactive Compound (from ABTRI 3)



After incubation, the culture broth was separated by centrifugation at 5000 rpm for 10 minutes. The bioactive compounds present in the cell free supernatant was extracted by adapting liquid-liquid extraction method using ethyl acetate and chloroform for 24 hours (Chellaram *et al*., 2004). The solvent portion was collected and concentrated by evaporation. In the current process, the potential strain ABTRI

3 was inoculated on the nutrient broth and incubated at 35°C for seven days. After incubation, the culture broth was separated by centrifugation at 10,000 rpm for 15 minutes. The bioactive compounds present in the cell free supernatant was been extracted by using ethyl acetate for 24 hours. The solvent layer was collected and concentrated by evaporation.

The crude extract was subjected to purification by thin layer chromatography (TLC) (Selvameenal *et al*., 2009) using commercially available silica gel coated chromatography sheets. To find out the best solvent system to separate the crude compound, the solvents such as methanol, chloroform, acetic acid, n-butanol, n-hexane and water were used in different proportions. The crude compound was dissolved in 200µl of ethyl acetate and the sample was spotted at the bottom of silica gel coated sheet with the help of capillary tube. Then the chromatogram was developed using different solvent systems. After running, the sheet was kept in room temperature for the complete drying of the plates. Then the sheet was kept in closed iodine chamber to visualize the separated compound as clear spots.

The present study was carried out with ethyl acetate as solvent. The crude extract was subjected to purification by thin layer chromatography using precoated silica gel sheets. The optimized solvent system fixed for the crude compound, was as Ethyl acetate and Hexane in varying ratio. The crude compound was dissolved in 100µl of ethyl acetate and the sample was spotted at the bottom of silica gel sheet with the help of capillary tube. Then the chromatogram was developed using Ethylacetate : Hexane solvent system. After running of the sample, the sheet was air drying process without any disturbance. Then the sheet was kept in UV-transilluminator to analyze the presence of compounds and number of distinct bands. The sheets were placed in closed iodine chamber to visualize the separated compound as clear spots and R_f values were calculated. From the results, it was found that the TLC profile of the crude extract of ABTRI 3 showed distinct band with R_f value 1.4, under UV illumination and iodine.

Crude ethyl acetate and chloroform extract was tested for antibacterial activity by disc diffusion method. 0.25 gm of crude extract was impregnated into the sterile filter paper disc, dried and placed over nutrient agar plates inoculated with test bacterial pathogens. The diameter of the inhibition zone was measured after 24 hours of incubation at 37°C (Balagurunathan and Subramanian, 1994). In the current study, Crude ethyl acetate extract was tested for antibacterial activity by well diffusion method. 10mg/1ml of DMSO (Dimethyl sulfoxide), and the extract was loaded in various concentrations in the wells over nutrient agar plates inoculated with test bacterial pathogens. The diameter of the inhibition zone was measured after 24 hours of incubation at 37°C and maximum inhibition was observed against *Bacillus subtilis* with ZOI of 14mm at a concentration of 1000µg/ml.

In conclusion, From the present study, it is evident that ABTRI 3 could exhibit both antibacterial activity and also antifungal activity. The crude compound (from ABTRI 3) extracted from ethyl acetate was effective against various bacterial pathogens. From the GC-MS technique it can be revealed that bioactive constituents are available with various activities. It can be concluded that the organism *Bacillus amyloliquefaciens* isolated from the soil has been found to possess appreciable antimicrobial activity. Further, the structural studies of bioactive compounds can be done for future research.

GCMS analysis is mainly performed to understand the presence of bioconstituents and the nature of the compound. Also, the studies possess with varying retention time and peak area in terms of percentage. Based on the presence of various compounds, biological activities of individual compounds can be known.

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