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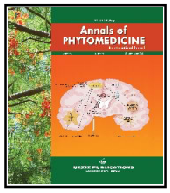

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Antimicrobial activity of *Haldina cordifolia* (Roxb.) Ridsdale and *Thevetia peruviana* (Pers.) Schum. leaf extract against multidrug resistant microbes

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Abstract

The major reason behind emergence of resistance among the various strains of microorganisms is the excessive uses of antimicrobial drugs in treating infections. Our aim of study is to find out the antimicrobial activities of methanolic and aqueous leaf extracts of *Haldina cordifolia* (Roxb.) Ridsdale and methanolic extracts of *Thevetia Peruviana* (Pers.) Schum. Antimicrobial activities for *H. cordifolia* and *T. peruviana* leaf extracts were estimated against number of multidrug resistant strains.The method used was Well Diffusion method for which nutrient agar was chosen for the growth of microbes. Growth curve assay was performed for the selected multidrug resistant microorganism. Cell surface hydrophobicity was determined for all the microorganisms using the plant extracts. From the study, antimicrobial activities were established to be quite rousing as both plant extracts in different concentration at 2.5 mg/ml, 5 mg/ml and 10 mg/ml shown bactericidal effect against all the MDR strains. Methanolic extracts of *T. peruviana* shown better effect compare to methanolic and aqueous extracts of *H. cordifolia*. Growth curve assay showed pronounce effect on *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *T. peruviana* extract elicit maximum cell surface hydrophobicity compared to the other extracts.

1. Introduction

Herbal drugs have an extended narration to treat a number of human infections and disorders from a different biological sources such as roots, flowers, bark, stem and leaves (Barnes *et al*., 2007). Medicinal plants were always the foundation for therapeutic uses throughout different time span of mankind and this conventional therapeutic entity always experienced till today. An advanced pharmaceutical drug recognizes natural source of medicine could be an options, the uses of plant and natural drugs is not sternly based on sustain systematic method. A large number of pharmaceutical industry directly connects with the physicians were using the natural medicines from a long duration likes of morphine, quinine, digitoxin and cinchonine, *etc*. From the data collected from WHO, 80% of the population of the world at present uses natural drugs for the maintenance of primary health. The research for herbal medicines have accelerated in last few years is astonishing. Researcher from different backgrounds comes together to study on the natural drugs which leads to the development and discovery of novel phytochemicals and that could be transformed into effective treatment of various diseases by the biomolecule. A large number of active biomolecules were discovered, out of which more than 85% were regularly used by the modern system of medicines now a days. A majority of plant species at which are nearly 40,000 which are predictable to have pharmaceutical importance originated from countries which are developing (Barnes *et al*., 2007).

From the literature data and reports, it was found that *H. cordifolia*, family Rubiaceae possesses several important medicinal activities such as anticancer (Sangameswaran and Saluja, 2012), antiulcer activities (Kasinadhuni *et al*., 1999), hepatoprotective effect (Agarwal *et al*., 2006), anti-inflammatory effect (Kausik *et al*., 2009), antifertility activities (Sabir and Razdan, 1970), antidiabetic activities (Chaudhary *et al*., 2012), antiamoebic (Iqbal *et al*., 2009), antinociptive (Jain *et al*., 2006), *etc*. In addition, this plant can also have a reputation such as rheumatism effects (Jain *et al*., 2010), relieve stomachache (Bhasker and Samant, 2012), used in headache (Singh *et al*., 2012), cold/cough (Pawar and Patil, 2011), toothache (Kambale *et al*., 2010), relieves fever (Padal *et al*., 2010), pain and swelling (Singh and Yadav, 2010), bacterial infection (Hossan *et al*., 2009), urinary problems (Mishra and Broker, 2009), conjunctivitis (Rahmatullah *et al*., 2009), miscarriage (Jadhav *et al*., 2006), *etc*. The reports about *T. peruviana*, belongs to family Apocyanaceae have displayed antispermatogenic activity (Gupta *et al*., 2011), antitermite (Kareru *et al*., 2010), antimicrobial (Ravikumar *et al*., 2008), antifungal properties (Gata *et al*., 2003), piscicidal activity (Singh and Yadav, 2010), anti-inflammatory (Thilagavathi *et al*., 2010), *etc*. From the above literature data with numerous effectiveness of both the plants, we were interested to evaluate the antimicrobial activity of this plant against multidrug resistant microbes. To the best of our knowledge, the antimicrobial activity

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from these plants against MDR microbes not reported till date. In the present paper, we would like to explore the antimicrobial activity of the methanolic extract of both plants employing various kinds of strains. It was found that majority of these strains employed, have displayed potential antibacterial activity.

2. Materials and Methods

2.1 Plant material

For the study, we have used the shade dried leaves of *H. cordifolia* and *T. Peruviana* which were collected from the local area of Lucknow, Uttar Pradesh, India. These plants were authenticated in the Faculty of Pharmacy, Integral University, Lucknow (Ref. No IU/PHAR/HRB/21/14 and IU/PHAR/HRB/21/15). First of all, fresh leaves were thoroughly washed in tap water, allowed to shade dried and further made to fine powder.

2.2 Preparation of methanol extract

The fine powder drugs of both the plants were first defattedusing petroleum ether using Soxhlet apparatus for 48 h. The remaining masses were extracted using Soxhlet apparatus by methanol for 48 h. The extracts were then allowed to cool, filtered and made concentrated by applying vacuum drying. The yield of both the plants was found to be 4.5 % and 4.35%, respectively. The marc left behind was kept in refrigerator for future applications.

2.3 Preparation of aqueous extract

The fine powders of *H. cordifolia* was extracted with double distilled water for 12 h by heating at lower temperature and then filtered with 6 layered muslin cloths in each 3 h after each steps of filtration, it was centrifuged for 20 min at 1500 rpm. Supernatant liquid was collected and the procedure was repeated twice. Finally, the supernatant liquid was concentrated, autoclaved and stored at 4^0C for further use.

2.4 Tested microorganisms

Different strains were used in this study. *Acinetobacter baumannii* (resistant to carbapenem), *Pseudomonas aeruginosa* (resistant to carbapenem), *Enterobacteriaceae* (resistant to carbapenem, ESBL producing), *Enterococcus faecium* (resistant to vancomycin), *Staphylococcus aureus* (resistant to methicillin, and vancomycin intermediate resistant), *Klebsiella pneumonia* (resistant to carbapenem), *Mycobacterium tuberculosis* (resistant to streptomycin) were clinical isolates collected from the hospital.

2.5 Antimicrobial activity

Antimicrobial activity was carried out using agar medium for bacterial culture and standard well diffusion method, 2.5 to 10 µl of suspension containing different colony forming unit's μl^{-1} of strains were inoculated over the nutrient agar medium plates using suitable apparatus such as sterile cotton buds.

Once the culture is prepared for different strains, three different concentration of extract were applied into each sample petridishes. These petridishes were then incubated at 37^0C for 24 h and the zone of inhibition was observed.

2.6 Minimum bactericidal concentration and zone of inhibition

The MBC refers to lowest concentration of antibiotics needed to kill bacterial strain. The plant extracts ranging from 2.5 mg/ml to 10 mg/ml were inoculated onto plates and allowed to incubate for 24 h at 37^0C. Serial dilution up to 6 dilutions is made for the MBC test. The MBC was evaluated by sampling all the clear tubes.

2.7 Minimum inhibitory concentration

MIC is the most common term used in microbiology, is defined as the lowest concentration of antimicrobial dugs that inhibits the growth of bacterial strains after incubating for 24 h. Different concentrations of the extracts were added to the bacterial culture in different tubes. All the tubes are incubated for 24 h at 37^0C individual tubes are evaluated against the control.

2.8 Growth curve assay

The growth curve assay is a process to evaluate the growth of bacterial strains used in the study. The growth of bacterial strains was checked at 0 h, 2 h, 6 h and 12 h and their turbidity (absorbance) was measured at 630 nm in a spectrophotometer. The bacterial cultures were prepared and were diluted 5 times using serial dilution method and was kept overnight at 37^0C for incubation. One ml of bacterial culture was added to all the three plant extracts and was kept for observation.

2.9 Cell surface hydrophobicity (CSH) assay

These types of assays are basically done to analyze the microbial adhesion to the surface of cells. For each strain, 2 ml suspensions were incorporated to different sterile test tubes, one of which is control and other is the test. Both the tubes are placed in a water bath at 37°C for 20 min to equilibrate. The hydrophobicity was measured in percentage reduction of turbidity of the test suspensions compared to control.

3. Results

The antimicrobial activity of all the three extracts against multidrug resistant strains shown excellent results which were comparable and better than the standard used in our study. The parameters used in our project are tabulated to compare the effectiveness of individual extracts and the positive control.

3.1 Minimum bactericidal concentration and zone of inhibition

Minimum bactericidal activities were observed with various degrees in all the three extracts. These effects were also observed in all the seven MDR strains for HME, TME and HAE. The most effective MBC value was observed in TME (14.8 µg/ml) for *Entero bacteriaceae* and least effective MBC value was observed in HME (24.9 µg/ml) for *K. pneumonia*. This value not more than four times greater than that of the MIC's on the corresponding microorganisms which indicates the antimicrobial potency of the used extracts. The results were given in the Table 1 and Figure 1.

Table 1: Minimum bactericidal concentration and zone of inhibition

Strains	Bacterial conc. (µg/ml)	HME	TME	HAE	Chloramphenicol (+ve control)
Acinetobacter baumannii	10	22.2 ± 1.5	16.7 ± 1.8	18.2 ± 0.9	19.2 ± 0.9
Pseudomonas aeruginosa	5	21.6 ± 2.1	17.8 ± 1.5	16.6 ± 2.3	18.6 ± 0.3
Enterobacteriaceae	10	23.8 ± 1.6	14.8 ± 1.9	16.5 ± 0.8	21.5 ± 0.8
Enterococcus faecium	5	21.8 ± 2.5	16.8 ± 2.1	18.8 ± 1.8	19.2 ± 0.7
Staphylococcus aureus	2.5	20.8 ± 1.6	18.9 ± 1.2	22.6 ± 1.5	18.8 ± 0.5
Klebsiella pneumonia	5	24.9 ± 1.4	23.9 ± 1.2	21.6 ± 2.1	23.6 ± 0.8
Mycobacterium tuberculosis	2.5	17.8 ± 1.7	17.9 ± 1.8	18.6 ± 1.2	19.2 ± 0 .3

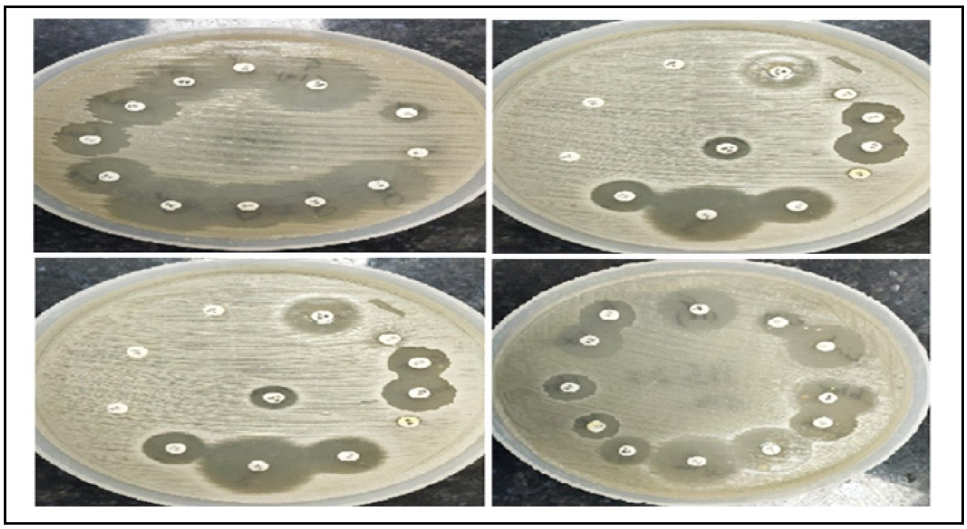

Figure 1: Minimum bactericidal concentration and zone of inhibition.

3.2 Minimum inhibitory concentration of different extracts

Our study shown the MIC values range from 3.5-10 µg/ml, for all the extracts. The lowest MIC values 3.5 µg/ml obtained with HAE were recorded on the one of the 7 microorganisms tested. The MIC values of 3.5 µg/ml obtained with extracts HAE against *Acinetobacter baumannii* was nearly 3 fold greater than that of reference antibiotic on the corresponding strains. Also, the MIC values of 4.5 3.5 µg/ml obtained with extract HME exhibited the double potency to reference antibiotic for *P. aeruginosa, Enterobacteriaceae and E. faecium* strains. TME has shown greater MBC potency in compare to its MIC. The results were shown in the Table 2.

Table 2: Minimum inhibitory concentration of different extracts

Strains	HME	TME	HAE	Chloramphenicol (+ve control)
Acinetobacter baumannii	7	7	3.5	9.5
Pseudomonas aeruginosa	4.5	7	3.9	10.0
Enterobacteriaceae	4.5	7	5	9.2
Enterococcus faecium	4.5	7	4.5	8.5
Staphylococcus aureus	5	10	5	7.3
Klebsiella pneumonia	6	9	4	9.1
Mycobacterium tuberculosis	9	10	6	10.6

3.3 Growth curve assay

The result shown in the growth curve assays indicated that TME has tremendous potential in controlling the growth of all the seven MDR strains used in the experiment. The minimum growth was observed in *A. baumannii* for TME after 12 h and maximum growth was observed in *P. aeruginosa* for HAE after 12 h incubation. All the results for this assay were given in the Table 3.

Table 3: Growth curve assay

Strains	0 h			2 h			6 h			12 h		
	HME	TME	HAE	HME	TME	HAE	HME	TME	HAE	HME	TME	HAE
Acinetobacter baumannii	0.3	0.2	0.3	0.38	0.39	0.4	0.2	0.3	0.9	0.18	0.29	1.2
Pseudomonas aeruginosa	0.32	0.31	0.32	0.32	0.31	0.32	0.31	0.32	0.85	0.32	0.31	1.4
Enterobacteriaceae,	0.22	0.21	0.22	0.41	0.45	0.4	0.21	0.22	0.89	0.41	0.45	0.86
Enterococcus faecium	0.3	0.34	0.3	0.45	0.42	0.41	0.34	0.3	0.78	0.45	0.42	0.84
Staphylococcus aureus	0.31	0.29	0.31	0.46	0.39	0.45	0.29	0.31	0.83	0.46	0.39	1.3
Klebsiella pneumonia	0.21	0.28	0.35	0.36	0.29	0.40	0.39	0.35	0.80	0.44	0.30	1.3
Mycobacterium tuberculosis	0.31	0.33	0.37	0.56	0.29	0.55	0.27	0.41	0.73	0.54	0.49	1.1

3.4 Cell surface hydrophobicity (CSH) assay

Hydrophobicity is one characteristic usually related with biofilm formation. Thus, hydrophobicity of pathogenic microbes cells was measured and was given in the Table 4.The cell surface hydrophobicity value of TME was shown highest in *E. faecium* (46.04) and lowest in *A. baumannii* (5.86).The overall lowest hydrophobicity shown in HAE extract with the minimal value 7.96 in *S. aureus*. It was also observed the all the extracts shown moderate cell surface hydrophobicity in *M. tuberculosis* and lowest in *A. baumannii*.

Table 4: Cell surface hydrophobicity (CSH) assay

Strains	HME	TME	HAE
Acinetobacter baumannii	9.28	10.26	5.86
Pseudomonas aeruginosa	28.78	48.01	37.03
Enterobacteriaceae	38.12	38.11	34.52
Enterococcus faecium	35.4	46.04	38.45
Staphylococcus aureus	9.48	11.42	7.96
Klebsiella pneumonia	15.56	21.23	27.56
Mycobacterium tuberculosis	21.20	31.23	19.97

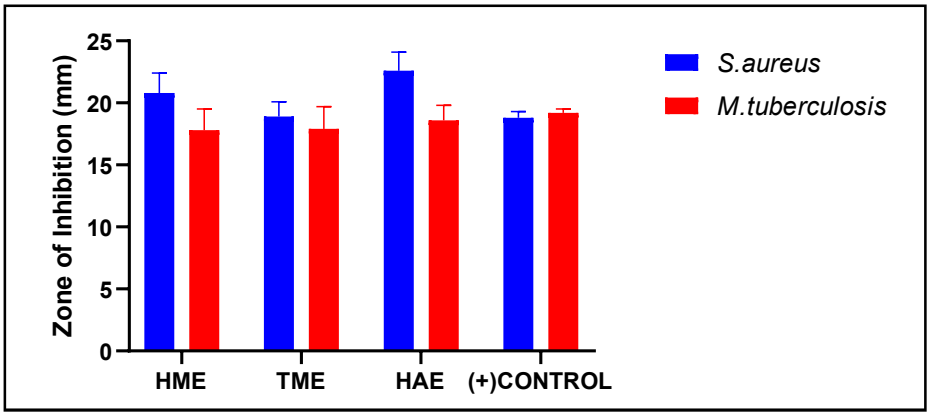

Figure 2: Zone of inhibition at 2.5 mg/ml concentration.

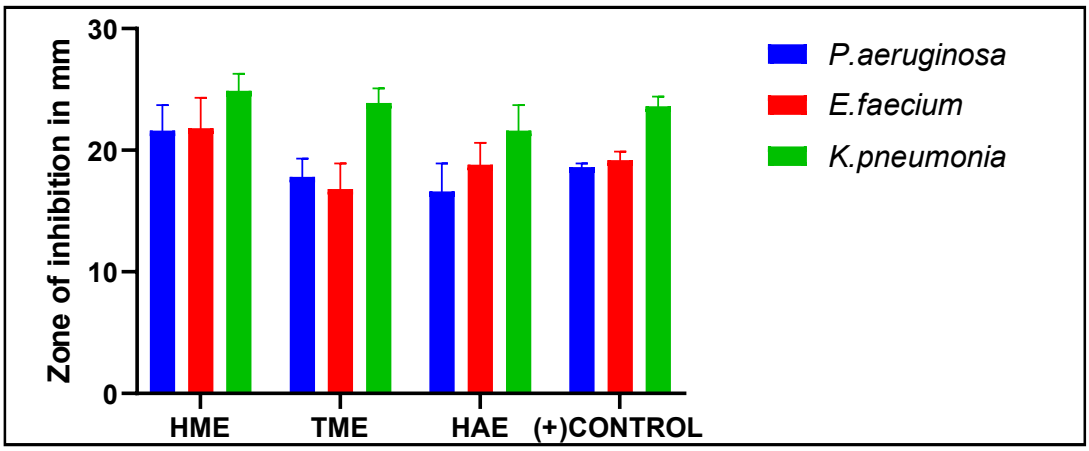

Figure 3: Zone of inhibition at 5 mg/ml concentration.

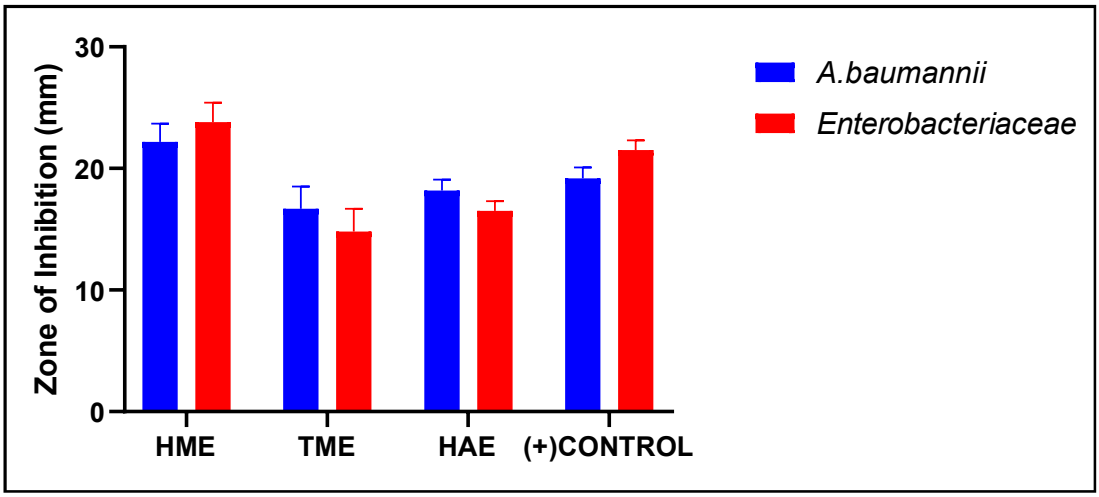

Figure 4: Zone of inhibition at 10 mg/ml concentration.

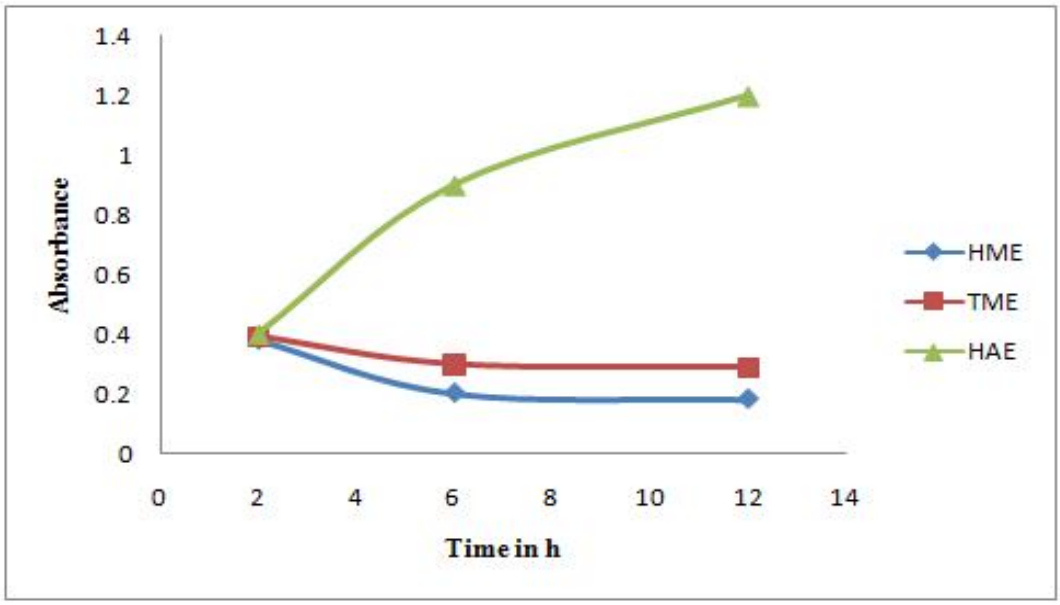

Figure 5: Growth curve of *A. baumannii.*

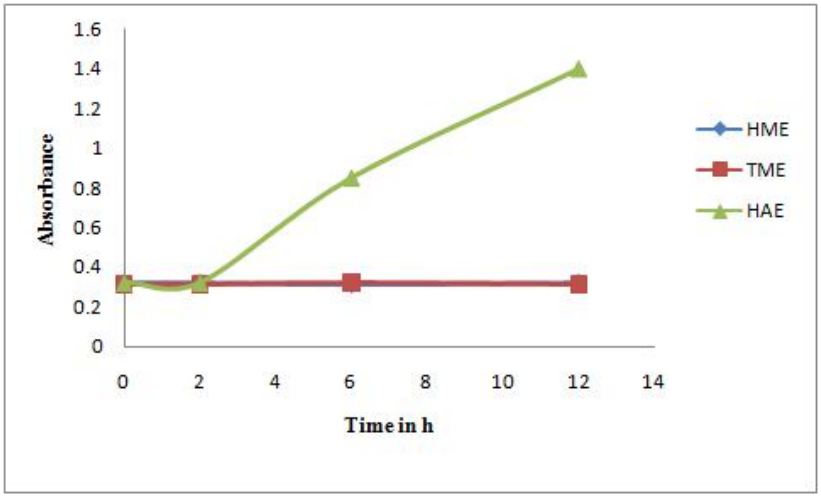

Figure 6: Growth curve of *P. aeruginosa.*

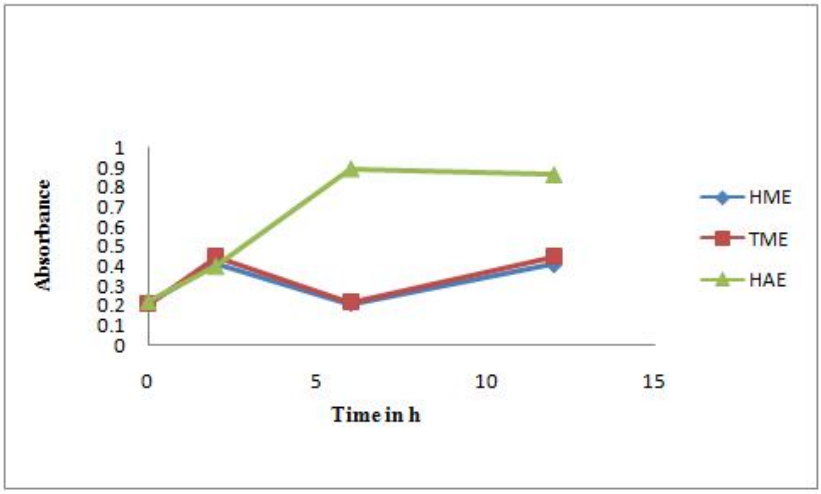

Figure 7: Growth curve of *E. faecium.*

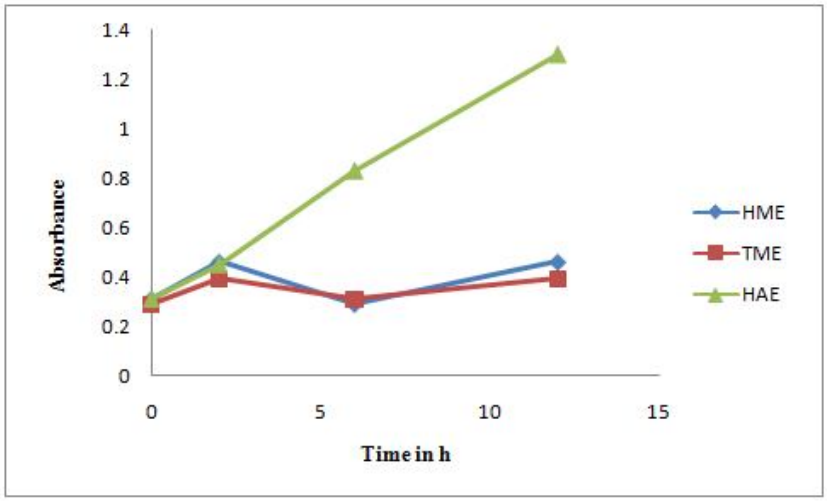

Figure 8: Growth curve of *S. aureus.*

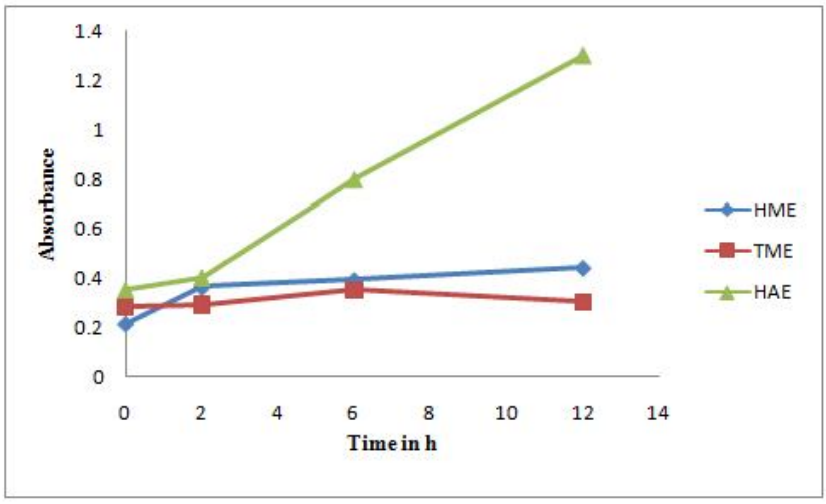

Figure 9: Growth curve of *K. pneumonia.*

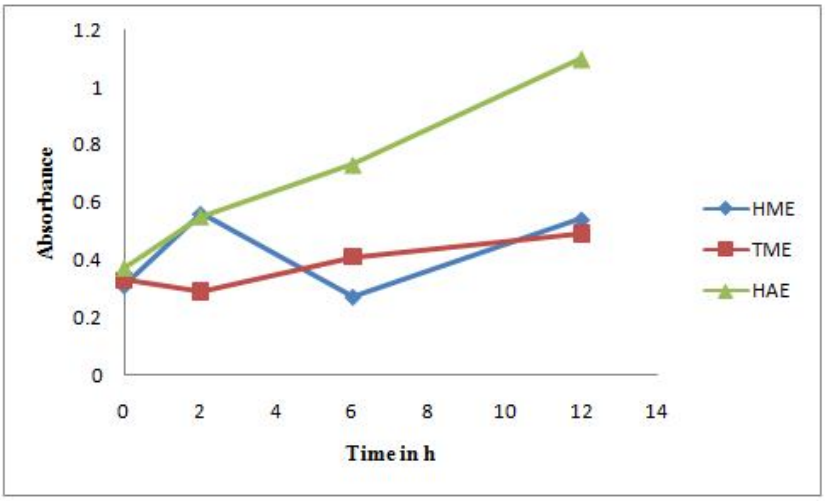

Figure 10: Growth curve of *M. tuberculosis.*

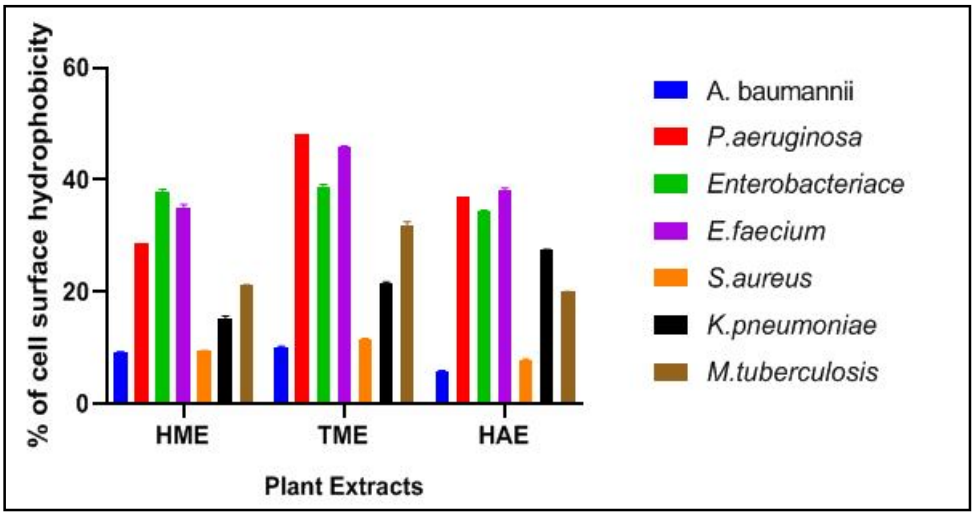

Figure 11: Cell surface hydrophobicity.

4. Discussion

From the above results, it was observed that methanolic and aqueous leaf extract of *H. cordifolia* had considerable effect against maximum number of strains used in the study like *A. baumannii* (carbapenem-resistant), *P. aeruginosa* (carbapenem-resistant), Enterobacteriaceae (carbapenem-resistant, ESBLproducing), *E. faecium* (vancomycin-resistant), *S. aureus* (methicillin-resistant, vancomycin-intermediate and resistant), *K. pneumonia* (carbapenem-resistant), *M. tuberculosis* (streptomycin) were the most vulnerable, with the inhibition zone is similar cephalosporin and penicillin for each tested concentrations. The result was supported by the work of Manivannan *et al.* (2015). *T. peruviana* leaf extract shown significant antimicrobial activity against each microorganism tested. By comparing with the standard drug (chloramphenicol), maximum effect of *T. peruviana* leaf extract (methanolic) was shown against *S. aureus* (22.6 ± 2.5 mm ZI) at 200 mg/ml followed, by *K. pneumonia* (21.6 ± 2.1mm ZI) at 5 µg /ml. MDR strains of *A. baumannii*, *P. aeruginosa*, Enterobacteriaceae, *E. faecium* and *M. tuberculosis* were established to be less vulnerable than the standard culture at all concentrations. *T. Peruviana* extract with 5 mg/ml and 10 mg/ml concentration showed significant activity against most experimental microorganisms, except for *Streptococcus and Klebsiella* (Rasool *et al.*, 2017). The result only differs for *Streptococcus* pyogens strain, with the report of Taye *et al.* (2011), the reports suggested the gram-positive microorganisms are more vulnerable than the gram-negative microorganism due to their difference in composition of cell wall. Minimum inhibitory concentration is a measure of concentration of drugs or antimicrobial entity that stop the growth of pathogens in 24 h of incubation. The minimum bactericidal concentration explains about the minimum drug/extract require to kill the microorganisms after culturing it in antibiotic free medium (Owuama *et al.*, 2015).The result shown in MIC of our study indicated that the *H. cordifolia* leaves extract (methanolic) have maximum potency against *K. pneumonia* and *A. baumannii*. A significant antimicrobial activity was also shown against *Enterobacteriaceae, E. faecium* and *P. aeruginosa* at a lower concentration of *H. cordifolia* leaf extract. The finding of our research resembles with the previous study (Kouadri *et al.*, 2015). The vulnerability difference among microorganisms may be due to variation in intrinsic resistance of microorganisms, or the different physicochemical properties of the plant extracts (Kouadri *et al.*, 2015). *H. cordifolia* leaf extracts have considerable bactericidal effects against *P. aeruginosa, Enterobacteriaceae, E. faecium, K. pneumonia, S. aureus* and *S. pyogenes,* and also shown bacteriostatic action against *S. aureus, P. aeruginosa, K. pneumonia, P. aeruginosa and M. tuberculosis*. Activity is measured to be high when MIC value is ≤ 10 µg/ml, average when MIC value is 10-100 µg/ ml and low when MIC value is ≥ 100 µg/ml (Bianco, 2015). *T. peruviana* extracts with MICs value from 5 ± 0.0 mg/ml to 10.0 ± 0.0 mg/ml, and 10.0 ± 0.0 to 20.0 ± 0.0 mg/ml, respectively, had moderate to low activity against the experimental microorganisms. Although, *T. peruviana* shown moderate to low effect against all tested microorganisms but it showed bactericidal activity against all tested strains, with MBC values ≥ 10 mg/ml against all tested strains except *S. aureus* and *S. pyogenes*. The presence of several bioactive molecules in both the plant extracts accounted for the broad-spectrum antimicrobial activities observed in this study, which is in agreement with the reports of the previous ones (Begashaw *et al.*, 2015).

The growth curve assay was a measure of growth of bacterial strain in the experimental antimicrobial agents. The result shown that the aqueous extract of *H. cordifolia* shown highest amount of growth in all MDR strain in compare to the other two methanolic extracts. If we study the growth curve of *H. cordifolia* methanolic extract and *T. peruviana* methanolic extract, there is a less growth of bacterial strain in both of them, but *T. peruviana* shown comparatively less growth than the *H. cordifolia extract* in almost all the strains. These results indicate TME (*T. peruviana* methanolic extract) has more efficiency than both HME and HAE (*H. cordifolia* methanolic extract and *H. cordifolia* aqueous extract).

Microbial adhesion has a great impact on the microorganism life cycle. The surface nature of cell allows the microorganism to form a biofilm and grow on that. The biofilm formation is better in the hydrophobic surface in compare to the hydrophilic surface. Our study result shown that all the three extracts used have shown significant effect on cell surface hydrophobicity for all the microbial strains. Maximum effect was shown on *A. baumannii, Enterobacteriaceae* and *P. aeruginosa.* There is also a significant effect on the *M. tuberculosis* which is a quite challenging mycobacterium strains. The best result was shown by *T. peruviana* extract which can be very efficient against MDR strains.

5. Conclusion

From the research, it was observed that all three plant extracts showed antimicrobial activities towards *A. baumannii*, *P. aeruginosa, Enterobacteriaceae*, *E. faecium, S. aureus*, *K. pneumonia*, *M. tuberculosis*. The result also show that *T. peruviana* methanolic leaf extracts have significant activity against all experimental microorganisms compared to methanolic and aqueous extracts of *H. cordifolia.* The study also concludes that methanolic extract *of T. peruviana* has excellent MBC and less effect on control of growth all the bacterial strains whereas great control over growth of microorganism in compare to other extracts. The cell surface hydrophobicity study shown excellent effect of *T. peruviana* methanolic extract. From our study, it was found that both the plant extracts shown excellent effect on all the MDR bacterial strain, so it can be considered for thorough research to find out the potential candidate for drugs which can used against MDR pathogens.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Abbreviations

HME: *Haldina cordifolia* methanolic extract

TME: *Thevetia peruviana* methanolic extract

HAE: *Haldina cordifolia* aqueous extract

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