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Antimicrobial Activity of *Lantana Camara*. LINN on Super Bugs: By *Invitro* Method

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ABSTRACT

To investigate the antimicrobial activity of *lantana camara* Linn.on NDM 1strain by using in- vitro method. The antimicrobial effect of leaf extract of *lantana camara* Linn (LELC) alone and with Gentamicin ,Ceftriaxone were evaluated by using in vitro methods namely disc diffusion and agar dilution method. Well characterized and laboratory stocked strains of *Escherichia coli* (ATCC25922), *staphylococcus aureus* (ATCC 25923) and well characterized NDM-1 strain of *Klebsiella pneumonia* were used for the evaluation. The antimicrobial activity of LELC by zone of inhibition against NDM1 Strain (1.4mm), *E.Coli* (3.6mm),*S.aureus* (4.2 mm). The additive inhibitory effect of the LELC and gentamicin against *E.coli* (5.6 mm), LELC and ceftriaxone against *S.aureus* (5.8 mm). Significant inhibitory effect was established with LELC with gentamicin against NDM1 strain with zone of inhibition (2.3 mm). By agar dilution method the Minimum Inhibitory Concentration (MIC) of LELC against *E.Coli* (500mcg), *S.aureus* (500mcg), but when we combine LELC and gentamicin (500mcg+100mcg) it showed inhibition of NDM1 growth. The LELC showed antibacterial effect against NDM1 producing bacteria in addition to gram negative bacteria. Hence LELC has the potential for the development of an ideal and futuristic antimicrobial agent against NDM1 producing organisms.

Keywords: *Lantana camara* Linn, antimicrobial effect, NDM1 strain.

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INTRODUCTION

Antibiotics the greatest invention of modern medicine, may not act on life threatening infections in the present scenario where careless use of antibiotics ended up in drug resistant microorganisms [1]. Wherever any crisis goes out of our hand we normally pray to God Nature to help us, Like wise we believe and pray that the nature has the answer for the antibiotic resistance crisis. If we do not discover new antibiotics to tide over the antibacterial resistance we will end up in facing serious and incurable diseases.

Lantana Camara.L originally called as weed or sage is a notorious ornamental plant. The genus *Lantana camara* as per Linnaeus consists of six species from South America and one from Ethiopia. *Lantana camara* from the Latin *lento* means to bend. It derived from the ancient Latin name of the genus *Viburnum*.as it resembles a small in foliage and inflorescence [2]. Lantana camara.L commonly known as weed or red sage, unniceeti (Tamil), pulikampa (Telugu) and caturang (Hindi) is a significant weed commonly found throughout in India [3]. It is ever green strong smelling shrub, with stout recurred prickles, leaves opposite, ovate , acute or sub acute, crenate , scab rid on both side [4]. It is a woody straggling plant with various flower colours, red, pink, white, yellow and violet. The plants stems and branches sometimes are armed with prickles or spines. It was introduced in India as an ornamental plant in the garden but entirely grown and spread throughout India (3),it was identified as one of the potential medicinal plants of the world. The fruits are useful in fistula, pustules, tumors and rheumatism [5]. In Asian countries, leaves were used for the treatment of cuts, join pains,ulcers and also used as a vermifuge. Extracts were applied topically for leprosy and scabies [6].It has been studied that a steroid, lancamarone, from the leaves showed cardiotoxic properties [7].

This study was carried out to investigate the antibacterial activity of Lantana camara Linn leaves on NDM1 producing strains which was not adequately explored previously.

MATERIALS AND METHODS

After getting the Institutional ethical committee approval the study was conducted at the central research lab of Sree Balaji Medical college & Hospital, Bharath university, Chennai.

Plant material

Leaves of Lantana camara Linn were collected in March 2012 from the Theosophical society garden, Adyar , Chennai, South India. The plant was indentified and certified by the research officer (Pharmacognosy) at Siddha Central Research Institute Arumbakkam, Chennai-600 106, India.

Preparation of extracts

Sample of fresh leaves of Lantana camara Linn were prepared using cold extraction method. A total of 500grams of fresh leaves were placed in a flask containing cold ethanol and left for 72 hours at an ambient temperature. A rotary vacuum pump extractor will be used to remove the methanol from the extracts (under reduced pressure.80° C).The extract was weighed and stored under refrigeration at < 4° C until further testing [8].

Investigation of antibacterial activity

Fully characterized known NDM₁ strains of Klebsiella pneumonia and fully characterized, stocked strains of Escherichia coli (ATCC 25922), Staphylococcus aureus (ATCC 25923) were utilized for the study. The antibiotics used for the study were Inj. Gentamicin 80mg (Aristo Pharmaceuticals, Mumbai, India), Inj. Ceftriaxone 250mg (Ranbaxy Pharmaceuticals, Mohali, India).

Disc diffusion method

6 mm filter paper discs (Whatman, no. 3) were impregnated with 20ml of each of the effective concentration of Lantana camara leaf extract (100mg/ml), Gentamicin (40mg/ml), Ceftriaxone (50mg/ml) .The discs were kept at room temperature till all the diluents evaporates and kept in refrigeration till they are ready to be used. The bacterial inoculums is adjusted to certain concentration, inoculated onto the entire

surface of a Mueller-Hinton agar (MHA) plate with a sterile cotton-tipped swab to form an even lawn. The loaded discs were placed onto the surface of the agar. Paper discs impregnated with 20ml of DMSO used to dilute natural products are used as the control. Tests were performed in duplicate [9].

The paper discs impregnated with diluted antibiotic solutions of Ceftriaxone, Gentamicin and LELC extract were placed on the surface of each MHA plate using a sterile pair of forceps. Paper discs impregnated with DMSO was placed in all the culture plates separately to rule out any activity. Then the plates were incubated aerobically and the diameter of zone inhibition was measured by a ruler or caliper. Based on the diameter of the inhibition zone and the CLSI interpretative criteria [10], the results are then assigned in two categories such as susceptible or resistant. If the diameter of the inhibitory zone is bigger the susceptibility of the microorganism to the antibacterial agent is high.

Agar dilution method and Minimum inhibitory concentration (MIC)

MIC by Agar dilution Technique:

It is a quantitative method for determining the minimum inhibitory concentration of the antibacterial agent against a given organism. It is mainly useful in testing isolates from serious bacterial infections or to verify equivocal results (eg. intermediate susceptibility).

The required dilutions of the antibiotics are made as follows:

The stock solution was prepared using antibiotic or plant extract to be tested viz., Ceftriaxone 50mg/ml, Gentamicin 40mg/ml and LELC extract 100mg/ml of DMSO. 0.5ml of above solution+9.5ml distilled water to produce (stock solution of 2500 micro gram/ml & 2000 micro gram/ml & 5000microgram respectively as solution-A).

The ethanolic extracts were dissolved in distilled water and dimethyl sulfoxide (DMSO) and made into a concentration of 5000 µg/ml. Further serial dilutions will be performed by the system for preparing dilutions for agar dilution method given by CLSI [9] and minimum inhibitory concentration (MIC) will be determined. The minimum inhibitory concentration (MIC) was defined as the lowest extract concentration of Leaf extract of *Lantana camara* required to inhibit the bacterial growth by agar dilution test method.

Preparation of agar plate with different concentration of the antibiotic/extract [11]

It is prepared by dispensing 2ml of the diluted antibiotic/extract solution into each of the marked sterile screw capped bottle. It is advisable to start with highest dilution so that single pipette can be used to dispense all the dilutions prepared. Sterile Muller-Hinton agar is cooled and maintained at 50-55 deg C in a water bath. This medium (18ml) is poured into the screw capped bottle containing the different concentration of antibiotic, shaken well and poured into sterile petri dish. By this method exact volume of medium is delivered into the screw capped bottles without the danger of the molten agar jellifying during transfer into dilution of the antibiotic. Poured plates after setting can be kept at 4°C.

Procedure:

1. The plates must be dry before performing the test.
2. A grid is marked on the bottom of the plates containing antibiotics
3. 20-25 strains can be test in plate control.
4. The organisms to be tested is inoculated into peptone water and kept at 37° C for 3-4 hours.
5. Turbidity adjusted with 0.5 Mac Farmland's Standard.
6. A loop calibrated to deliver 0.001-0.002 (1-2 µl) of the culture is spot inoculated on the surface of the medium, indicated by the square marked below. In each case 10⁴ is delivered to a spot 5-8 mm in diameter.
7. Inoculation is done starting with the plates containing highest dilution of the antibiotic.
8. A control plate without antibiotics is simultaneously inoculated.
9. Allow the drops to dry and incubate the plates without inverting at 37⁰ C for 16-18 hours.

Antibacterial potentiating effect of *Lantana camara* leaf extract by using Disc diffusion method :

In order to evaluate the antibiotic potentiating effect of leaf extract of *Lantana Camara*. L the MIC of aminoglycosides (Gentamicin) and 3rd generation Cephalosporin (Ceftriaxone) against fully characterized stocked strains of gram negative bacteria (*Staphylococcus aureus* , *Escherichia coli* (ATCC), NDM strains were determined [12]. The paper discs impregnated with diluted antibiotic solutions of equal concentrations (100mg/ml) of LELC with Gentamicin 40mg/ml and Ceftriaxone 50mg/ml respectively were placed on the surface of each MHA plate using a sterile pair of forceps. Then the plates were incubated aerobically and the diameter of zone inhibition was measured by a ruler or caliper. Based on the diameter of the inhibition zone and the CLSI interpretative criteria, the results are then assigned and the antibacterial potentiating effect of LELC determined.

PCR Sequencing study [13] to determine the effect of *Lanata camara* Linn. on the NDM1 genes

The bacterial isolates with the known resistance pattern and fully characterized for the genes responsible multidrug resistance (NDM1) were exposed to the Leaf extract of *Lanata camara* Linn. Further, the isolates were checked for the change in the susceptibility pattern and analyzed for the changes responsible at the molecular level by using PCR.

RESULTS

The antibacterial properties of leaf extract of *lantana camara* Linn was demonstrated by using two methods viz., Disc diffusion method , agar dilution method.

Disc diffusion method :

- I. *Ecoli* shows zone of inhibition of 4.6mm for Gentamicin,5.1mm for Ceftriaxone, 3.6mm for LELC, 5.6mm for LELC+ Gentamicin and 5.6mm for LELC+Ceftriaxone (Table.1) indicating there is antimicrobial activity LELC , Which is enhanced when combined with gentamicin than with Ceftriaxone.(Fig.1&4)
- II. *Staph.aureus* shows zone of inhibition of 6.5mm, 5.6mm, 4.2mm,6.2mm and 5.8mm respectively (Table.1) indicating antimicrobial activity of LELC which is mildly enhanced when combined with gentamicin and Ceftriaxone.(Fig.2&5)
- III. NDM1 strain shows nil inhibition for Gentamicin, 1.7mm for Ceftriaxone ,1.4 mm for LELC, 2.3mm for LELC +Gentamicin and 1.2mm for LELC+Ceftr (Table.1) indicating antimicrobial activity of LELC which is enhanced when combined with gentamicin than with Ceftriaxone. (Fig.3&6) The enhanced activity of Gentamicin when combined to LELC shows its potentiating effect by overcoming the antimicrobial resistance of NDM1.

TABLES

1.Agar diffusion method

Organisms	Zone of inhibition (mm)				
	Gentamicin	Ceftriaxone	LELC	LELC+Gentamicin	LELC+Ceftriaxone
<i>E.Coli</i>	4.6	5.1	3.6	5.6	5.6
<i>Stap.aureus</i>	6.5	5.6	4.2	6.2	5.8
NDM1 <i>Kleb.pneumoniae</i>	nil	1.7	1.4	2.3	1.2

Table: 1 .Showing zone of inhibition of LELC alone and in combination with Gentamicin and Ceftriaxone

2. Agar dilution method

Organisms	MIC (µgm)				
	Gentamicin	Ceftriaxone	LELC	LELC+Gentamicin	LELC+Ceftriaxone
E.Coli	0.5	10	500	5+1	5+1
Stap.aureus	0.5	10	500	2.5+0.5	10+4
NDM1					
Kleb.pneumoniae	nil	nil	nil	500+100	nil

Table: 2 Shows antibacterial activity and minimal inhibitory concentration (MIC) by agar dilution method.

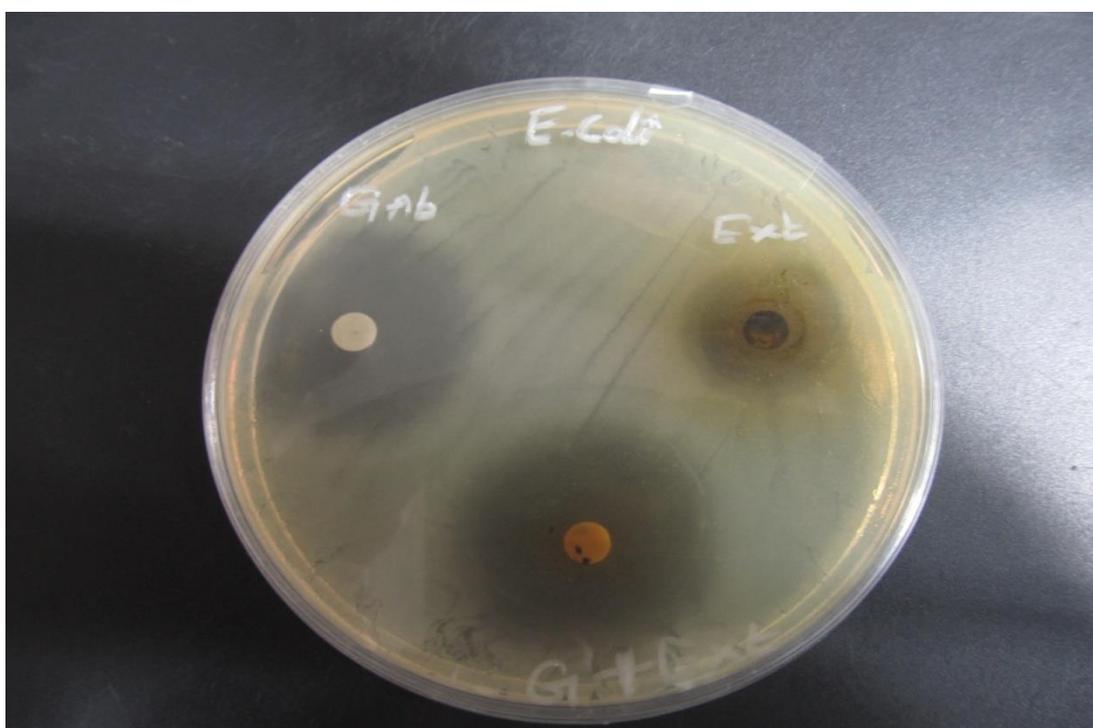


Fig.1 Disc diffusion method showing zone of inhibition of Gentamicin, LELC and Gentamicin with LELC in culture plate with E.coli .

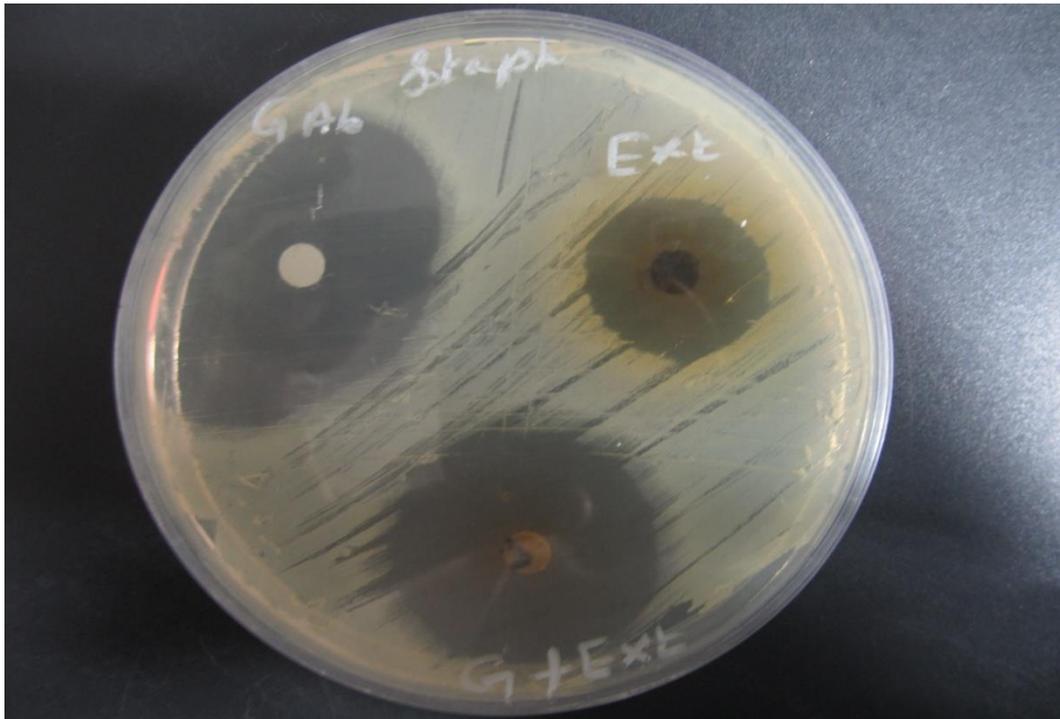


Fig.2. Disc diffusion method showing zone of inhibition of Gentamicin, LELC and Gentamicin with LELC in culture plate of *Staphylococcus aureus* .



Fig.3. Disc diffusion method showing zone of inhibition of Gentamicin, LELC, Gentamicin with LELC in NDM-1 strains of *Klebsiella pneumonia* culture plate .



Fig.4. Disc diffusion method showing zone of inhibition of Ceftriaxone, LELC and Ceftriaxone with LELC in culture plate with E.coli.



Fig.5. Disc diffusion method showing zone of inhibition of Ceftriaxone, LELC and Ceftriaxone with LELC in culture plate with staphylococcus aureus.



Fig.6. Disc diffusion method showing clear zone of inhibition of Ceftriaxone, LELC, and Ceftriaxone with LELC in the culture plate of NDM-1 strains with *Klebsiella pneumonia*



Fig.7. LELC showing minimum inhibition of *E. coli*, *Staphylococcus aureus* and nil inhibition of MDM strains by agar dilution method.



Fig.8. Agar dilution method showing inhibition of E.coli, Staphylococcus aureus but nil inhibition of NDM at 5000mcg concentration of LELC and Ceftriaxone.



Fig.9. Agar dilution method showing inhibition of E.coli, Staphylococcus aureus and also considerable inhibition of NDM1 strains at 500mcg LELC + 100mcg of gentamicin.

Agar dilution method:

- I. For E.Coli the minimum inhibitory concentration (MIC) of gentamicin 0.5mcg, Ceftriaxone 10mcg, LELC 500mcg, LELC(5mcg)+Gentamicin (1mcg), LELC (5mcg) +Ceftriaxone (1mcg) . (Table.2, Fig.7)
- II. Staph.aureus the minimum inhibitory concentration (MIC) of gentamicin 0.5mcg, Ceftriaxone 10mcg, LELC 500mcg, LELC(2.5mcg)+Gentamicin (0.5mcg), LELC(10mcg)+ Ceftriaxone (4mcg).(Table.2, Fig.8)
- III. NDM strain was not inhibited by gentamicin, Ceftriaxone, LELC alone but the combination of LELC(500mcg)+Gentamicin (100mcg) showed considerable inhibition where as the combination of LELC+Ceftriaxone did not show any inhibition. This clearly indicates the antimicrobial enhancing effect of Gentamicin +LELC.(Table.2, Fig.9).

DISCUSSION

The disc diffusion method clearly revealed the antibacterial effect of LELC against NDM1 strain, E.Coli, and Staph.aureus . In the case of NDM1 strains gentamicin alone showed no activity but when combined with LELC it showed enhanced antimicrobial activity where as the ceftriaxone showed mild activity against NDM1 strains but when combined with LELC there was no significant activity noted. This shows that gentamicin and LELC both may have similar mechanisms of antimicrobial activity which requires further research to find out the exact mode of action [14].

The agar dilution method showed the MIC of Gentamicin, Ceftriaxone against E.Coli, Staph.aureus, but none of them inhibited NDM1 strain growth, Whereas the combination of LELC (500mcg)+Gentamicin (500mcg) showed considerable inhibition of NDM1 strains. This finding again confirms the synergistic antibacterial effect of gentamicin and LELC [15]. Further studies are required to evaluate the mechanism of action [16] of LELC against resistant organisms like MDM1 strains of bacteria.

A research article titled "Inhibition of NDM-1 in superbugs by flavonoids- an Insilico Approach" [17] which demonstrated a bioinformatics method and found out a suitable chemical structure. It predicted by using three docking softwares and suggested that the flavonoid [18] Quercetin and its analog penta-O-ethylquercetin are potential inhibitors of NDM-1. BAPTA (1,2-bis-o-aminophenoxy) ethane-N,N',N'-tetra acetic acid) having higher affinity towards zinc and showed best inhibition activity against NDM -1 strains. Hence a flavonoid Quercetin [19] with a zinc chelating agent could be an ideal antimicrobial agent in the present scenario to inhibit the activity of NDM strains . Based on this we attempted to evaluate the activity of chelating agent EDTA with our compound LELC against NDM1 strains by disc diffusion method .To our surprise EDTA [20] alone showed inhibition of NDM1 strains and when EDTA combined [21,22] with LELC it showed enhanced inhibition of NDM1 strains,. Further detailed research is mandatory to establish the antimicrobial effect of the above compounds against the most threatening super bugs [23,24].

CONCLUSION

Disc diffusion as well as agar dilution invitro methods demonstrated the antimicrobial activity of LELC especially against NDM1 strains where as the combination of Gentamicin +LELC showed more antimicrobial activity than Ceftriaxone + LELC. This small finding throws some light on the natural product like LELC which may have the potential to overcome the threat posed by the highly resistant microbes such as the NDM1 strains. Further in depth research is required to establish the effectiveness of LELC especially against highly resistant microbes. Future ideal therapeutic agent to curb the emerging super bugs would be a combination of a plant product like LELC with a zinc chelating agent.

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