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A Discussion on Scientific Validation on Antibiotic Resistance Reversal of Euphorbia hirta L. With A Short Note on Its Bio-Autographic Studies

Gayathri A. Research scholar, Department of Plant Biology and Plant Biotechnology, Quaid-e-Millath Government College for Women, Chennai 600002, Tamil Nadu, South India

Vijaya Ramesh K.* Assistant Professor, Department of Plant Biology and Plant Biotechnology, Quaid-e-Millath Government College for Women, Chennai 600002, Tamil Nadu, South India

Abstract: A common weed, *Euphorbia hirta* L. has been studied for its antibacterial activity with special reference to reversal of antibiotic resistance. Preliminary studies with aqueous and ethanolic extract of the inflorescence corroborated the antibacterial efficacy of the plant as already reported but reversal of antibiotic resistance is a new report by our group. *Pseudomonas* and *E. coli* were chosen for the study and of the two,*Pseudomonas* reveals much clear picture of reversal of resistance against both the chosen antibiotics tetracycline and chloramphenicol. We have presented a photographic evidence of bioautographic study on the ethanolic extract of *E. hirta* L. on the above two bacterial genera which is reported here for the first time. *E. Hirta* L. is a promising medicinal plant that can be further studied as a drug resistance modifier and can be used in conjunct with antibiotics for effective control of multidrug resistant bacterial infections.

Keywords: *Euphorbia hirta* L.; *Pseudomonas, E. coli* ESBL.; Bioautography; Reversal of resistance; Synergistic.

1. Introduction

A plant showing fast growth that spurges into activity with a little rain, (commonly the plant is known as "*garden spurge*" in English, *"Ammaan Pachirisi*" in Tamil and "*Duddhi*" in Sanskrit) *E. hirta* L. has been a favourite weed plant with a good number of scientific proof for its medicinal properties [1-4]. The plant has been an attraction for many laboratories that have pioneered its antibacterial potentials against diarrhoegenic entero pathogens [5]. Further, a number of reports re-iterating its anti-inflammatory, antioxidant and immune modulatory activities have been thrown open into the scientific arena, compelling for research into its phytochemical analysis using chromatographic techniques.

On the other front, it is disheartening to note the emergence of bacteria, both gram positive and gram negative that have long since become resistant to affordable antibiotics. In the present scenario, it has thus become imperative to report various levels of antibacterial activity of the medicinal plants against the antibiotic resistant bacterial strains.

2. Materials and Methods

2.1. Collection of Plant Specimen

The dicot plant *Euphorbia hirta* L. of the family Euphorbiaceae was collected from gradens in and around Adyar, Chennai. A voucher specimen has been deposited at the herbarium repository of Presidency College, Chennai 5 (Voucher No. 8413). The powder obtained after appropriate shade drying of the inflorescence part of the plant was used for the study.

2.2. Bacterial Cultures

Bacterial genera used for the study were provided by Sri Ramachandra Medical College and Research Institute, Porur, Chennai. We have used two antibiotic resistant bacteria *viz.* Extended β lactamase *Escherichia coli* and Multidrug resistant *Pseudomonas aeruginosa*.

2.3. Antibiotics Used For the Study

Following antibiotics were chosen since they are very affordable and they have long since become useless as antibacterial agents against these strains. We have used chloramphenicol (30µg) and Tetracycline (50µg) in powder form (HiMedia).

*Corresponding Author

A preliminary study indicated the higher efficacy of ethanol extract of the plant as antibacterial agent when compared to hot and cold aqueous extracts. Hence further studies were performed using ethanol extract. Methodology of Vijaya, *et al.* [6] was used for preparation of ethanol extract. Micro-broth Tube Dilution technique was used to elaborate the Minimum Inhibitory Concentrations of the extract and the chosen antibiotics individually and in combination by the methodology of Perumal, *et al.* [7]. Fractional Inhibitory Concentrations of the plant extract individually as well as in combination with the antibiotics was calculated as per the methodology of Adikwu, *et al.* [8].This study indicated the synergistic, additive or indifferent activities of both the extract and the antibiotics used against the bacterial strains tested.

2.4. Reversal of Antibiotic Resistance

Agar well diffusion assay was performed to ascertain the reversal of antibiotic resistance of the bacterial strains against the chosen antibiotics. Briefly, the bacterial culture load equated to McFarland tube No. 1 was mixed with equal volumes of the ethanolic extract of plant (of various concentrations) and after time intervals ranging from1hto 3 h, bacterial lawn was spread over Mueller Hinton Agar that was overlaid with 0.01ml of antibiotics at fixed concentrations of 50µg/ml for tetracycline and 30µg/ml for chloramphenicol in the diluent 10% DMSO. Appropriate extract free controls were maintained and incubated for development of zones of inhibition (ZOI).

2.5. Bioautography of Ethanolic Extract of E. hirta L. [9]

2.5.1. Development of the Chromatogram

Thin layer chromatography was used for preparation of the chromatogram. Pre-coated TLC silica get sheets (SD Fine chemicals) were procured and cut as required. Extract was spotted singly and run using ethyl acetate, ethanol, xylene and 1,2,4, trichlorobenzene at equal ratios. All the basic developing conditions were maintained.

The developed chromatogram was placed on the base agar surface. The bacterial cultures were prepared in 0.5% agar (10^5cfu/ml) and poured over the developed chromatogram. After a brief period of refrigeration (10-15 min), the set up was incubated at 37^{o}C overnight. 1% TTC (2,3,5 Triphenyl-tetrazoliumchloride) was poured over the incubated chromatogram and the ZOI was visualized as clearance areas (due to non- growth of bacteria) limited by pinkish red colouration due to the reduction of the TTC to TPF formazan by the living bacterial cells.

The Rf values of the areas showing clearance zones could be earmarked after comparing with the untreated chromatogram.

3. Results and Discussion

There are nevertheless many reports on antibacterial potentials of *E. hirta* L with various levels of inhibitions that are quite low when compared to our results possibly because of the extraction solvents [10, 11] but this report discusses the synergistic interaction of the plant extract on the activity of chosen antibiotics against ESBL *E.coli* and MDR *Pseudomonas* for which no previous work has been reported

3.1. Preliminary Antimicrobial Study

Preliminary studies (Table 1 & 2) show that aqueous extract of the inflorescence can also be used as an antibacterial agent. Needless to say that ethanol may be a better solvent than water (Table 3), aqueous extracts also reveal some amount of bacterial inhibition, only thing is they can have a comparatively high MICs.

3.2. MIC Test for Ethanol Extract Individually and In Combination with Antibiotics

Even though with drug resistant *E. coli*, ethanolic extract of the plant material exhibited a very high MIC i.e., 22.5 mg/ml when tested individually, in combination with the antibiotics tetracycline and chloramphenicol, its MIC decreased considerably i.e., there was a 4 time decrease in the MIC of the ethanol extract . Tetracycline and chloramphenicol revealed MICs at concentrations of 25µg when tested individually but in combination with the ethanol extract, their MICs decreased considerably i.e., 6 µg for tetracycline and 12.5µg for chloramphenicol, i.e., a 4 times decrease for tetracycline and 2 times decrease for chloramphenicol (Table 4 & 5). When we take the case of MDR *Pseudomonas aeruginosa*, there was a 16 times decrease in the MIC of the ethanol extract in combination with the antibiotics than when checked individually, and the MICs of both the antibiotics fell down to nanogram level which reveals the efficacy of the plant extract in improving the activity of the antibiotics (Table 4 & 5). This result is further strengthened by the fact that with MDR *Pseudomonas*, the chosen antibiotics and the plant extract reveal a synergistic interaction when used together, ie., we can hypothesize that the plant extract and the regular antibiotic dosage can be taken orally to make the antibiotic efficient in controlling certain bacterial infections. Nevertheless, with *E. coli*, chloramphenicol along with the extract revealed an additive effect only. From the FIC results it is clear that compared to *Pseudomonas*, *E. coli* seems to be difficult bacteria to control (Table 6).

3.3. Reversal of Resistance

Result of the reversal of tetracycline resistance was met with intermediate success as far as *E.coli* ESBL was concerned. At a concentration of 50µg/ml of tetracycline, *E.coli* showed intermediate sensitivity after 3 hours of incubation in the presence of 17mg/ml concentration of the ethanolic extract (Table 7). With MDR *Pseudomonas*, there was a clear reversal of resistance as seen in Table 8 even at time T0 and the sensitivity to the antibiotic increased after 3 hours of incubation of the culture with the plant extract even at a concentration of 8.5 mg/ml.

E. coli when tested with 17.5mg/ml concentration of plant extract for resistance reversal against chloramphenicol (Table 9) revealed intermediate sensitivity after 3 hours of incubation with the plant extract. *Pseudomonas* in this case, revealed complete reversal of resistance as seen in the (Table 10), with extract concentrations as low as 4.2 mg/ml. This clearly indicates that in the presence of the plant extract, the bacterial cultures can become more sensitive to the common antibiotics that are not in use today.

3.4. Bio Autography

The bio-autographic results clearly highlight the compounds with R_f values indicating inhibition by way of growth clearance (Table 11) as visualized as colour change in the indicator (Plate 1 & 2). We can go ahead in purifying the compounds and further study and compare their activities individually and in consortia. This method definitely saves time and labour for ear marking the active principles of any natural product. Our study results are quite unique in that the zones are clearer when compared to few other publications that we have with *E. hirta* L. [12] which do not show any photographic evidence of bioautography.

4. Conclusion

We can conclude that with more refinement in methodology and choice of antibiotics, we can hope to control gram negative bacterial infections caused by *E.coli* and *Pseudomonas*, when we include *E. hirta* L., extracts along with common antibiotics that have been replaced with much stronger and costlier ones in recent times.

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Preliminary Antimicrobial Study by Agar Well Diffusion Method:

Table-1. ZOI at various concentrations of cold aqueous extract

ESBL *E.coli*	MDR *Pseudomonas aeruginosa*	Concentration mg/ml
13mm	13mm	37 mg/ml
11mm	11mm	18.5 mg/ml
10mm	10mm	9.25 mg/ml
9mm	9mm	4.625 mg/ml
8mm	8mm	2.312 mg/ml

Table-2. ZOI at various concentrations of hot aqueous extract

ESBL *E.coli*	MDR *Pseudomonas aeruginosa*	Concentration mg/ml
13mm	13mm	57 mg/ml
11mm	11mm	28.5 mg/ml
10mm	10mm	14.25 mg/ml
9mm	9mm	7.125 mg/ml
8mm	8mm	3.562 mg/ml

Table-3. ZOI at various concentrations of ethanol extract

ESBL *E.coli*	MDR *Pseudomonas aeruginosa*	Concentration mg/ml
18mm	18mm	57 mg/ml
14mm	14mm	28.5 mg/ml
12mm	12mm	14.25 mg/ml
10mm	10mm	7.125 mg/ml
9mm	9mm	3.562 mg/ml

Table-4. MIC of ethanol extract and antibiotics individually

Organisms	Ethanol (90mg/ml)	Tetracycline (0.1mg/ml)	Chloramphenicol (0.1mg/ml)
ESBL *E.coli*	22.5	0.025	0.025
MDR *Pseudomonas*	5.625	0.0125	0.025

Table-5. MIC of ethanol extract and antibiotics in combination

Organisms	Ethanol (90mg/ml)	Tetracycline (0.1mg/ml)	Chloramphenicol (0.1mg/ml)
ESBL *E.coli*	5.625	0.00625	0.0125
MDR *Pseudomonas*	0.351	0.000390	0.000195

Table-6. Calculation of FIC index

Organisms	Tetracycline	Chloramphenicol
ESBL *E.coli*	0.5 synergistic	1 additive
MDR *Pseudomonas*	0.093 synergistic	0.038 synergistic

Table-7. Reversal of resistance - Tetracycline-50µg/ml, Organism- ESBL *E.coli*

Time in hours	Plant extract concentrations					
	17mg/ml		8.5 mg/ml		4.2mg/ml	
	Test	Control	Test	Control	Test	Control
T0	16mm	14mm	15mm	14mm	15mm	14mm
T1	16mm	13mm	15mm	13mm	15mm	13mm
T2	17mm	13mm	15mm	13mm	15mm	13mm
T3	17mm	13mm	15mm	13mm	15mm	13mm

Table-8. Reversal of resistance- Tetracycline 50µg/ml ,Organism- Multidrug resistant P*seudomonas aeruginosa*

Time In hours	Plant extract concentrations					
	17mg/ml		8.5 mg/ml		4.2mg/ml	
	Test	Control	Test	Control	Test	Control
T0	22mm	16mm	15mm	16mm	15mm	16mm
T1	25mm	16mm	21mm	16mm	20mm	16mm
T2	26mm	15mm	25mm	15mm	20mm	15mm
T3	27mm	15mm	25mm	15mm	20mm	15mm

Table-9. Reversal of resistance- Chloramphenicol 30µg/ml, Organism- ESBL *E.coli*

Time In hours	Plant extract concentrations					
	17mg/ml		8.5 mg/ml		4.2mg/ml	
	Test	Control	Test	Control	Test	Control
T0	14mm	13mm	13mm	13mm	10mm	13mm
T1	15mm	10mm	13mm	10mm	12mm	10mm
T2	15mm	10mm	15mm	10mm	13mm	10mm
T3	15mm	9mm	15mm	9mm	14mm	9mm

Table-10. Reversal of resistance-Chloramphenicol 30µg/ml, organism- Multidrug resistant *Pseudomonas aeruginosa*

Time In hours	Plant extract concentrations					
	17mg/ml		8.5 mg/ml		4.2mg/ml	
	Test	Control	Test	Control	Test	Control
T0	20mm	18mm	20mm	18mm	18mm	18mm
T1	28mm	15mm	25mm	15mm	20mm	15mm
T2	28mm	15mm	25mm	15mm	22mm	15mm
T3	30mm	12mm	25mm	12mm	22mm	12mm

Table-11. Chromatogram pattern of ethanolic extract of *E.hirta* L.inflorescence

Bioactive components	Rf values	Areas of clearance around the Rf values	
		ESBL *E.coli*	MDR *Pseudomonas*
A	0	+	+
B	0.46	+	+
C	0.53	+	+
D	0.65		+
E	0.69		
F	0.73		
G	0.76		
H	0.84	+	+
I	0.96	+	+

Plate-1. Bio - autogram for *E. coli* (direct contact method)

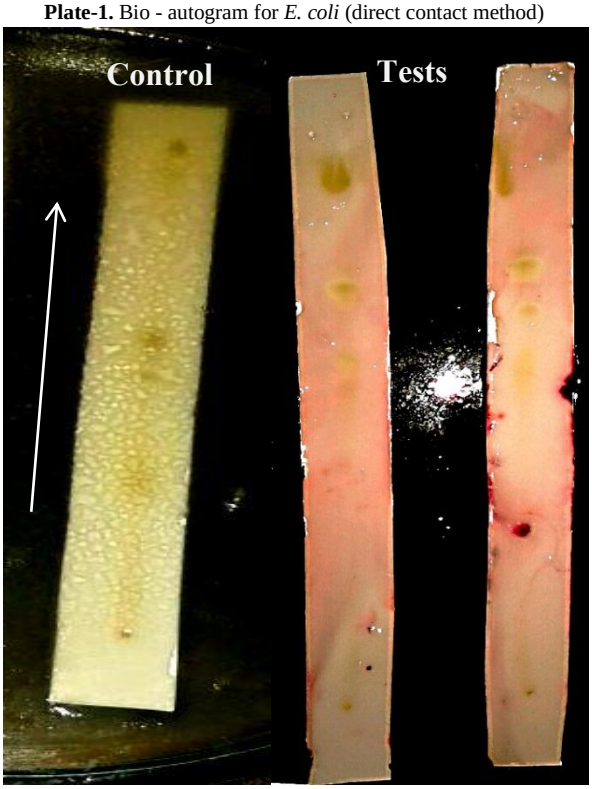

Plate-2. Bio-autogram for *Pseudomonas aeruginosa* (Contact method)