Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2357

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **4 (4):** 205-211 (2016)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Antibacterial Activity of *Evolvulus nummularius* Against Standard ATCC Gram Positive and Gram Negative Strains: Studies on MIC, MBC, Growth Curve Analysis and ROS Generation

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Received: 15.08.2016 | Revised: 22.08.2016 | Accepted: 24.08.2016

ABSTRACT

Evolvulus nummularius is a widely used ethnomedicinal plant of North-East India. The whole plant of E. nummularius is used as a medicine for hysteria, to cure burns, cuts, wounds and scropion 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 standard gram positive and two gram negative strains of bacteria (ATCC strains). This plant extract showed both bacteriostatic and bactericidal activity against all bacterial strains. The extract was most active against gram positive bacterial strain S. pyogenes and B. subtilis. In growth analysis study, it was obtained that, after treatment with IC_{50} 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 (40-55%) in presence of IC_{50} dose of Evolvulus nummularius.

Key words: *Evolvulus nummularius, Gram-positive bacteria, Gram negative bacteria, Growth curve, ROS.*

INTRODUCTION

Plants for decades had been used as a natural therapeutics for maintaining human health[1].The frequency of infections by pathogenic microorganisms has increased worldwide and is an important factor of morbidity and mortality in developing countries[2]. So, there is a continuous and urgent need to discover new antimicrobial compounds with diverse mechanism of actions that can be used against novel and re-emerging infectious diseases[3].

Tripura, small state of the north-eastern region of India is rich in biodiversity with vast resource of medicinal plants[4,5,6].

Cite this article: Saha, S., Deb, B., Mullick, J.B., Choudhury, P.R., Saha, P., Ghosh, B., and Sil, S.K., Antibacterial Activity of *Evolvulus nummularius* against Standard ATCC Gram Positive and Gram Negative Strains: Studies on MIC, MBC, Growth Curve Analysis and ROS Generation, *Int. J. Pure App. Biosci.* **4(4):** 205-211 (2016). doi: http://dx.doi.org/10.18782/2320-7051.2357

Evolvulus nummularius, the ethnomedicinal plant of north- east India, has antihelminthic activity[7], wound healing activity[8], poor sedative and anticonvulsant properties[9]. But the antibacterial activity of *Evolvulus nummularius* had not been reported yet.

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[10,11]. Reactive by- products of oxygen, such as superoxide anion radical (O_2), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals (·OH), are generated continuously in cells grown aerobically[10]. These species cause damage to proteins, lipids, and nucleotides, negatively impacting the organism[11].

Therefore, in this study the antibacterial activity of *Evolvulus nummularius* against standard bacterial strains will be evaluated with mechanism of action of by estimating the percentage of ROS in both standard gram positive and gram negative strains in presence and absence of methanol extract of *Evolvulus nummularius* (MEEN).

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[12]. 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

Both gram negative bacterial species: *Escherichia coli*. (ATCC 11229), *Pseudomonas aeroginosa* (ATCC 10145) and gram positive bacterial species: *Staphylococcus aureus* (NCTC 6571), *Bacillus subtilis* (ATCC 6633), *Sreptococcus pyrogenes* (ATCC 12384) were grown, cultured and maintained on Muller Hinton Broth and stored at 4 ° C[13]. For long time storage 15% glycerol solution was used and vial was stored at -80 ° 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[14]. Then, % of Inhibition was calculated by following formula,

% of Inhibition = [1- {(Exp. - Blank) / (Positive Control – 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, IC_{50}, IC_{100} and $>IC_{100}$ 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. IC_{100} 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[14].

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 IC_{50} 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 OD_{600}[13].

Estimation of Reactive Oxygen Species (ROS)

0.1 ml of each bacterial suspension (where OD_{600} = 1.0) in Hank's balanced salt solution (HBSS) was incubated with respective IC_{50} 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)[15].

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

Determination of Minimum Inhibitory Concentration (MIC)

Antibacterial activity of MEEN on both gram positive and gram negative bacterial species were obtained by determining the minimum inhibitory concentrations. As shown in table 1 and Fig 1, the growth of gram positive bacteria *B. subtilis* and *S. pyogenes* were inhibited completely at lower concentrations of MEEN (2.5 mg/ml), but growth of *E. coli* was completely inhibited at too higher concentration of MEEN (10 mg/ml). The order of observed sensitivity on 5 different bacterial strains were, *B. Subtilis* ≈ *S. pyogenes* > *P. aeruginosa* ≈ *S. aureus* > *E. coli.*

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 inhibit the bacterial growth but also can kill both gram-positive and gram negative 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. Both the bacterial strains were exposed to MEEN separately, at a concentration of IC_{50} 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 *S. pyogenes* was mostly affected by MEEN.

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 IC_{50} 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 *S. pyogenes*, in which ROS production increased about 55% in 3 hours compared to control, whereas in *E. coli*, ROS production increased about 40%. The order of observed ROS production on 5 different bacterial strains were, *S. pyogenes* > *B. Subtilis* ≈ *S. aureus* ≈ *P. aeruginosa* > *E. coli.*

Table 1: MIC values for both gram positive and gram negative bacterial species. This data is significant at a level of $p < 0.05$

	IC_{50}(mg/ml)	IC_{100}(mg/ml)
Gram negative bacteria		
E. coli	5 ± 0.32	10 ± 0.27
P. aeruginosa	2.5 ± 0.17	5 ± 0.34
Gram positive bacteria		
B. subtilis	1.25± 0.32	2.5± 0.32
S. aureus	2.5 ± 0.17	5 ± 0.34
S. pyogenes	1.25± 0.32	2.5± 0.32

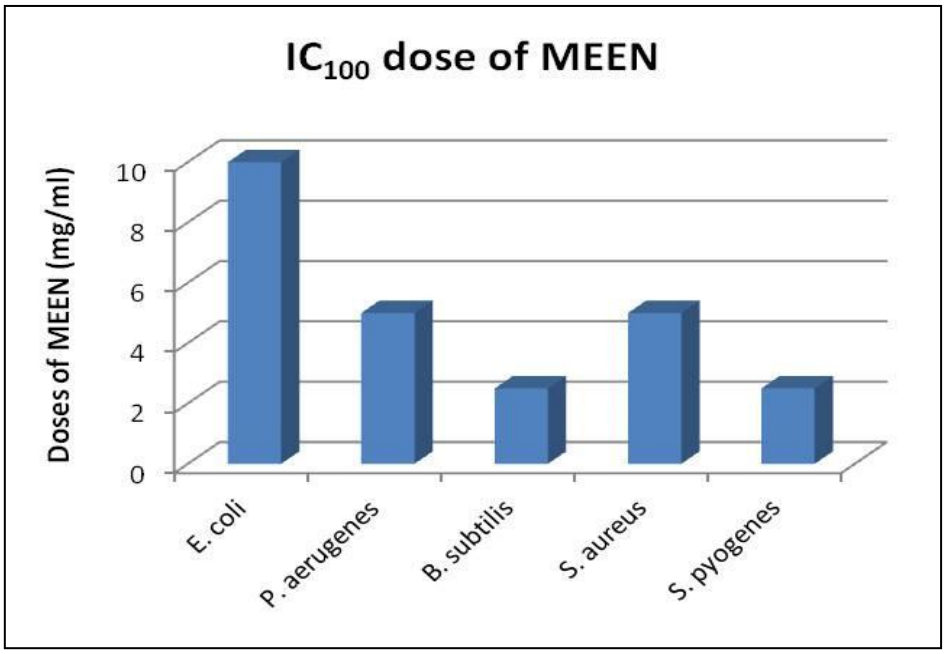

Fig. 1: Column diagram IC_{100} dose of MEEN

Table 2: MBC values for gram negative bacterial species

Gram (-) Bacteria	MBC	IC_{100}/MBC
E. coli	10 mg/ml	1
P. aeruginosa	5 mg/ml	1

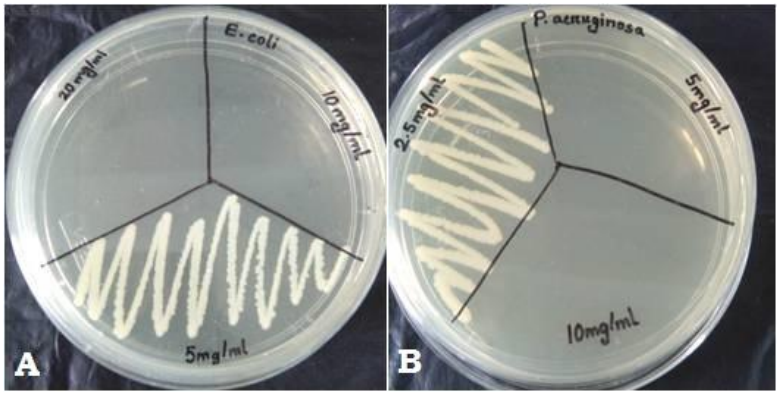

Fig. 2A: Muller Hinton Agar Plate showing MBC of Gram negative Bacteria (A) *E. coli*, (B) *P. aeruginosa*

Table 3: MBC values for gram positive bacterial species

Gram (-) Bacteria	MBC	IC_{100}/MBC
B. subtilis	2.5 mg/ml	1
S. aureus	10 mg/ml	1
S. pyogenes	2.5 mg/ml	1

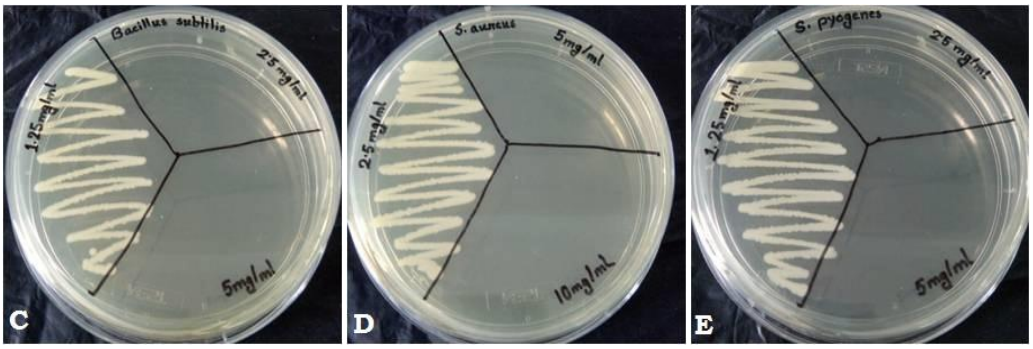

Fig. 2B: Muller Hinton Agar Plate showing MBC of Gram positive Bacteria (E) *B. Subtilis*, (F) *S. aureus*, (G) *S. pyogenes*

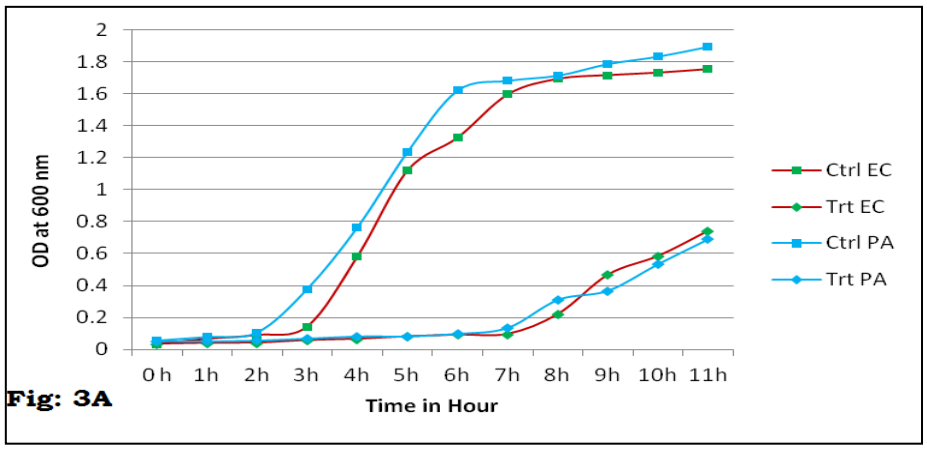

Fig. 3A: Growth curve of gram negative bacterial strains in presence and absence of MEEN. This data is significant at a level of $p < 0.05$

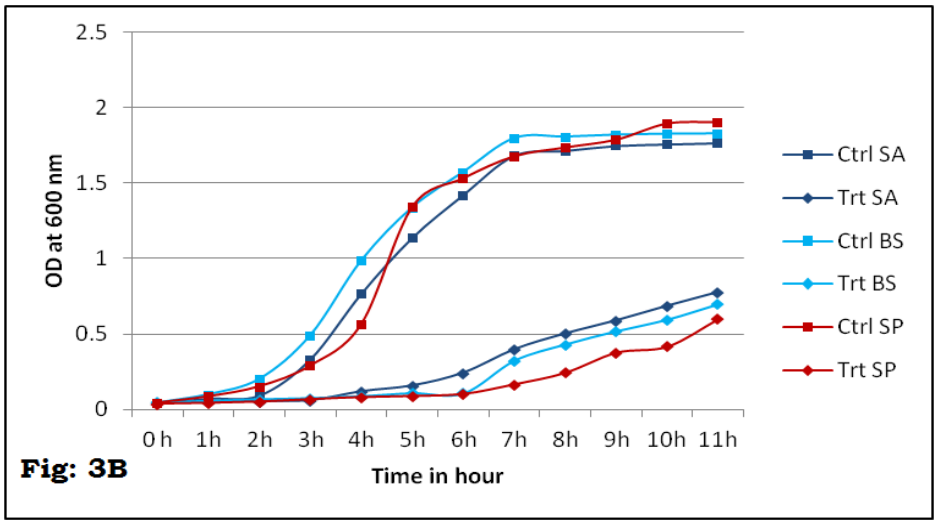

Fig 3B: Growth curve of gram positive bacterial strains in presence and absence of MEEN. This data is significant at a level of $p < 0.05$

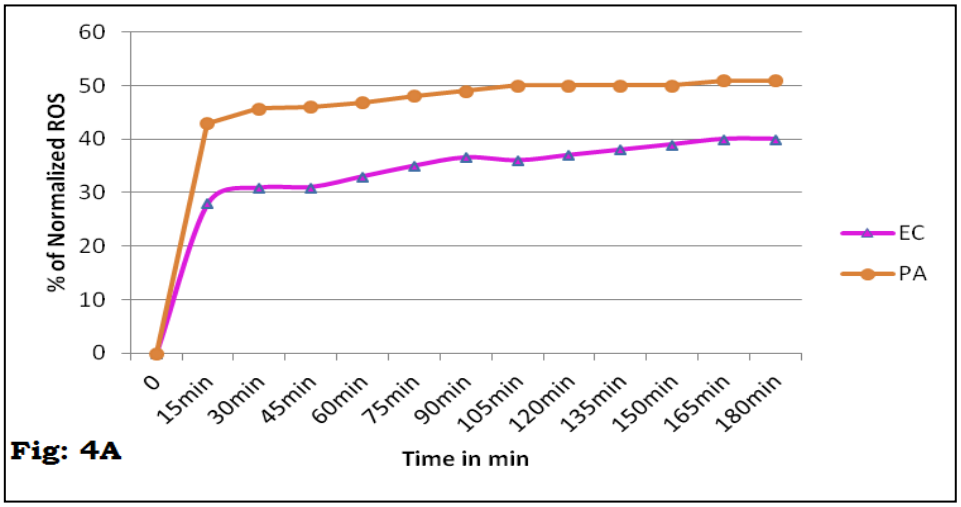

Fig. 4A: Normalized % of ROS produced by gram negative bacterial strains in presence of MEEN. This data is significant at a level of $p < 0.05$

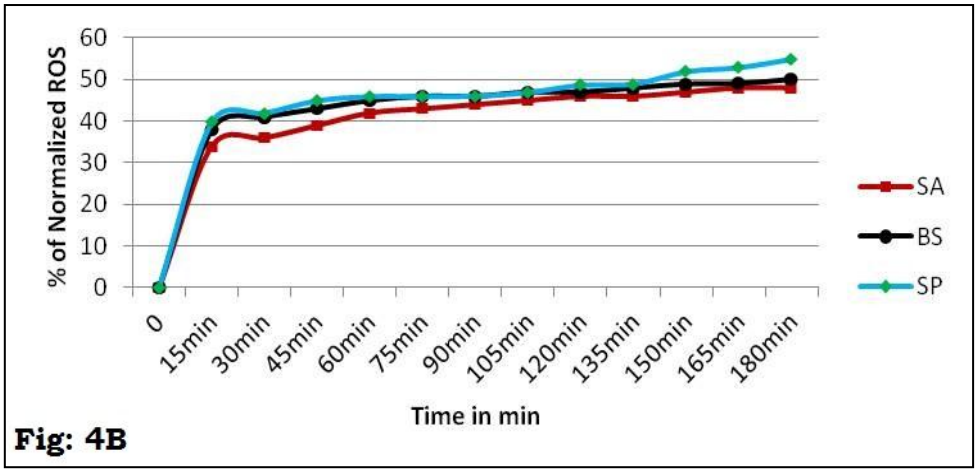

Fig. 4B: Normalized % of ROS produced by gram positive bacterial strains in presence of MEEN. This data is significant at a level of $p < 0.05$

DISCUSSION

In present study the antibacterial activity of *Evolvulus nummularius* was evaluated to explore it's potentiality as antibacterial agent. The growth of gram positive bacteria *B. subtilis* and *S. pyogenes* were inhibited completely at lower concentrations of MEEN (2.5 mg/ml), but growth of *E. coli* was completely inhibited at too higher concentration of MEEN (10 mg/ml). 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 for standard ATCC strains.

For growth kinetics studies, all the bacterial strains were exposed to MEEN separately, at a concentration of IC_{50} 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 *S. pyogenes* 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 IC_{50} dose. After treatment of MEEN, the production of ROS was increased drastically with time. It was highest in *S. pyogenes*, in which ROS production increased about 55% in 3 hours compared to control, whereas in *E. coli*, ROS production increased about 40%. The order of

observed ROS production on 5 different bacterial strains were, *S. pyogenes* > *B. Subtilis* ≈ *S. aureus* ≈ *P. aeruginosa* > *E. coli.* Therefore, the crude methanol extract of *Evolvulus nummularius* may be used as a potent source of antibacterial agent. MEEN is more effective against gram positive bacteria. So, this antibacterial study is justified to explore the proper use of this plant in traditional medicine.

Acknoledgement

We acknowledge Prof. B. K. Datta, Department of Botany, Tripura University for identification of plant species. We also acknowledge State Biotech Hub, Tripura University for technical support and Department of biotechnology (DBT).

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