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Research Article

# ANTIBACTERIAL EFFECT OF LANTANA CAMARA LINN. ON GRAM NEGATIVE BACTERIA AND NDM-1 STRAIN: AN INVITRO STUDY

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**ABSTRACT**

Objective: To evaluate the antibacterial effect of lantana camara Linn. on gram negative bacteria and NDM-1 strain by in vitro method.

Methods: The antibacterial activities of leaf extract (LELC) of lantana camara alone or in combination with Gentamicin, Ceftrioxone were determined by in vitro study namely disc diffusion and agar dilution methods. Fully characterized, stocked strains of Gram negative bacteria of Escherichia coli (ATCC25922), staphylococcus aureus (ATCC 25923) and fully characterized known NDM-1 strains of Klebsiella pneumonia were used for the study.

Results: The results showed the inhibitory activity of LELC against E.Coli (3.5mm), S.aureus (4 mm), and NDM1 Strain (1.2mm). The synergestic effect was observed by LELC and gentamicin (5.5 mm) against E.coli, LELC and ceftrioxone (6 mm) against S.aureus. Notable effect was obtained with LELC with gentamicin against NDM1 strain with zone of inhibition (2.2 mm). In the agar dilution method MIC of LELC against E.Coli ( 500mcg), S.aureus (500mcg ), but the combination of LELC and gentamicin only showed MIC of (500mcg+100mcg ) against NDM1.

Conclusion: The LELC showed antibacterial activity against gram negative bacteria especially the NDM1 producing bacteria which may be developed as a future antimicrobial agent against resistant gram negative organisms.

**Keywords:** Lantana camara, antibacterial effect, gram negative bacteria, NDM1 strains

## INTRODUCTION

Antibiotics once hailed as the bedrock of modern medicine, may not work on infections in the present scenario where indiscriminate use of antibiotics resulted in drug resistant microorganisms (1). Whenever any problem goes out of our hand we used to pray to God nature to help us, similarly we believe that the nature has the answer for the antibiotic resistance also. If we do not develop new antibiotics to deal with the problems of resistance we will end up in serious trouble.

Lantana Camara.L commonly known as weed or red sage is described as a notorious as well as an ornamental plant. The genus *Lantana camara* (Verbenaceae) as described by Linnaeus in 1753 contained seven species, six from South America and one from Ethiopia. *Lantana camara* from the Latin *lento* means to bend. Probably derives from the ancient Latin name of the genus *Viburnum* which it resembles a little 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 or ovate –oblong, acute or sub acute, crenate -serrate, scab rid on both side (4). It is a woody straggling plant with various flower colors, red, pink, white, yellow and violet. The stems and branches are sometimes armed with prickles or spines. It was introduced in India as an ornamental plant but entirely naturalized and found throughout India3, it was listed one of the important medicinal plants of the world. The fruits are useful in fistula, pustules, tumors and rheumatism (5). In Asian countries, leaves were used to treat cuts, rheumatisms, ulcers and as a vermifuge. Decoctions were applied externally for leprosy and scabies. It has been claimed that a steroid, lancamarone, from the leaves exhibited cardiotonic properties (6).

This study was undertaken to investigate the antimicrobial activity of Lantana camara. Linn leaves on gram negative organisms especially the NDM1 producing strains which were not explored previously.

## MATERIALS AND METHODS

The study was approved by the Institutional ethical committee and 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) Siddha Central Research Institute Arumbakkam, Chennai-600106, 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 was used to remove the methanol from the extracts (under reduced pressure 80 $^{o}$ C).The extract was weighed and stored under refrigeration at < 4$^{o}$ C until further testing (7).

### Investigation of antibacterial activity

Fully characterized, stocked strains of Gram negative bacteria Escherichia coli (ATCC 25922), staphylococcus aureus (ATCC 25923) and fully characterized known NDM$_1$ strains of *Klebsiella pneumonia* were used for the study. The antibiotics used for the study were Inj.Gentamicin 80mg (Aristo Pharmaceuticals, Mumbai, India), Inj.Ceftrioxone 250mg (Ranbaxy Pharmaceuticals, Mohali, India).

### Disc diffusion method

6 mm filter paper discs (Whatman, no. 3) were impregnated with 20 ml of each of the effective concentration of *Lantana camara* leaf extract (100mg/ml), Gentamicin(40mg/ml), Ceftrioxone (50mg/ml).

The discs were allowed to remain at room temperature until complete diluents evaporation and kept under refrigeration until ready to be used.

The bacterial inoculum is adjusted to certain concentration, and inoculated on to 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 20 ml of diluents used to dilute natural products were used as control. Tests were performed in duplicate (8).

The paper discs impregnated with diluted antibiotic solutions of Ceftrioxone,Gentamicin and LELC extract were placed on the surface of each MHA plate using a sterile pair of forceps. A Paper disc 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 inhibiton zone and the CLSI interpretative criteria (9), the results are then assigned in two categories such as susceptible or resistant. The bigger the diameter of the inhibition zone, the more susceptible is the microorganism to the antimicrobial.

**Agar dilution method. 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 (e.g. intermediate susceptibility).

The required dilutions of the antibiotics are made as follows:

The stock solution was prepared using antibiotic/plant extract to be tested viz., Ceftrioxone 50mg/ml, Gentamicin 4omg/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 methanolic extract was 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 (8) 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**

Dispense 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 $4^0$C.

**PROCEDURE**

- The plate must be dry before performing the test.
- A grid is marked on the bottom of the plates containing antibiotics
- 20-25 strains can be test in plate control.
- The organisms to be tested is inoculated into peptone water and incubated at 37° C for 3-4 hours.
- Turbidity adjusted with 0.5 Mac Farmland's Standard.
- 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 $10^4$ is delivered to a spot 5-8 mm in diameter.
- Inoculation is done starting with the plates containing highest dilution of the antibiotic.
- A control plate without antibiotics is simultaneously inoculated.
- Allow the drops to dry and incubate the plates without inverting at $37^0$ 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 LELC the MIC of aminoglycosides (Gentamicin) and 3rd generation Cephalosporin(Ceftrioxone) against fully characterized stocked strains of gram negative bacteria ( Staphylococcus aureus, Escherichia coli (ATCC), NDM strain were determined (10). The paper discs impregnated with diluted antibiotic solutions of equal concentrations (100mg/ml) of LELC with Gentamicin 40mg/ml and Ceftrioxone 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 inhibiton zone and the CLSI interpretative criteria, the results were then assigned and the antibacterial potentiating effect of LELC was determined.

**PCR Sequencing study 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 LELC. 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**

**Agar diffusion method**

**Table: 1.Showing zone of inhibition of LELC with and without combination of Gentamicin and Ceftrioxone.**

| | Zone of inhibition (mm) | | | | |
|---|---|---|---|---|---|
| **Organisms** | **Gent** | **Ceftr.** | **LELC** | **LELC+Gent** | **LELC+Ceftr.** |
| E.Coli | 4.5 | 5 | 3.5 | 5.5 | 5.5 |
| Stap.aureus NDM1 | 6 | 5.5 | 4.5 | 6 | 6 |
| Kleb.pnem. | nil | 1.6 | 1.7 | 2.2 | 1 |

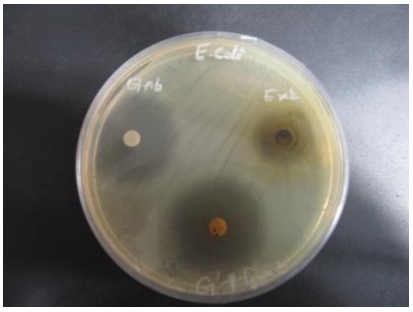


**Fig.1: Disc diffusion method showing zone of inhibition of Gentamicin, LELC, Gentamicin with LELC in E.coli culture plate.**

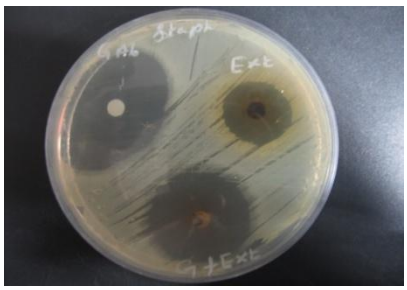


**Fig.2.Disc diffusion method showing zone of inhibition of Gentamicin, LELC, Gentamicin with LELC in Staphylococcus aureus culture plate.**

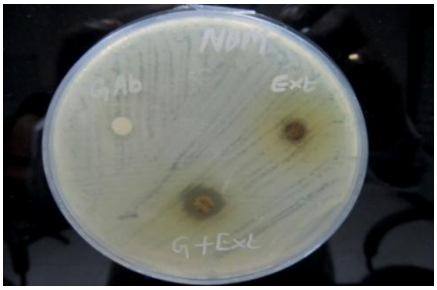


**Fig.3: Disc diffusion method showing zone of inhibition of Gentamicin, LELC, Gentamicin with LELC in NDM-1 strain of Klebsiella pneumonia culture plate.**


**Fig.4: Disc diffusion method showing zone of inhibition of Ceftrioxone, LELC, Ceftrioxone with LELC in E.coli culture plate.**

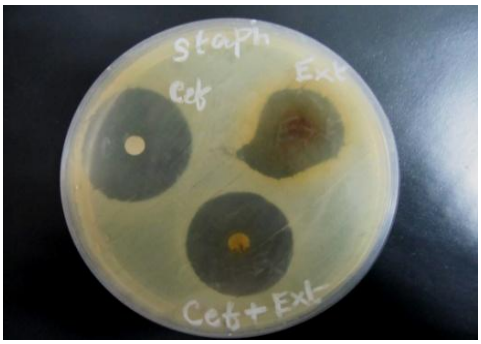


**Fig.5: Disc diffusion method showing zone of inhibition of Ceftrioxone, LELC, Ceftrioxone with LELC in Staph.aueus culture plate.**

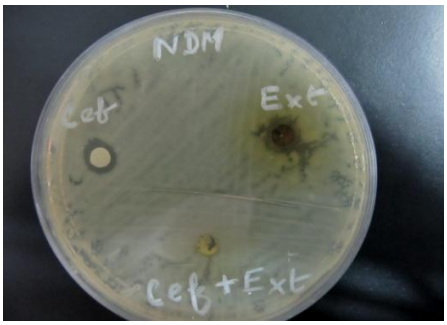


**Fig.6: Disc diffusion method showing zone of inhibition of Ceftrioxone, LELC, Ceftrioxone with LELC in NDM-1 strain of Klebsiella pneumonia culture plate.**

**Table2: Investigation of antibacterial activity and minimal inhibitory concentration (MIC) by agar dilution method.**

| Organisms | MIC (µgm) | | | | |
|---|---|---|---|---|---|
| | Gent. | Ceftr. | LELC | LELC+Gent. | LELC+Ceftr |
| E.Coli | 0.5 | 10 | 500 | 5+1 | 5+1 |
| Stap.aureus | 0.5 | 10 | 500 | 2.5+0.5 | 10+4 |
| NDM1 | | | | | |
| Kleb.pnem. | nil | nil | nil | 500+100 | Nil |


**Fig.7: LELC showing minimal inhibition of Ecoli, staphylococcus aureus and no inhibition of MDM strains by agar dilution method.**

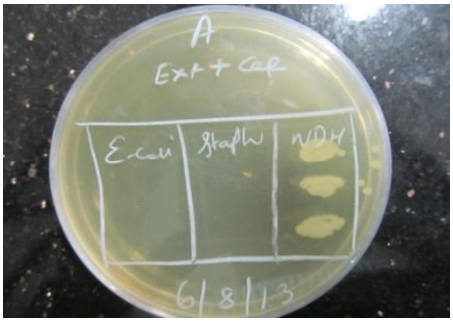


**Fig.8: Agar dilution method showing inhibition of E.coli, Staphylococcus aureus but no.inhibition of NDM at 5000mcg concentration of LELC and Ceftrioxone.**

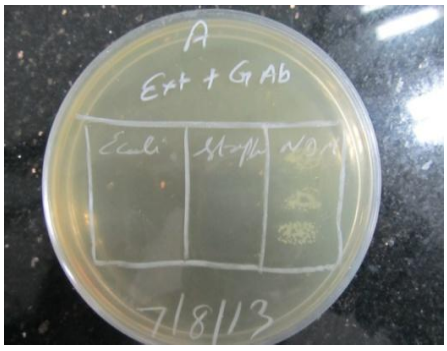


**Fig.9.Agar dilution method showing inhibition of E.coli, Staphylococcus aureus but there is mild inhibition of NDM at 500mcg LELC and 100mcg of gentamicin.**

The antimicrobial properties of leaf extract of lantana camara were demonstrated by using two methods Viz., Disc diffusion and agar dilution methods.

**Disc diffusion method**

- Ecoli shows zone of inhibition of 4.5mm for Gentamicin,5mm for Ceftrioxone, 3.5mm for LELC, 5.5mm for LELC+ Gentamicin and 5.5mm for LELC+Ceftrioxone (Table.1) indicating there is antimicrobial activity LELC which is enhanced when combined with gentamicin than with Ceftrioxone.( Fig.1&4)
- Staph.aureus shows zone of inhibition of 6mm, 5.5mm, 4.5mm,6mm and 6mm respectively (Table.1)indicating antimicrobial activity of LELC which is mildly enhanced when combined with gentamicin and Ceftriozone.(Fig.2&5)
- NDM strain shows nil inhibition for Gentamicin and 1.6mm for Ceftrioxone ,1.7 mm for LELC, 2.2mm for LELC +Gentamicin and 1mm for LELC+Ceftr (Table.1) indicating antimicrobial activity of LELC which is enhanced when combined with gentamicin than with Ceftriozone. (Fig.3&6)
- The enhanced activity of Gentamicin when combined to LELC shows its potentiating effect by overcoming the antimicrobial resistance of NDM.

**Agar dilution method**

- For E.Coli the minimum inhibitory concentration(MIC) of gentamicin 0.5mcg, Ceftrioxone10mcg, LELC 500mcg, LELC(5mcg) + Gentamicin(1mcg), LELC(5mcg) + Ceftrioxone (1mcg). (Table.2, Fig.7)
- ii)Staph.aureus the minimum inhibitory concentration (MIC) of gentamicin 0.5mcg, Ceftrioxone10mcg, LELC 500mcg, LELC(2.5mcg) + Gentamicin (0.5mcg), LELC (10mcg) + Ceftrioxone (4mcg). (Table.2, Fig.8)
- NDM strain was not inhibited by gentamicinl,Ceftrioxone, LELC alone but the combination of LELC(5ooomcg)+Gentamicin (100mcg) showed considerable inhibition where as the combination of LELC+Ceftrioxone 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 demonstrated the antimicrobial activity of LELC against E.Coli, Staph.aureus and especially against NDM1 strains. Gentamicin alone shows no activity against NDM1 strains but when combined with LELC it shows enhanced antimicrobial activity. Ceftrioxone shows mild activity against NDM1 strains but when combined with LELC there was no activity observed. This clearly shows that gentamicin and LELC both may have similar antimicrobial activity against NDM1 strains which requires further research to know the exact mode of action (11).

The agar dilution method shows the MIC of Gentamicin, Ceftrioxone against E.Coli, Staph.aureus. but none of them showed inhibition of NDM1 strain growth except LELC (500mcg)+Gentamicin (500mcg) which showed considerable inhibition of NDM1 strains. This finding again confirms the synergistic antibacterial effect of gentamicin and LELC (12). Further studies are required to evaluate the mechanism of action of LELC against resisitant oraganisms like MDM1 strains.

A research article titiled 'Inhibition of NDM-1 in superbugs by flavonoids- and Insilico Approach' (13) demonstrated a bioinformatics method by using three docking softwares and suggested that the flavonoid Quercetin and its analog penta-O-ethylquercetin are potential inhibitors of NDM-1. BAPTA (1,2-bis-o-aminophenoxy) ethane-N,N,N',N'-tetra acetic acid) having higher affinity towards zinc and showed best inhibition activity against NDM -1 strains. Hence a flavonoid (14) Quercetin with a zinc chelating agent could be an ideal antimicrobial agent in the present scenario to inhibit the activity of NDM1 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 alone showed inhibition of NDM1 strains and when combined with LELC it showed enhanced antimicrobial activity. However further detailed research is mandatory to establish the antimicrobial effect of the above compounds against the most threatening super bugs (15,16).

**CONCLUSION**

Both disc diffusion and agar dilution methods demonstrated the antimicrobial activity of LELC especially against NDM1 strains where as the combination of Gentamicin +LELC showed more antimicrobial activity than Ceftrioxone + LELC. This small attempt throws some light on the fact that the natural products like LELC have the potential to overcome the antimicrobial resistance of highly resistant microbes like the NDM1 strains. Further in depth research is required to establish the exact mechanism of action 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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