



Identification of Phytoconstituents using GC-MS and Determination of Antimicrobial and Antimycobacterial Activity of *Boerhaavia diffusa* L. Leaves

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Background: To identify bioactive phytoconstituents and evaluate antimicrobial and antimycobacterial potential of *Boerhaavia diffusa* leaves against selective human pathogens.

Methods: The extract of *Boerhaavia diffusa* leaves was carried out by using methanol. Bioactive compounds was identified by GC-MS. Antimicrobial and antimycobacterial activity of methanolic extract *Boerhaavia diffusa* leaves was tested *in vitro* by Kirby-Bauer well diffusion method and rapid culture - MGIT™ DST method against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* & *Pseudomonas aeruginosa* and *Candida*, *Aspergillus* and *M. tuberculosis* (H37RV) and *Mycobacterium tuberculosis* bacteria resistant to Isoniazid & Rifampicine.

Results: The obtained results of the GC-MS of *Boerhaavia diffusa* led to the identity of 16 bioactive compounds. The crude extract showed antimicrobial activity against *E.coli* (Sensitive), *Pseudomonas aeruginosa* Sensitive and *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* (Sensitive) but extract did not show antimicrobial effect against, *E.coli* (ESBL), *Klebsiella pneumonia* (Sensitive), *Klebsiella pneumonia* (ESBL), *Pseudomonas aeruginosa* (Resistant), *Candida albicans* and *Aspergillus fumigates*. The result of anti-mycobacterial activity

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showed that extract not showed antimycobacterial activity against *M. tuberculosis* (H37RV) and *Mycobacterium tuberculosis* bacteria resistant to Isoniazid & Rifampicine.

Conclusion: The study result proved that methanolic extract of *Boerhaavia diffusa* leaves has antimicrobial potential due to the presence of the bioactive compounds.

Keywords: *Boerhaavia diffusa*; GC-MS; antimicrobial and antimycobacterial activity.

1. INTRODUCTION

An infectious disease is a leading cause of morbidity and mortality in world. The clinical efficacy of leading antibiotics is suffered by the emergence of multi-drug resistant pathogens, so it needs to find out new drug molecule. The mechanisms of resistance are inactivation of antibiotics; reduce the membrane permeability, modification of target site, efflux or transport of antibiotic.

Escherichia coli are usually present in warm blooded organisms especially in the gastrointestinal tract. It commonly causes urinary tract infection and, Gastro-intestinal diseases [1]. *Klebsiella pneumoniae*, mostly present in the human gastro-intestinal, is a Gram-negative, non-sporulating, aerobic shaped rod bacterium. The development of an infection is contributed by adhesion to a mucosal surface [2]. *Staphylococcus aureus* generally lives in human skin and mucosa and it enters the body can led to disease spreading. It produces food poisoning, which gives to severe vomiting and cramps. *Pseudomonas aeruginosa* is a pathogen exploits break in the host defense system to start an infection [3]. *Candida albicans* lives in the human digestive tract, mouth, and genital region that contributes in fungal infections [4]. *Aspergillus* species is second most widespread systemic fungal infection. *Aspergillus* primarily affects lungs and causes bronchopulmonary aspergillosis, chronic necrotizing pulmonary aspergillosis, aspergilloma and invasive aspergillosis.

In recent decades bioactive compounds, previously with unknown biological activities, have been extensively investigated as a source of therapeutic active substances [5].

Plant secondary metabolites such as tannins, alkaloids, phenolic compounds, and flavonoids, which have been found antimicrobial activity [6,7].

Boerhaavia diffusa L. is a perennial creeping weed, prostrate or ascending herb belong

Nyctaginaceae family. *Boerhaavia diffusa* L., known as 'Punarnava', are used in traditional medicine for the treatment of diabetes, stress, dyspepsia, abdominal pain, inflammation, jaundice, enlargement of spleen, cardiac diseases [8,9,10,11].

Pharmacological studies have reported in *Boerhaavia diffusa* as anticonvulsant [12], cardioprotective [13], anti-inflammatory [14], anti-diabetic [15], anti-arthritic [16], hepatoprotective [17], anxiolytic activity.18

Considering immeasurable potentiality of phytoconstituents as sources for antimicrobial and antimycobacterial drugs, the aim of the study was to evaluate phytoconstituents screening and antimicrobial and antimycobacterial extracts of *Boerhaavia diffusa* leaves on Gram-positive and Gram-negative microorganisms.

2. MATERIALS AND METHODS

2.1 Preparation of Extract

Plant material leaves were collected from Amer forest region, Jaipur. The shade dried powder of leaves was used for the extraction with methanol for 24 hours by Soxhlet equipment and filtered through membrane filter. This filtrate was evaporated under reduced pressure and dried in a rotator evaporator at 55°C [19].

2.2 Gas Chromatography- Mass Spectrometry (GC-MS) Analysis

GC -MS analysis was carried out on an Agilent system equipped with Mass Spectrometer detector and split/splitless injection system. The GC was fitted with a HP-5MS capillary column (30m X 250m; film thickness: 0.25m). The temperature program was as follows: injector temperature 280°C, initial oven temperature at 50°C, then increased at 25°C/min to 300°C and was hold for 10 mins. Helium was used as carrier gas at 17.69 psi pressure with flow 2.1 ml/min. Samples were dissolved in methanol and 1 µl aliquot were injected automatically. The

biological active compounds were identified through the comparison of their mass spectra with the reference mass spectra of library of the NIST (National Institute of Standards and Technology) [20,21].

2.3 Antimicrobial Study

Antimicrobial activity study of crude extract of *Boerhaavia diffusa* leaves including antibacterial and antifungal activity was carried by Kirby-Bauer well diffusion method at C.I.R.D (Centre for Innovation, Research & Development) Dr. B. Lal Institute of Biotechnology and Research Centre, Malviya Nagar, Jaipur, Rajasthan.

2.3.1 Selection of microorganisms

For the purpose of Antimicrobial evaluation, selection of Microorganisms were done by taking two different set (sensitive and resistant) for Gram positive, Gram negative and Mycobacterium. For Antifungal testing *Candida albicans* and *Aspergillus fumigatus* were selected. All the pure microbial cultures utilized in this study were procured from Microbial Culture Collection Division (MCRD) established at CIRDC.

2.3.2 Processing of samples

Two concentrations of the crude extract of *Boerhaavia diffusa* leaves under study were prepared from the stock solution (200 mg/ml and 100 mg/ml) and then the dilution series was prepared for the compound, out of which 50 µl was used in each well. Streptomycin was used as positive control (5 mg/ml concentration) for antibacterial activity and Itraconazole was used

as positive control (5 mg/ml concentration) for antifungal activity.

2.4 Antimicrobial Susceptibility Testing (Kirby-Bauer Well Diffusion Method)

2.4.1 Agar plates were prepared for the antibacterial activity

Mueller-Hinton agar medium and Sabouraud dextrose agar medium is the only susceptibility test medium that has been validated by CLSI for screening the antimicrobial activity by disk/ well diffusion susceptibility testing.

2.4.2 Preparation of inoculums

Cultures of sensitive and resistant strain of *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* & *Pseudomonas aeruginosa* and *Candida* were inoculated in Peptone water and were kept for incubation for 30 minutes at 37°C and *Aspergillus* was inoculated in Normal Saline and was kept for incubation for 48 hours at 28°C.

2.4.3 Inoculums size of bacteria was adjusted using McFarland turbidity standard as reference

The bacterial suspensions were compared to 0.5 McFarland Turbidity Standard.

2.4.4 Swabbing of the liquid cultures

Bacterial cultures were swabbed onto the Mueller Hinton Agar surface and *Candida* and *Aspergillus* cultures were swabbed onto the Sabouraud dextrose agar surface.

Chart 1. Pure bacterial and fungal cultures

Organisms	Details
<i>E.coli</i> (Sensitive)	ATCC 25922
<i>E.coli</i> (ESBL)	ATCC 35218
<i>Klebsiella pneumoniae</i> (Sensitive)	MTCC 3384
<i>Klebsiella pneumoniae</i> (ESBL)	ATCC 7000603
<i>Staphylococcus aureus</i> (Sensitive)	ATCC 25923
<i>Staphylococcus aureus</i> (MRSA)	ATCC 4330
<i>Pseudomonas aeruginosa</i> (Sensitive)	ATCC27853
<i>Pseudomonas aeruginosa</i> (Resistant)	Clinical isolate-CIRD-MCRD
<i>Mycobacterium tuberculosis</i>	H37Rv
<i>Mycobacterium tuberculosis</i>	Clinical isolate-CIRD-MCRD
<i>Candida albicans</i>	Clinical isolate-CIRD-MCRD
<i>Aspergillus fumigatus</i>	Clinical isolate-CIRD-MCRD

2.4.5 Loading of different test solutions into the wells

50 µl from different dilutions prepared from stock (200 mg/ml and 100 mg/ml of the compound) was loaded into the respective wells.

2.4.6 Incubation

The bacterial and *Candida* plates were kept for incubation at 37°C for 24 hours and plates of *Aspergillus* were kept for incubation at 28°C for 7 days.

2.5 Antimycobacterial Susceptibility Test

The antimycobacterial activity of crude extract of *Boerhaavia diffusa* leaves was determined against *M. tuberculosis* (H37RV) and *Mycobacterium tuberculosis* bacteria resistant to Isoniazid & Rifampicine both by rapid culture - MGIT™ DST method.

The crude extract of *Boerhaavia diffusa* was diluted to the 10mg/ml concentrations. Total seven MGIT tubes were labeled and 0.8 ml supplement was added to each tube. 1st tube was then kept a side and extract 100 µl from the stock of 100 mg / ml were added to the respective tubes. Tubes were mixed properly and kept a side. 1:100 dilution of DST inoculum (*M. tuberculosis* (H37RV) was prepared for the Growth control tube (1st tube) and 1:5 dilution of DST inoculum (*M. tuberculosis* (H37RV) & *M. tuberculosis* (MDR) were prepared for tubes. 0.5 ml of 1:100 dilution was added to the 1st tube (Growth control tube). 0.5 ml of 1:5 dilution was added to other respective tubes. All tubes were incubated in MGIT-320 instrument at 37°C.

3. RESULTS

Due to emerging resistant it needs to find out alternative chemotherapeutic agents. Plants and herbs have rich amount of active phytoconstituents they are responsible for many pharmacological activities. The aims of this study find out bioactive compound which are responsible for antimicrobial activity. In additionally explore antimicrobial potential of *Boerhaavia diffusa* leaves.

3.1 GC-MS Profiling of Extract of *Boerhaavia diffusa* Leaves

The obtained results of the GC-MS analysis of *Boerhaavia diffusa* led to the identification of 16

chemical constituents (Table 1) 3-Amino-2-oxazolidinone, 1,2-Cyclopentanedione, 2-Hydroxygammabutyrolactone, Undecane, Acetic acid, pentyl ester, 2-Methoxy-4-vinylphenol, 2,4-Di-tert-butylphenol, 3-tert-Butyl-4-hydroxyanisole, Myo-Inositol, 4-C-methyl, Myo-Inositol, 2-C-methyl, 9-Octadecenoic acid (2-phenyl-1,3-dioxolan-4-yl) methyl ester, Hexadecanoic acid, methyl ester, Methyl 9-cis,11-transoctadecadienoate, 9,12,15-Octadecatrienoic acid, methyl ester (Linolenic acid), Phytol, Hexadecanoic acid, 2,3-di hydroxyl propylester. The chromatogram of *Boerhaavia diffusa* leaves presented in form of retention time (RT), molecular formula, molecular weight (MW), Peak area, peak height, % area. (Fig. 1)

Mass spectra of bioactive compounds obtained from GCMS analysis, presented in from Figs. 2 to 16.

3.2 Antimicrobial Activity

The results of antibacterial activity of the Methanolic of *Boerhaavia diffusa* are presented in Table 2. The Methanolic extract at 100 mg/ml concentration showed antibacterial activity against *Staphylococcus aureus* (Sensitive), *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa* (Sensitive) in respective of 26 mm, 25 mm, 27 mm zone of inhibition. (Figs. 17,18) The Methanolic extract at 200 mg/ml extract concentration showed antibacterial activity against *Staphylococcus aureus* (Sensitive), *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* (Sensitive) in respective of 28 mm, 26 mm, 30 mm zone of inhibition. On the contrary *E.coli* (Sensitive), *E.coli* (ESBL), *Klebsiella pneumoniae* (Sensitive), *Klebsiella pneumonia* (ESBL, *Pseudomonas aeruginosa* (Resistant)), *Candida albicans* and *Aspergillus fumigatus* shows no zone of inhibition (ZOI). (Figs. 17, 18) The overall Result found that methanolic extract exhibited highest antimicrobial against *Pseudomonas aeruginosa* (Sensitive). Antimicrobial activity depends upon concentration manner and highest antimicrobial activity shows on 200mg/ml concentration.

3.3 Antimycobacterial Activity

The antimycobacterial activity was evaluated by rapid culture - MGIT™ DST method. The result showed that methanolic extract of *Boerhaavia diffusa* leaves not inhibited growth of mycobacteria given in Table 3.

Table 1. GC-MS analysis of methanolic extract of *Boerhaavia diffusa* leaves

RT	Peak Area	Peak Height	% Area	Mol. Wt.	Formula	Compound name
4.41	75335014.29	14086286.79	1.26	102	C ₃ H ₆ N ₂ O ₂	3-Amino-2-oxazolidinone
7.61	70567046.98	14000831.84	1.18	98	C ₅ H ₆ O ₂	1,2-Cyclopentanedione
9.45	63775433.33	13261568.93	1.07	102	C ₄ H ₆ O ₃	2-Hydroxygammabutyrolactone
12.87	167166756.3	33029207.05	2.81	156	C ₁₁ H ₂₄	Undecane
15.71	237189946.8	39212191.87	3.98	130	C ₇ H ₁₄ O ₂	Acetic acid, pentyl ester
18.92	238952145.3	75373004.72	4.01	150	C ₉ H ₁₀ O ₂	2-Methoxy-4-vinylphenol
24.34	84069089.43	21624304.77	1.41	160	C ₁₄ H ₂₂ O	2,4-Di tertbutylphenol
25.81	91120430.48	26847993.41	1.53	180	C ₁₁ H ₁₆ O ₂	3-tert-Butyl-4-hydroxyanisole
29.39	79628080.25	14656284.53	1.34	194	C ₇ H ₁₄ O ₆	Myo-Inositol,4-C-methyl
29.67	1617421473	163763471.6	27.15	194	C ₇ H ₁₄ O ₆	Myoinositol,2-C-methyl
30.12	69460194.69	21499353.84	1.17	444	C ₂₈ H ₄₄ O ₄	9-Octadecenoic acid (2phenyl1,3dioxolan4yl) methyl ester
33.16	373921717.5	134115899.1	6.28	270	C ₁₇ H ₃₄ O ₂	Hexadecanoic acid, methyl ester
36.01	205087691.3	82803133.7	3.44	294	C ₁₉ H ₃₄ O ₂	Methyl 9-cis,11-transoctadecadienoate
36.1	962814789.5	239650611.5	16.16	292	C ₁₉ H ₃₂ O ₂	9,12,15-Octadecatrienoic acid, methyl ester
36.26	609159447.7	238869090.5	10.23	296	C ₂₀ H ₄₀ O	Phytol
42.09	175776566.8	57449452.83	2.95	330	C ₁₉ H ₃₈ O ₄	Hexadecanoic acid, 2,3-di hydroxyl propylester

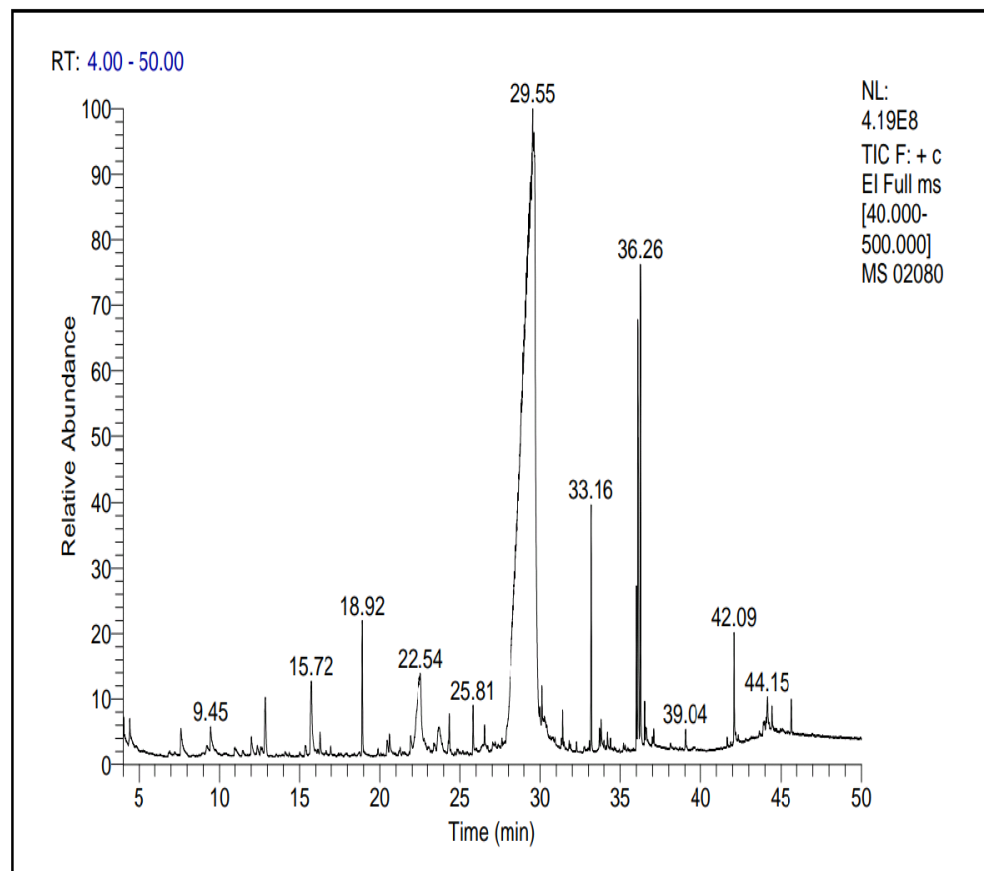


Fig. 1. GC-MS chromatogram of methanolic extract of *Boerhaavia diffusa* leaves

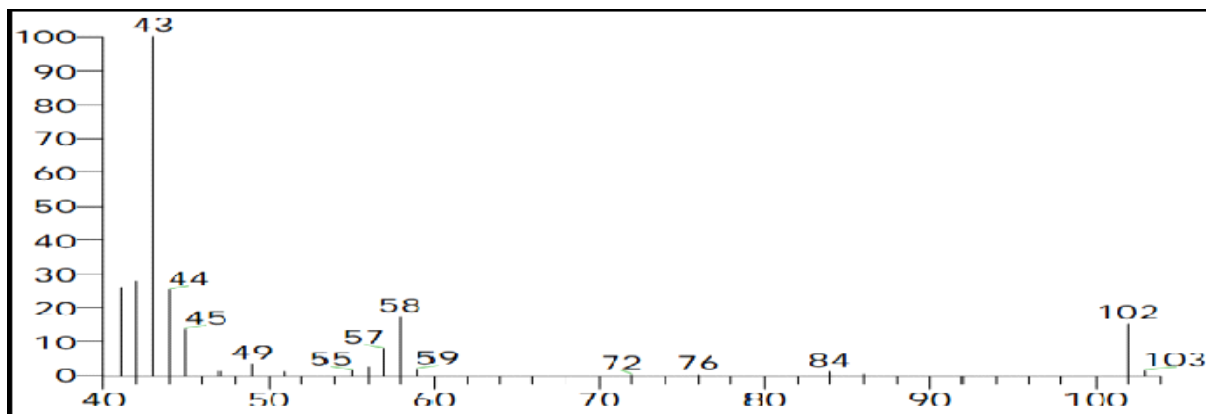


Fig. 2. Mass spectra of 3-Amino-2-oxazolidinone

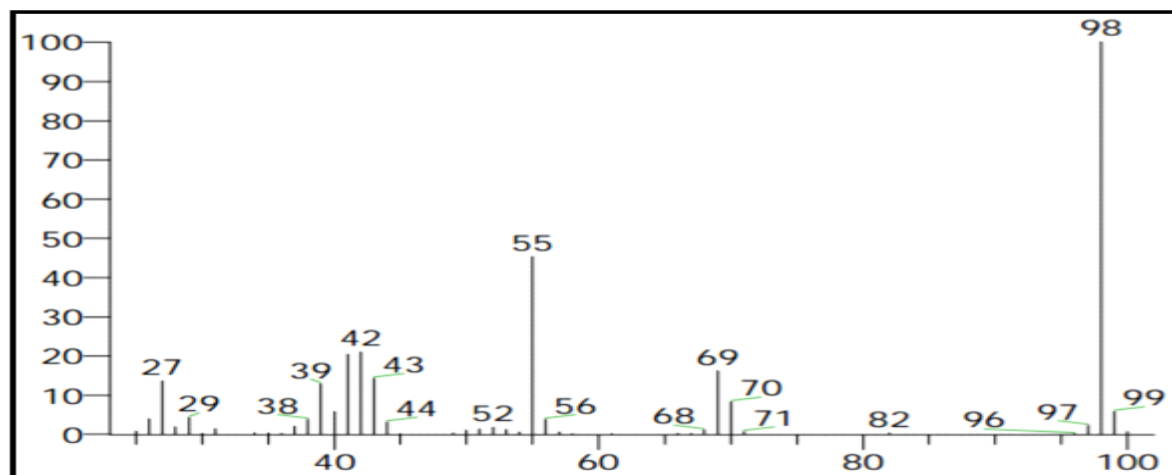


Fig. 3. Mass spectra of 1,2-cyclopentanedione

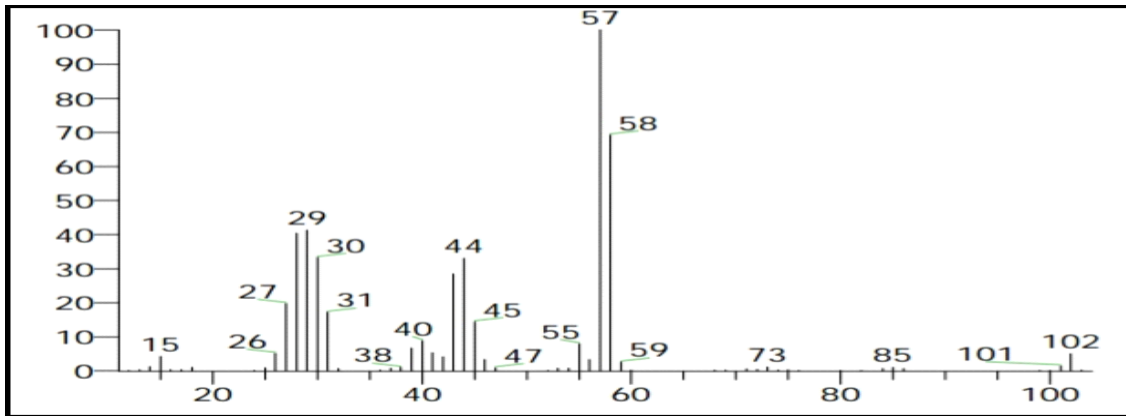


Fig. 4. Mass Spectra of 2-Hydroxygamma-butyrolactone

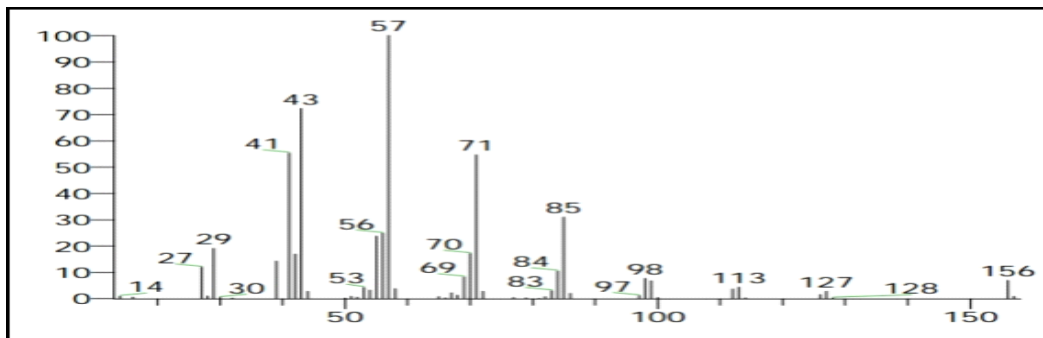


Fig. 5. Mass spectra of undecane

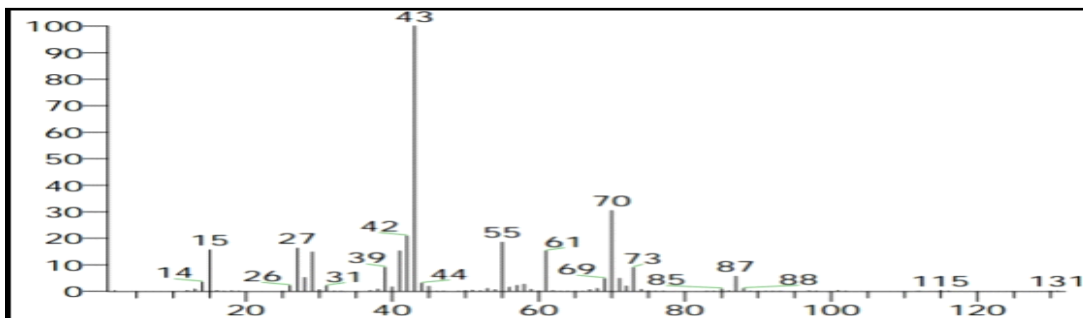


Fig. 6. Mass spectra of acetic acid, pentyl ester

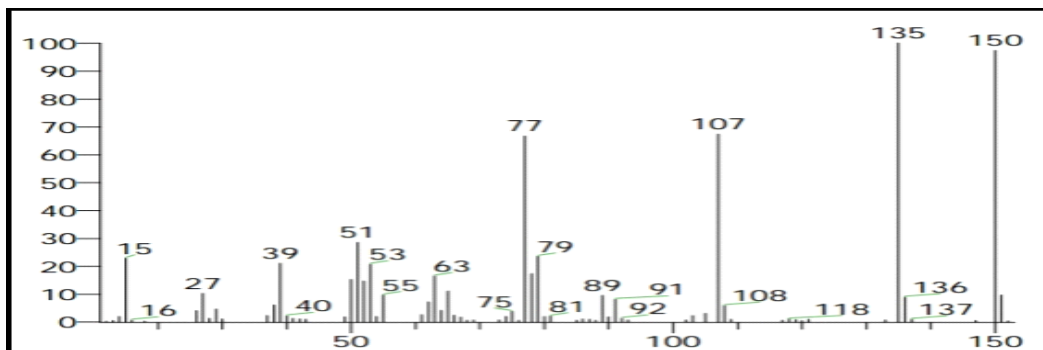


Fig. 7. Mass spectra of 2-methoxy-4-vinylphenol

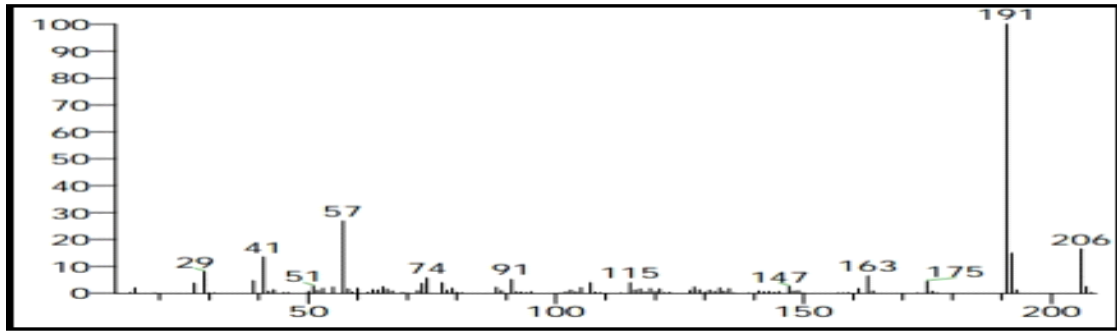


Fig. 8. Mass spectra of 2,4-Di tertbutylphenol

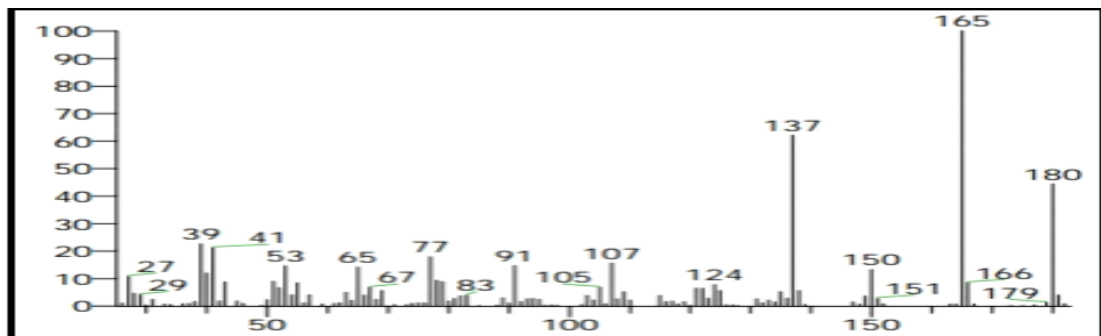


Fig. 9. Mass spectra of 3-tert-butyl-4-hydroxyanisole

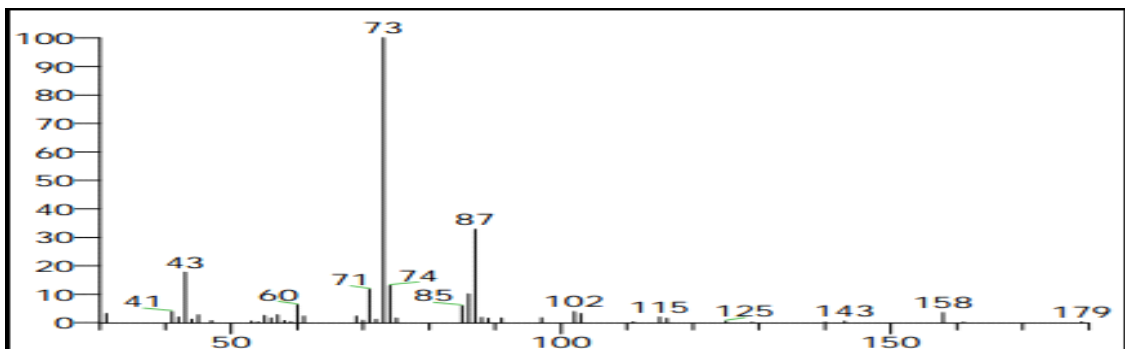


Fig. 10. Mass spectra of myo-inositol,4-C-methyl

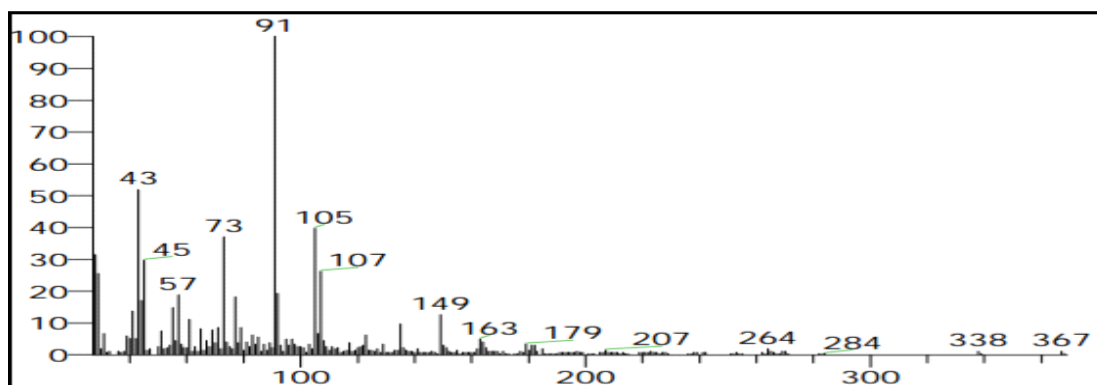


Fig. 11. Mass spectra of 9-octadecenoic acid (2phenyl1,3dioxolan4yl) methyl ester

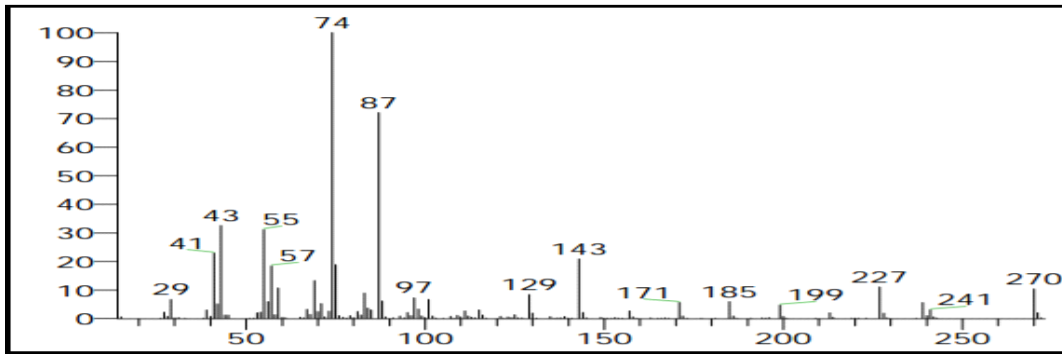


Fig. 12. Mass spectra of hexadecanoic acid, methyl ester

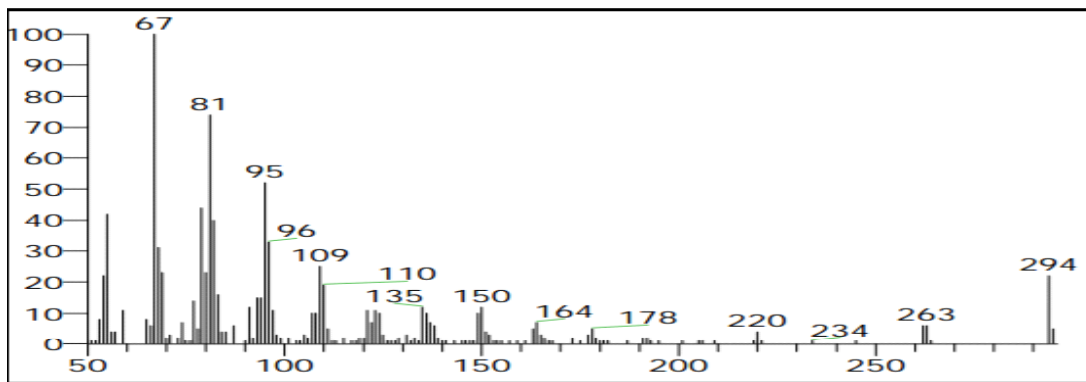


Fig. 13. Mass spectra of Methyl 9-cis,11-transoctadecadienoate

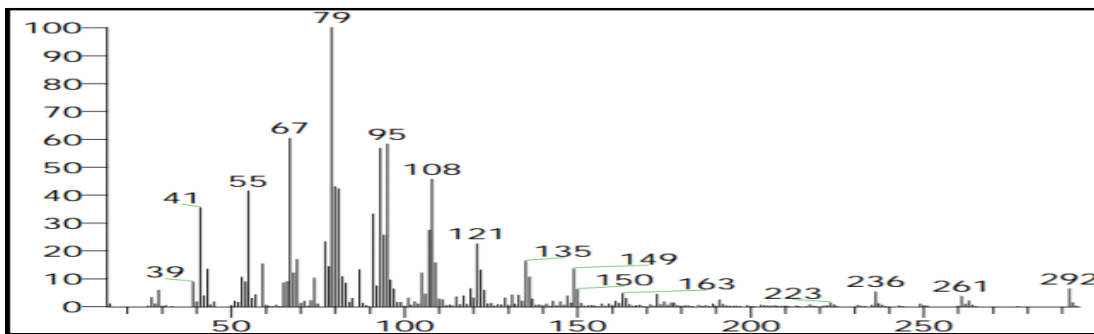


Fig. 14. Mass spectra of 9,12,15-octadecatrienoic acid, methyl ester

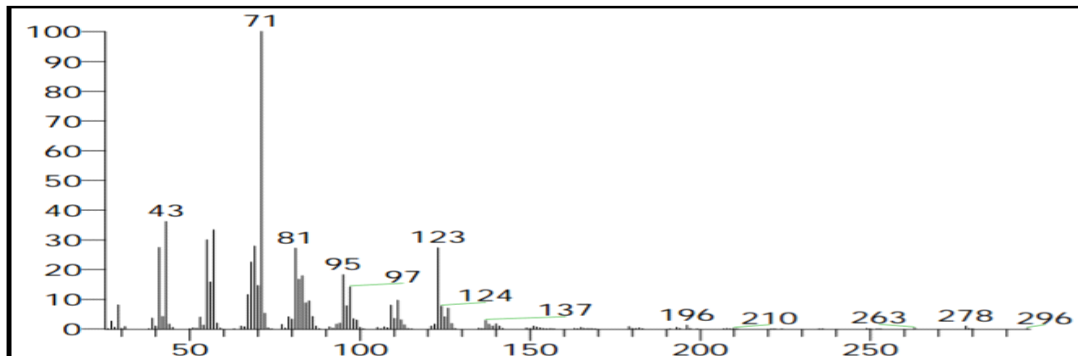


Fig. 15. Mass spectra of phytol

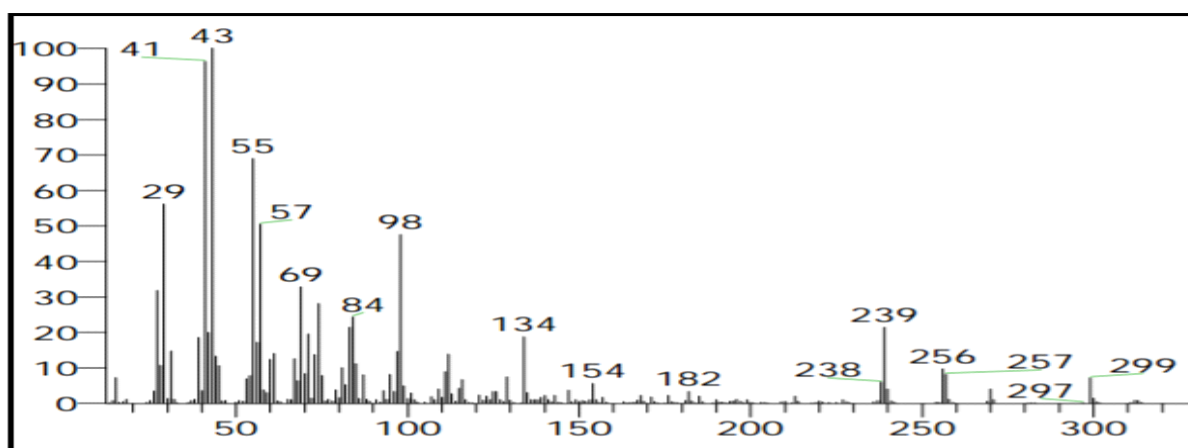


Fig. 16. Mass spectra of hexadecanoic acid, 2,3-di hydroxyl propylester

Table 2. Antimicrobial activity of extract of *Boerhaavia diffusa*

Name of organism	Zone of inhibition (mm)		
	Test (MEBD)*		(Streptomycin, Itraconazole) 5 mg/ml
	100 mg/ml	200 mg/ml	
<i>E.coli</i> (Sensitive)	No ZOI	No ZOI	28mm
<i>E.coli</i> (ESBL)	No ZOI	No ZOI	21mm
<i>Klebsiella pneumoniae</i> (Sensitive)	No ZOI	No ZOI	20mm
<i>Klebsiella pneumoniae</i> (ESBL)	No ZOI	No ZOI	18mm
<i>Staphylococcus aureus</i> (Sensitive)	26mm	28mm	26mm
<i>Staphylococcus aureus</i> (MRSA)	25mm	26mm	25mm
<i>Pseudomonas aeruginosa</i> (Sensitive)	27mm	30mm	28mm
<i>Pseudomonas aeruginosa</i> (Resistant)	No ZOI	No ZOI	22mm
<i>Candida albicans</i>	No ZOI	No ZOI	26mm
<i>Aspergillus fumigatus</i>	No ZOI	No ZOI	16mm

* Methanolic extract of *Boerhaavia diffusa*

Table 3. Antimycobacterial activity Of *Boerhaavia diffusa* leaves

Tube Number	Compound Name	Extract Type	Concentration used per vial	Growth Observed <i>M. tuberculosis</i> (H37Rv)	Growth Observed <i>M. tuberculosis</i> (MDR)
1	Growth / Positive Control	Nil	Nil	YES	YES
2	Negative Control 1 (NTC-1)	Methanol	100 µl methanol	YES	YES
4	<i>Boerhaavia diffusa</i>	Methanolic extract	100 µl (volume) from the stock of 10 mg/ml	YES	YES

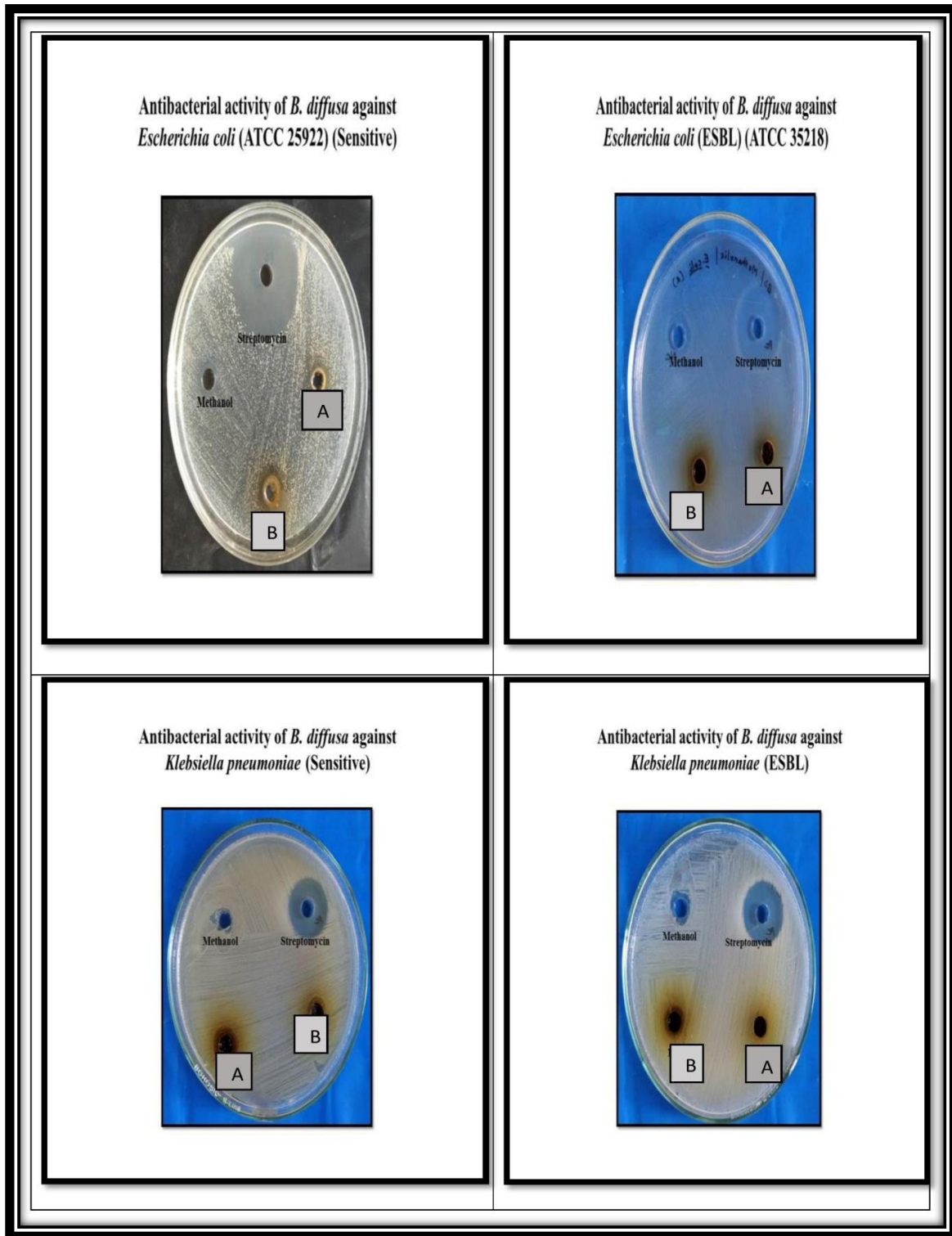


Fig. 17. Antimicrobial activity of extract of *Boerhaavia diffusa* leaves against *E.coli* (Sensitive), *E.coli* (ESBL), *Klebsiella pneumoniae* (Sensitive), *Staphylococcus aureus* (Sensitive)

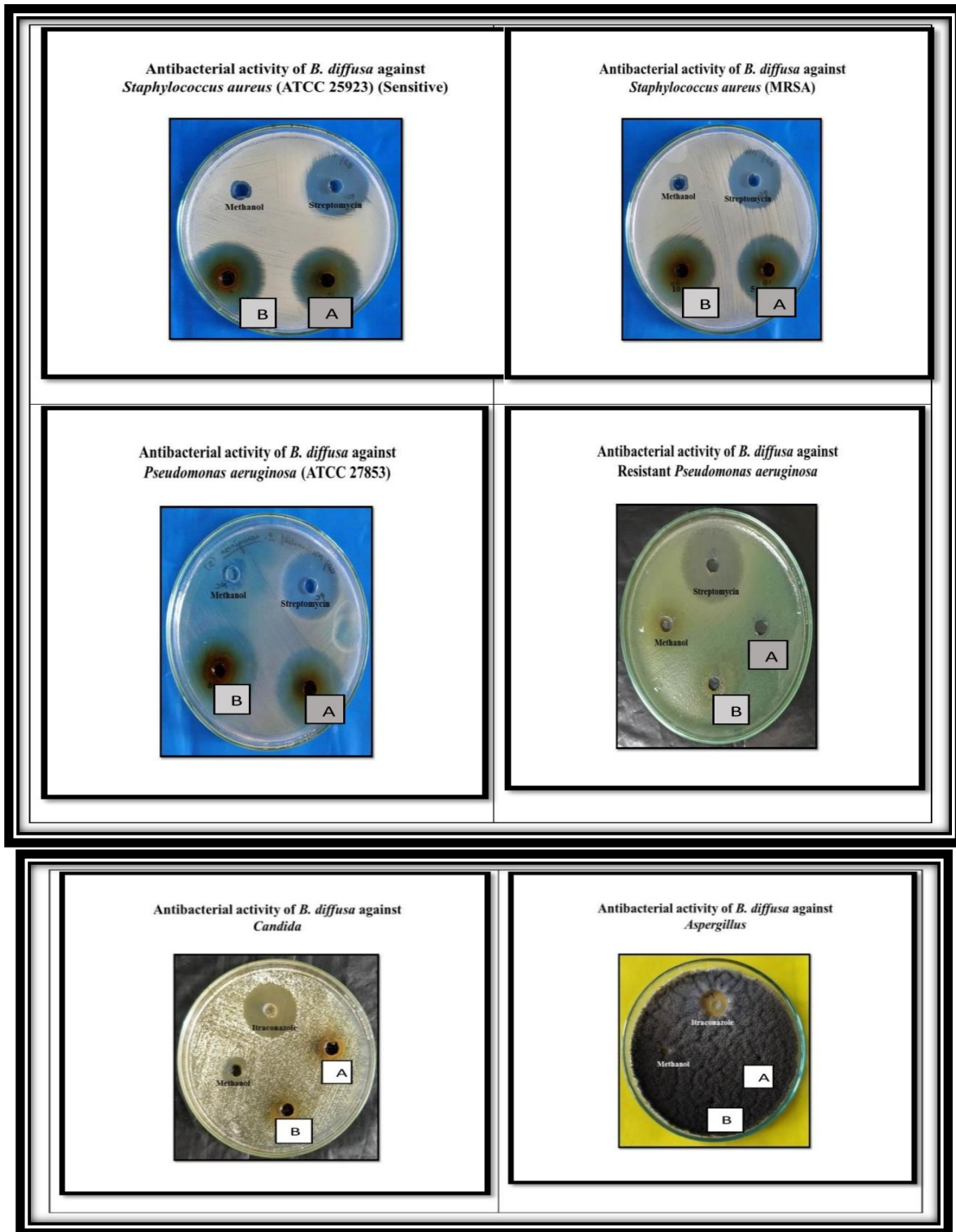


Fig. 18. Antimicrobial activity of extract of *Boerhaavia diffusa* leaves against *Staphylococcus aureus* (Sensitive), *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* (Sensitive), *Pseudomonas aeruginosa* (Resistant), *Candida albicans* and *Aspergillus fumigatus*

4. DISCUSSION

The potential of leaves extract of *Boerhaavia diffusa* as antimicrobial and antimycobial along with GC-MS profiling was investigated. The sixteen bioactive compounds were identified using GC-MS in methanolic extract of *Boerhaavia diffusa* leaves.

From GC-MS analysis bioactive compounds like 9-Octadecenoic acid [22], phytol [23,24], 2, 4-Di tertbutylphenol [25] Myo-Inositol, 4-C-methyl, Hexadecanoic acid, methyl ester [26,27] have showed antimicrobial activity.

The antimicrobial activity evaluated against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* & *Pseudomonas aeruginosa* and *Candida*, *Aspergillus* by Kirby-Bauer well diffusion method and measuring inhibition zone diameters.

The antimycobacterial activity evaluated against *M. tuberculosis* (H37RV) and *Mycobacterium tuberculosis* bacteria resistant to Isoniazid & Rifampicine by rapid culture - MGIT™ DST method.

The methanolic extract of *Boerhaavia diffusa* leaves exploited antimicrobial activity against *Staphylococcus aureus* (Sensitive), *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa* (Sensitive) in both concentrations at 100mg/ml and 200 mg/ml. It is observed during the study extract showed the highest antimicrobial activity against *Pseudomonas aeruginosa* (Sensitive) compare with standard (Streptomycin). Additionally, the antimicrobial effect was found to totally depend upon concentration. The result was found that extract was not effective against *Candida*, *Aspergillus*. The antimycobacterial activity was evaluated by the MGIT™ DST method. Similarly, the extract has not inhibited the growth of *M. tuberculosis* (H37Rv) and, *M. tuberculosis* (MDR). The phytoconstituents could be responsible for antimicrobial activity and indicated plants could be used in the evolution of novel antimicrobial agents.

5. CONCLUSION

Inspired by the huge medicinal potential of the herbal wealth of Indigenous plants, the present study was conducted to find out the Antimicrobial and Antimycobacterial activity potential of *Boerhaavia diffusa* leaves. *Boerhaavia diffusa* is a famous herb used in the treatment of many

ailments traditionally. The obtained result of the GC-MS showed the presence of many secondary metabolites or phytoconstituents in *Boerhaavia diffusa* leaves. The study result showed that extract exhibited an antimicrobial effect against few pathogens. Further study will necessary to required to find out the molecular mechanism of the antimicrobial effect of the extract.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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