

Original Research Article

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Increased ROS Generation: Implication in Antibacterial Activity of *Evolvulus nummularius* against Multidrug Resistant Gram Negative Bacterial Strains

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ABSTRACT

Keywords

Evolvulus nummularius, Gram negative bacteria, MDR strains, Growth curve, ROS.

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Evolvulus nummularius, is ethnomedicinally used as a medicine for hysteria, to cure burns, cuts, wounds and scorpion stings. This plant has antihelminthic activity, wound healing activity, poor sedative and anticonvulsant properties. In this study, the antibacterial activity of crude methanol extract of *E. nummularius* was studied against three gram negative multidrug resistant strains *E. aerogenes*, *P. aeruginosa* and *K. pneumoniae*. This plant extract showed both bacteriostatic and bactericidal activity against all these bacterial strains. The extract was most active against *E. aerogenes*. In growth analysis study, it was obtained that, after treatment with IC₅₀ dose for each bacterial strains, the lag phase become extended compared to untreated bacterial cells. To understand the mechanism of action of *Evolvulus nummularius* the reactive oxygen species (ROS) was estimated and result showed that, reactive oxygen species were increased (~ 35-60%) in presence of IC₅₀ dose of *Evolvulus nummularius*.

Introduction

The frequency of infections by pathogenic microorganisms has increased worldwide and is an important factor of morbidity and mortality in developing countries (Al-Bari *et al.*, 2007).

As the number of multidrug resistant bacterial strains are increasing day-by-day, so, the identification of new infection-fighting strategies is necessary (Zy *et al.*, 2005, Rojas *et al.*, 2006). Therefore, the demand for plant based therapeutics in both

developed and developing countries is increasing.

Tripura, small state of the north-eastern region of India is rich in biodiversity with vast resource of medicinal plants (Deb, 1983; Das *et al.*, 2009; Roy *et al.*, 2010). *Evolvulus nummularius* has antihelminthic activity (Dash *et al.*, 2003), wound healing activity (Saini *et al.*, 2007), poor sedative and anticonvulsant properties (Chitralkha *et al.*, 1964). Phytochemical analysis of aerial parts

of *E. nummularius* revealed that, it had three new compounds, 1-3 along with β -sitosterol and its glucoside, stigmasterol, *d*-mannitol, ursolic acid and oleanolic acid (Dinda *et al.*, 2007).

Aerobic bacteria use molecular oxygen (O_2) for respiration or oxidation of nutrients to obtain energy. During the whole life cycle bacterial species are remains in continuous contact with reactive oxygen species (ROS) generated by endogenously, as a product of aerobic metabolism, or exogenously during ionizing and nonionizing (UV) irradiation, that produces number of radical and peroxide species through the ionization of intracellular water (Cabiscol *et al.*, 2000; Lucana *et al.*, 2012). Reactive by-products of oxygen, such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals ($\cdot OH$), are generated continuously in cells grown aerobically (Cabiscol *et al.*, 2000). These species cause damage to proteins, lipids, and nucleotides, negatively impacting the organism (Lucana *et al.*, 2012). Therefore, in this study we will examine the antibacterial activity of of *Evolvulus nummularius* in MDR strains with mechanism of action by estimating the increased percentage of ROS in presence and absence of methanol extract of *Evolvulus nummularius*.

Materials and Methods

Preparation plant extract

Fresh whole plant of *Evolvulus nummularius* was collected from Suryamaninagar, Tripura. After washing with water this plant materials were allowed to dry in shade. Then whole plants were cut in to small pieces. Then 100 gm of powdered plant materials were soaked in 500 ml of methanol and kept in a shaker for 48 hours. After that the solution was filtered through Whatman filter paper no. 1

for 3 times. Then these solutions were dried in rotary evaporator at 70°C (Nicolic *et al.*, 2012). 100 mg of dried extract was dissolved in 1ml of distilled water and filtered by a 0.22 μm syringe filter and stored at -20°C.

Bacterial Culture and growth condition

MDR Strains *klebsiella pneumonia* (ATCC BAA-1705), *Pseudomonas aeruginosa* (ATCC 10145), *Enterobacter aerogenes* (ATCC 13048) were grown, cultured and maintained on Muller Hinton Broth and stored at 4°C (Bhattacharya *et al.*, 2012). For long time storage 15% glycerol solution was used and vial was stored at -80°C.

Bacterial Sensitivity Testing

All three bacterial strains were cultured on Muller Hinton broth for overnight. Then 2 ml of bacterial culture was poured into the Muller Hinton agar plate, incubated for 5 minutes and excess amount of culture was pipette out from each plate. After that, 8 different antibiotic discs (purchased from HIMEDIA) were kept on respective zone of Muller Hinton agar plate and incubated for overnight at 37°C.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined by serial dilution technique, with an inoculum of 10^6 CFU/ml of both Gram positive and Gram negative bacteria in separate 96 well plate, in presence of increasing concentrations of MEEN. The bacterial cultures were incubated at 37°C and shaken at 200 rpm for 24 hours. Then the bacterial cell viability was determined by measuring the OD value at 600 nm. Here, MEEN with media, used as blank; media MEEN and bacterial culture, used as experiment; media with bacterial culture and distilled water, used as positive control; and

media with only distilled water, used as negative control (Demetrio *et al.*, 2015). Then, % of Inhibition was calculated by following formula,

$$\% \text{ of Inhibition} = [1 - \{(\text{Exp.} - \text{Blank}) / (\text{Positive Control} - \text{Negative Control})\}] * 100]$$

Determination of Minimum Bactericidal Concentration (MBC)

After determining the MIC values, MBCs for each bacterial strains were determined by treating the bacteria with 3 different doses, IC₅₀, IC₁₀₀ and >IC₁₀₀ dose. After incubation with these 3 doses, one loop full bacterial culture from each tube was streaked on Muller Hinton agar plate in respective zone and again these plates were incubated at 37 °C for overnight. IC₁₀₀ value indicates the concentration which inhibits 100% of bacterial growth, whereas, MBC value indicates the concentration at which a drug can kill the bacterial species (Demetrio *et al.*, 2015).

Growth Kinetics Studies

To determine the bacterial growth kinetics, in presence of MEEN, each bacterial species were grown in Muller Hinton Broth in presence and absence of MEEN separately, at 37 °C at 200 rpm for 12 hours. Here, bacterial cells were treated with respective IC₅₀ dose. Then, the bacterial concentration in presence and absence of MEEN were determined by measuring the OD at 600 nm in every 1 hour interval. Bacterial growth kinetics was plotted graphically with time versus OD₆₀₀ (Bhattacharya *et al.*, 2012).

Estimation of Reactive Oxygen Species (ROS)

0.1 ml of each bacterial suspension (where OD₆₀₀ = 1.0) in Hank's balanced salt solution (HBSS) was incubated with respective IC₅₀

dose of MEEN for 3 hours with 15 min interval at 37 °C. Then 500 µl of 1 mg/ml NBT was added and again incubated for 30 min at 37 °C. After incubation, 0.1 (M) HCl was added and tubes were centrifuged at 3000 rpm for 10 min. The pellets were treated with 0.6 µl of DMSO to extract the reduced NBT. Then, 0.5 µl of HBSS was added and OD was measured at 575 nm (intracellular ROS) (Pramanik *et al.*, 2012).

Statistical Analysis

We repeated these experiments for 3 times and data were expressed by calculating the standard deviation of all 3 experiments. ANOVA single factor (using Microsoft Office Excel) was used to determine statistical significance for multiple comparisons. *P* < 0.05 was accepted as statistically significant.

Results and Discussion

Bacterial sensitivity testing

The sensitivity these 3 bacterial strains were evaluated by using 8 different antibiotic discs. All these strains were resistant to more than 3 antibiotics. As shown in Fig 1 and Table 1 *P. aerugens* was resistant to ampicillin, tetracycline and penicillin-G, whereas, *E. aerogenes* and *K. pneumoniae* were resistant to ampicillin, ciprofloxacin, chloramphenicol, rifampicin and penicillin-G. Therefore, these three bacterial strains are multidrug resistant strains.

Determination of Minimum Inhibitory Concentration (MIC)

Antibacterial activity of MEEN against MDR strains were obtained by determining the minimum inhibitory concentrations. As shown in table 1 and Fig 1, the growth of *E. aerogenes* was inhibited completely at lower

concentrations of MEEN (2.5 mg/ml), but growth of *K. pneumoniae* were completely inhibited at too higher concentration of MEEN (10 mg/ml). The order of observed sensitivity on 3 different bacterial strains were, *E. aerogenes* > *P. aeruginosa* > *K. pneumoniae*.

Determination of MBC

Minimum bactericidal concentration of MEEN on each bacterial strain was also determined, shown in Fig. 2A and 2B. Table 2 and 3 showed that, the ratio between MBC and MIC for each bacterium is same (~1, for all bacteria). This result indicated that, MEEN is a bactericidal agent. It not only

inhibits the bacterial growth but also can kill multi drug resistant bacterial strains.

Bacterial Growth Kinetics Studies

We next measured the growth curve of both gram negative and gram positive bacterial strains to examine whether MEEN has any effect on growth pattern of each bacterium. All three bacterial strains were exposed to MEEN separately, at a concentration of IC₅₀ dose for each bacterium. As shown in Fig 3A and 3B, the lag phase of all MEEN treated bacteria were extended compared to control. Among all these bacteria, the growth curve of *E. aerogenes* is mostly affected by MEEN.

Table.1 Effect of different antibiotics on gram negative multidrug resistant bacterial strains. This data is significant at a level of p < 0.05

Bacteria \ Antibiotics	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>E. aerogenes</i>
Streptomycin	+	+	+
Ampicillin	-	-	-
Tetracyclin	+	-	+
Chloramphenicol	-	+	-
Rifampicin	-	+	-
Gentamycin	+	+	+
Copprofloxacin	-	+	-
Penicillin G	-	-	-

“+” = Sensitive; “-” = Resistant

Table.2 MIC values for gram negative MDR bacterial strains. This data is significant at a level of p < 0.05.

Bacteria	IC ₅₀ (mg/ml)	IC ₁₀₀ (mg/ml)
<i>K. pneumoniae</i>	5 ± 0.38	10 ± 0.45
<i>P. aeruginosa</i>	2.5 ± 0.17	5 ± 0.34
<i>E. aerogenes</i>	1.25± 0.32	2.5± 0.32

Table.3 MBC values for gram negative bacterial species

Gram (-) Bacteria	MBC	IC ₁₀₀ /MBC
<i>K. pneumonia</i>	10 mg/ml	1
<i>P. aeruginosa</i>	5 mg/ml	1
<i>E. aerogenes</i>	2.5 mg/ml	1

Fig 1 Muller Hinton Agar Plate showing antibiotic sensitivity of Gram negative Bacteria (A, B) *K. pneumoniae*, (C, D) *P. aeruginosa*, (E) *E. aerogenes*

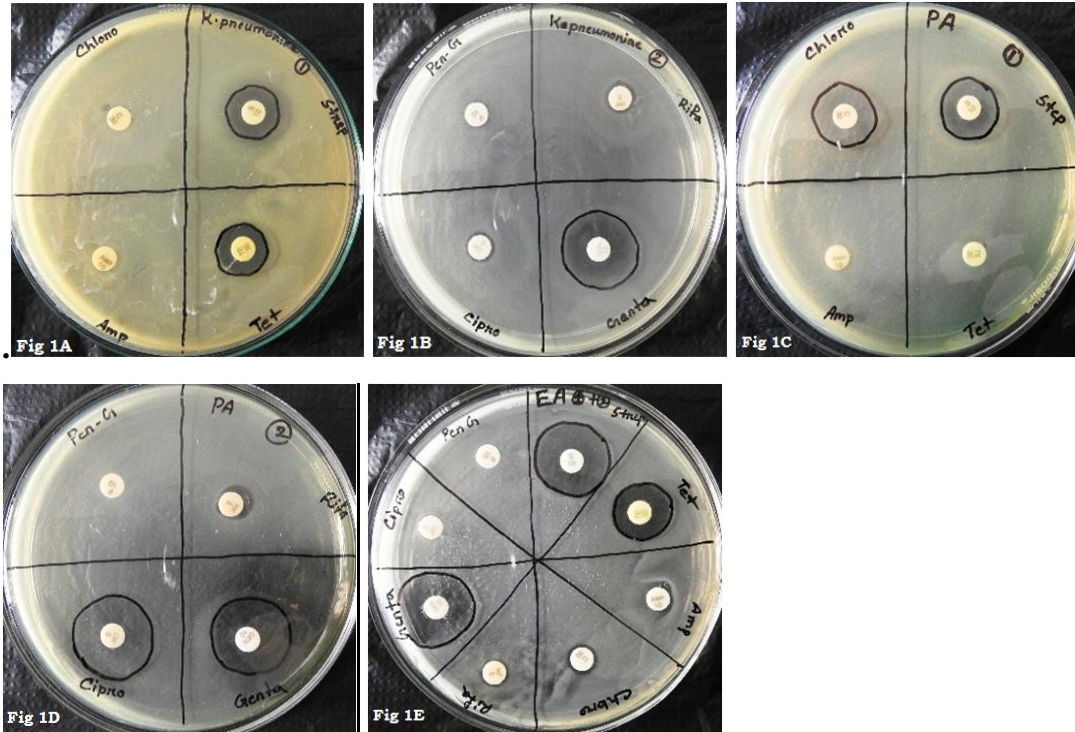


Fig 2 Column diagram IC100 dose of MEEN

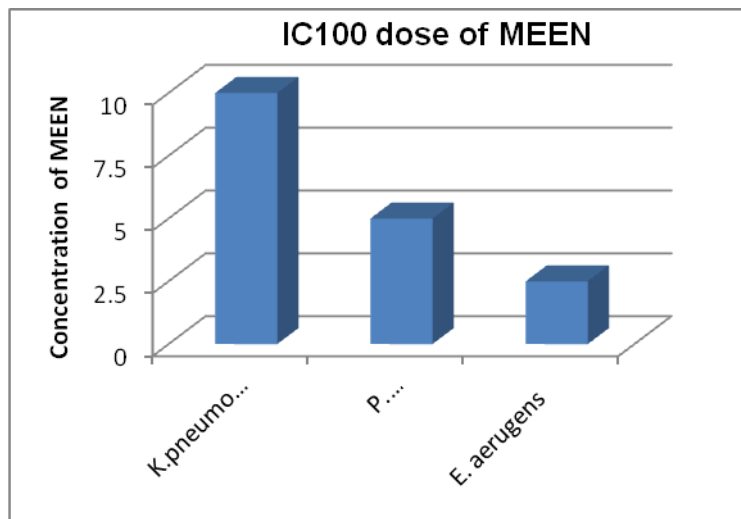


Fig 3 Muller Hinton Agar Plate showing MBC of Gram negative Bacteria
 (A) *K. pneumoniae*, (B) *P. aeruginosa*, (C) *E. aerogenes*

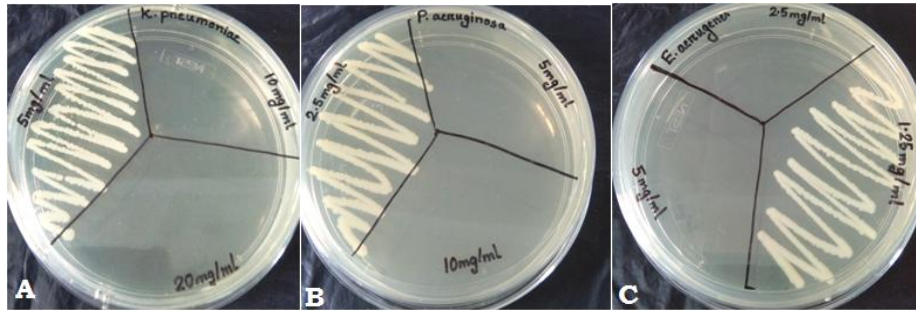


Fig 4 Growth curve of MDR strains in presence and absence of MEEN. This data is significant at a level of $p < 0.05$

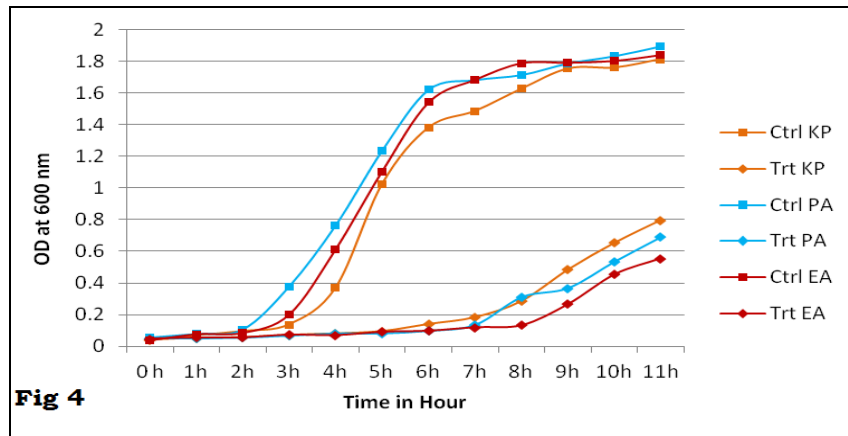
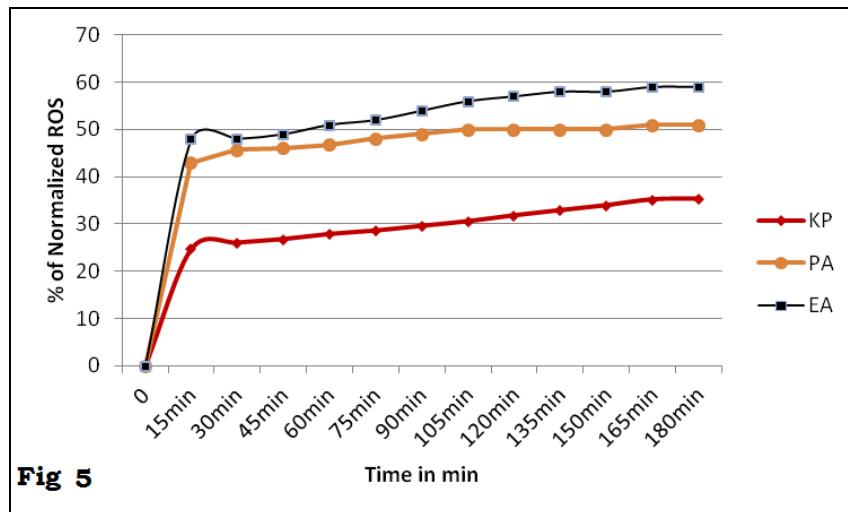


Fig 5 Normalized % of ROS produced by gram negative MDR bacterial strains in presence of MEEN. This data is significant at a level of $p < 0.05$



Estimation of ROS

Finally, to understand the mechanism of antibacterial activity of MEEN, intracellular reactive oxygen species (ROS) was estimated after treatment with MEEN at IC₅₀ dose.

As shown in Fig 4A and 4B, after treatment of MEEN, the production of ROS was increased drastically with time. It was highest in *E. aerogenes*, in which ROS production increased about 60% in 3 hours compared to control. The order of observed ROS production on 3 MDR strains were, *E. aerogenes* > *P. aeruginosa* > *K. pneumoniae*.

In present study the effect of *Evolvulus nummularius* on generation of ROS in MDR strains were evaluated to explore the mode of action of antibacterial activities of this plant. The growth of gram negative multidrug resistant bacterial strains were inhibited completely at lower concentrations of MEEN (2.5 -10 mg/ml). The IC₁₀₀ dose of MEEN for *E. aerogenes*, *P. aeruginosa* and *K. pneumoniae* were 2.5mg/ml, 5mg/ml and 10mg/ml respectively. Minimum bactericidal concentration (MBC) of MEEN on each bacterial strain was also determined. The result showed that, the ratio between MBC and MIC for each bacterium is same (~1, for all bacteria). Therefore, MEEN showed both bacteriostatic and bactericidal activity against multidrug resistant strains of bacteria.

For growth kinetics studies, all the bacterial strains were exposed to MEEN separately, at a concentration of IC₅₀ dose for each bacterium and the lag phase of all MEEN treated bacteria were extended compared to control. Among all these bacteria, the growth curve of *E. aerogenes* is mostly affected by MEEN.

Finally, to understand the mechanism of antibacterial activity of MEEN, intracellular

reactive oxygen species (ROS) was estimated after treatment with MEEN at IC₅₀ dose. After treatment of MEEN, the production of ROS was increased drastically with time. It was highest in *E. aerogenes*, in which ROS production increased about 60% in 3 hours compared to control. The range of increased ROS production in MDR strains was about 35% to 60%. This was the sufficient concentration to kill the bacterial strains.

Therefore, the crude methanol extract of *Evolvulus nummularius* may be used as a potent source of antibacterial agent because, MEEN was effective against MDR strains, which are resistant to commercially available antibacterial agents.

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