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ETHANOL EXTRACT OF EUPHORBIA HIRTA LINN MODIFIES THE RESISTANCE OF METHICILLIN RESISTANT BETA LACTAMASE POSITIVE STAPHYLOCOCCUS AUREUS - AN IN VITRO STUDY

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ABSTRACT

The synergistic effect of *Euphorbia hirta* L. (inflorescence) ethanol extract (EHE) and antibiotics on drug resistant bacteria were investigated by determining the fractional inhibitory concentration (FIC) of the extract and antibiotics individually and in combination. Gram positive (β-lactamase positive *Staphylococcus aureus*- MRSA) bacteria was included in the study. Micro-titre plate dilution assay (96 well) was performed to calculate the MIC and FIC of the extract and for study of resistance reversal in the selected bacteria. Bio autography, a scientific method which combines chromatography with bioassay was attempted to determine the bioactive components in the ethanol extract of the inflorescence of *Euphorbia hirta L*inn. and Rf value calculated for the active compound.

KEYWORDS: *Euphorbia hirta* L, resistance, antibiotics, MRSA,

TLCType equation here.

INTRODUCTION

Antibiotics are indeed a priceless substance that is bestowed by nature to very few living organisms be it plants, animals or microorganisms- both flora and fauna. Since the discovery of antibiotics and their uses as chemotherapeutic agents, there was a belief in the medical fraternity that this would lead to the eradication of infectious diseases. This study can

definitely be replicated with clinical isolates which are bound to give better results since they are more susceptible to multi drug resistance of *Euphorbia hirta* L. with antibiotic agents**.** However diseases and disease agents that were once thought to have been controlled by antibiotics are returning in new forms resistant to antibiotic therapies.[11] Incidents of epidemics due to such drug resistant microorganisms are now a common global problem posing enormous public health concerns.[8] The global emergence of multi-drug resistant bacterial strains is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure of infections.[6] Examples include methicillin-resistant staphylococci, pneumococci resistant to penicillin and macrolides, vancomycin-resistant enterococci as well as multi drug resistant gram-negative organisms.[14] As resistance to old antibiotics spreads, the development of new antimicrobial agents have been expedited if the problem is to be contained. However, the past record of rapid, widespread and emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents should have a short life expectancy.[3] It becomes imperative therefore that alternative approaches are explored. Targeting and blocking resistance processes could be an attractive approach. The presence of efflux pumps and multidrug resistance (MDR) proteins in antibiotic resistant organisms contribute significantly to the intrinsic and acquired resistance in these pathogens. The discovery and development of new compounds that either block or circumvent resistance mechanisms could improve the containment, treatment, and eradication of these strains.[16] Owing to their popular use as remedies for many infectious diseases, searches for substances with antimicrobial activity in plants are frequent.[2] Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties.[12] There is reason therefore to believe that, plants could be a source of compounds that can increase the sensitivity of bacterial cells to antibiotics. Such compounds could be useful particularly against antibiotic resistant strains of pathogenic bacteria. The rich chemical diversity in plants promises to be a potential source of antibiotic resistance modifying compounds and has yet to be adequately explored.

MATERIALS AND METHODS

Preparation of inflorescence extract

Euphorbia hirta L. whole plant was collected from gardens in and around Tindivanam and Chennai, Tamil Nadu. The fresh part of inflorescence were carefully cleaned, dried under

shade at room temperature for 10-15 days and ground into fine powder using a mixer grinder and stored in air tight bottles. The powder was subjected to extraction with ethanol extracts.

Euphorbia hirta Linn.

Preparation of ethanol extract

Inflorescence powder (10g) was taken and 100 ml of hexane added to it and kept for 48h. The extract was filtered and residual matter was air dried and immersed in 100ml of ethanol for another 48h. The extract was filtered and air dried. The dried residue was weighed was dissolved in Dimethyl sulfoxide and stock solution was prepared and stored at 4°C for further study.

Preparation of reference antibiotics

Tetracycline, Cotrimoxazole, and penicillin were separately dissolved in 10% DMSO and stored at 4°Cfor further study.

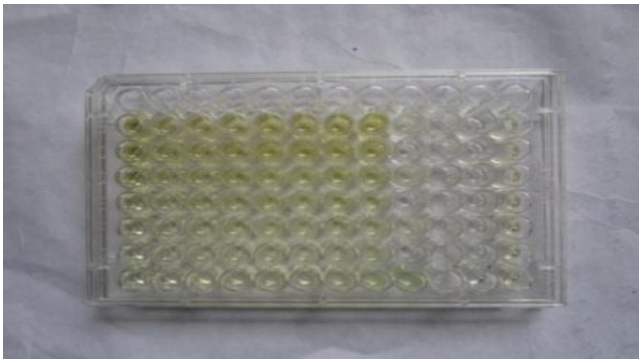
Test microorganisms

Staphylococcus aureus (β MRSA). The cultures were obtained from Sri Ramachandra Medical College and Research Institute, Porur, Chennai-116.

Microbroth Dilution Asssay for calculating the MIC of each extract

The ethanol extract was dissolved in 10% DMSO giving a concentration of 1 mg /ml which was used as a neat dilution. The chosen reference antibiotics ie., Tetracycline, Co-trimoxazole and Penicillin (1mg/ml) for *Staphylococcus aureus* (β MRSA) were also diluted with 10% DMSO. This assay was performed in flat bottom 96-well clear micro-titre plates. The wells in column A of each row was left blank and the last seven wells from column B to H was filled with 100 µl of 10% DMSO. Working solution of plant extracts were added to the wells in column A and B of each row and an identical two fold serial dilution was made from column B to the column G. The last wells in column H served as drug free control. An

appropriate solvent blank (DMSO) was included as negative control. Lastly, 100µl of bacterial inoculum was added to all the wells from column A to H and mixed thoroughly. Tests were performed in triplicates. The cultured micro-plates were sealed with parafilm and incubated at 37^{o}C for 24h. The MIC of samples were visually detected following addition of 0.2 mg/ml of TTC (100 µl) in all the wells. After incubation at 37^{o}C for 30 min, the growth or no-growth was detected by observing the change of colour of TTC in the micro-plate wells (pinkish red formazan where there is growth and clear solution where there is no growth). MIC was calculated as the lowest sample concentration showing no colour change thereby exhibiting complete inhibition of bacterial growth. Positive and negative controls were included in the appropriate wells. [Fig-1]



96 well micro-titre plate assay [Fig-1].

Calculation of fractional inhibitory concentration index (FIC)

The MICs of extracts and the chosen antibiotics were determined in combinations by varying proportions of the extract and antibiotics. Each proportion of the extract and antibiotic combination were serially diluted (2 fold) and inoculated with 0.1 ml of test isolates, incubated at 37°C for 24 h. The interaction or synergism effect was assessed algebraically by determining the Fractional Inhibitory Concentration (FIC) indices according to the equation:

$FIC_{index} = FIC_{extract} + FIC_{antibiotic}$, where

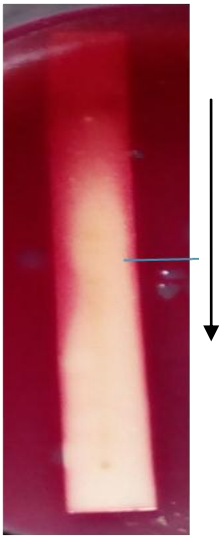
$FIC_{extract}$ = MIC of ethanol extract in combination with antibiotic / MIC of ethanol extract alone

$FIC_{antibiotic}$ = MIC of antibiotic in combination with ethanol extract / MIC of antibiotic alone

FIC was calculated to know whether the plant extract showed synergism (≤ 0.5), additive effect (0.5 ≤ 1.0), indifferent effect (≥ 0.5-≤ 4.0) and antagonism (≥ 4.0) towards the chosen antibiotics.[10,15]

To perform Bio-Autography of the fractionated extract of *E. hirta* L

The effective extract of *E. hirta* L. showing significant antibacterial activity was investigated by Thin Layer Chromatography (TLC) using the Agar Overlay Method. For this, a known concentration of the extract was applied on pre formed TLC plates (Merck) and developed in ethanol alcohol (1:1) mixture. Bacterial inoculum was prepared by suspending the bacteria in nutrient broth with an approximate concentration of 10^6 cfu/ml. The TLC plates was put in a sterile petri dish and covered with 10 ml of inoculum. After solidification, the plates were incubated at 37^{o}C for 15 h after which the plates was sprayed with an aqueous solution of phenyl tetrazolium chloride. Clear zones on the chromatogram after 30 min - 1 h of incubation at 37^{o}C, will indicate inhibition of growth. [Fig-2]



Bio-autography of EHE for β MRSA- Methicillin resistant *Staphylococcus aureus* [Fig-2]

RESULTS AND DISCUSSION

The study using the ethanol extract of the chosen plant revealed promising antibacterial efficacy as MIC and FIC as shown [Table 1-4].

Table 1: MIC of ethanol extract and antibiotic individually.

S.no	Bacterial culture	Ethanol extract mg/ml	Tetracycline mg/ml	Co-Trimoxazole mg/ml	Penicillin mg/ml
1.	β MRSA	2.75	0.039	1.25	0.625

Table 2: MIC of ethanol extract and antibiotic Combination- Ethanol concentration.

S.no	Bacterial culture	EHE+ Tet mg/ml	EHE+ CoT mg/ml	EHE+P mg/ml
1.	β MRSA	0.021	0.168	1.375

Table 3: MIC of antibiotic with ethanol extract Combination- Antibiotic Concentration.

S.no	Bacterial culture	Tet+EHE mg/ml	CoT+EHE mg/ml	P+ EHE mg/ml
1.	β MRSA	0.039	0.312	0.312

βMRSA- Methicilin Resistant *Staphylococcus aureus*

Ehe- Ethanol extract, Tet- Tetracycline, CoT- Co-trimoxazole, P- Penicillin,

Table 4: FIC Calculation.

S.no	Bacterial culture	Tetracycline	Co-trimoxazole	Penicillin
1.	β MRSA	1 Additive	0.31 Synergistic	0.99 Additive

Table 5: Different components of ethanol extract revealed by TLC.

S.No	Solvent System	Sample	Spot
1.	Ethanol: Ethyl acetate: xylene:1,2 Trichloro benzene: 124 Dichloro bencence (1:1:1:1:1) (β MRSA)	ethanol extract	1

Table 6:

Bio active components	Rf Values Presence of active zone β MRSA	
A	0.12	
B	0.37	
C	0.44	+
D	5.44	+
E	0.56	+
F	0.6	+
G	0.64	+
H	0.88	+

R_f = Distance traveled by the compound

Distance traveled by the solvent

The bio-autographic studies clearly show inhibition zone near the solvent front indicating the presence of an active principle that is possibly soluble in ethanol. [Table-6, Fig-2]

The crude ethanol extract EHE chromatogram revealed a clear inhibition area at Rf 0.8 against β MRSA using bio-autography, our study showed similar results as reported by Demetrio *et al.*[5] Another study using agar overlay bio-autography method of the isolated compounds demonstrated clearance zone at Rf values 0.86 against MRSA.[2] Our study also revealed zone of clearance at Rf 0.88 against MRSA.

CONCLUSION

To conclude, *Euphorbia hirta* L. is definitely a promising plant for drug discovery research against bacterial pathogens since it has shown a well marked reversal of antibiotic resistance in the studies. Further, it is decided to continue studies related to proving its efficacy as an antibacterial agent by studying its mode of action pertaining to enzyme regulation.

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