

Original Research Article

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Anti-Vibrio Activity of *Parkia javanica*: Studies on MIC, MBC, Growth Curve Analysis and ROS Generation on Four *Vibrio cholerae* Strains

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Parkia javanica, a medicinal plant, of North-East India, was screened for anti cholera activity against 4 *Vibrio cholerae* strains. Fresh stem barks of *Parkia javanica* was collected and crude methanol extract of *Parkia javanica* (MEPJ) was prepared by Hahnshin rotary evaporator. The minimum inhibitory concentration of MEPJ was evaluated on *Vibrio cholerae* strains by serial dilution technique. 10mg/ml of MEPJ is the IC₁₀₀ dose for *Vibrio cholerae* strains. MEPJ also showed bactericidal activity against all four *Vibrio cholerae* at the same concentration. The lag phase become extended after treatment with IC₅₀ dose of MEPJ. Intracellular reactive oxygen species (ROS) was increased (40% - 60%) in presence of MEPJ indicating ROS mediated mechanism of growth inhibition.

Introduction

Vibrio cholerae, a Gram-negative, facultative anaerobic, comma shaped bacterium, is the causative agent of life threatening diarrheal disease called cholera (Pacini, 1854). In developing countries, infectious diseases are the important factors for morbidity and mortality in common people (Mattana *et al.*, 2012). *Parkia javanica* is a very large tree and has found traditional use as food and ethnomedicine in North- East India (Maiti, 2004). It is generally used by tribal population of North-East India to cure stomach ache, dysentery, food poisoning (Majumder *et al.*, 2009).

Saha *et al.*, 2007, reported that, *Parkia javanica* has antibacterial activities against both standard Gram positive and Gram negative bacterial strains. But there is no report, on anti-vibrio activity of *Parkia javanica* against *Vibrio cholerae* strains. Therefore, this work has been undertaken to explore the anti-vibrio activities of *P. javanica* using crude methanol extract.

Materials and Methods

Preparation Plant Extract

Fresh stem barks of *Parkia javanica* was collected from Suryamaninagar, Tripura,

India. After washing with water these barks were allowed to dry in shade. Then barks were cut into small pieces. Then 500 gm of powdered bark was soaked in 2000 ml of methanol to prepare the crude extract and kept in a shaker for 48 hours. After that the solutions were filtered through Whatman filter paper no. 1 for 3 times. Then these solutions were dried in rotary evaporator at 70°C. Finally these solutions were freeze-dried and stored at -20°C (Nikolic *et al.*, 2014).

100 mg of dried extract was dissolved in 1ml of 25% DMSO-water mixture and filtered by a 0.22 µm syringe filter and stored at -20°C.

Bacterial Strains and Growth Conditions

Vibrio cholerae 097 VTE 2357, *Vibrio cholerae* 06 VTE 2523, *Vibrio cholerae* classical Y 1254, *Vibrio cholerae* 01 hybrid 02459 strains were grown, cultured and maintained in Muller Hinton Broth and Muller Hinton agar. For long time storage 15% glycerol solution was used and vial was stored at -80 °C (Bhattacharya *et al.*, 2012).

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined by serial dilution technique, with an inoculum of 10⁶ CFU/ml of both Gram positive and Gram negative bacteria in separate 96 well plates, in presence of increasing concentrations of MEPJ. 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, MEPJ with media, used as blank; media, MEPJ and bacterial culture, used as experiment; media with bacterial culture and 25% DMSO, used as positive control; and media with only 25% DMSO, used as negative control (Demetrio *et al.*,

2015). Then, % of Inhibition was calculated by following formula,

% of Inhibition =

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

Determination of Minimum Inhibitory Concentration (MBC)

After determining the MIC values, MBCs for each bacterial species were examined by treating the each bacterial species 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 absence of extract 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).

Measurement of Bacterial growth Kinetics

To determine the bacterial growth kinetics, in presence of MEPJ, each bacterial species were grown in Muller Hinton Broth in presence and absence of MEPJ 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 MEPJ 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)

The bacterial suspension (0.1 ml) (where OD₆₀₀ = 1.0) in Hank's balanced salt

solution (HBSS) was incubated with respective IC₅₀ dose of MEPJ 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

Minimum Inhibitory Concentration

Antibacterial activity of MEPJ on *Vibrio cholerae* strains were obtained by determining the minimum inhibitory

concentrations. The growth of *Vibrio cholerae* strains were inhibited completely at concentrations of 10 mg/ml of MEPJ, but the order of observed IC₅₀ dose on 4 bacterial strains were, VC 097 VTE 2357 > VC 06 VTE 2523 > VC classical Y 1254 > VC 01 hybrid 02459 [Table 1].

Minimum Bactericidal Concentration

Minimum bactericidal concentration of MEPJ on each bacterial strain was also determined as shown in Fig. 1. The ratio between MBC and MIC for each bacterium is same (~1, for all bacteria) [Table 2]. This result indicated that, MEPJ is a bactericidal agent rather than bacteriostatic agent.

Bacterial Growth Kinetics Studies

We next measured the growth curve of all these bacterial strains to examine whether MEPJ kill or inhibit the growth of these bacteria. Both the bacterial strains were exposed to MEPJ separately, at a concentration of IC₅₀ dose for each bacterium. As shown in Fig 2, the lag phase of all MEPJ treated bacteria were extended compared to control.

Table.1 MIC values for *Vibrio cholerae* Strains. (This data is significant at a level of $p < 0.05$)

	IC ₅₀ (mg/ml)	IC ₁₀₀ (mg/ml)
<i>Vibrio cholerae classical Y 1254</i>	5.5 ± 0.43	10 ± 0.22
<i>Vibrio cholerae 06 VTE 2523</i>	5 ± 0.18	10 ± 0.24
<i>Vibrio cholerae 097 VTE 2357</i>	4.5 ± 0.34	10 ± 0.27
<i>Vibrio cholera 01 hybrid 02459</i>	6 ± 0.41	10 ± 0.49

Table.2 MBC values for *Vibrio cholerae* Strains. (This data is significant at a level of $p < 0.05$)

	MBC (mg/ml)	MBC/MIC
<i>Vibrio cholerae</i> classical Y 1254	10 ± 0	1
<i>Vibrio cholerae</i> 06 VTE 2523	10 ± 0	1
<i>Vibrio cholerae</i> 097 VTE 2357	10 ± 0	1
<i>Vibrio cholera</i> 01 hybrid 02459	5 ± 0	1

Fig.1 Muller Hinton Agar Plate showing MBC of *Vibrio cholerae* strains. A- *Vibrio cholerae* classical Y 1254, B- *Vibrio cholerae* 06 VTE 2523, *Vibrio cholerae* 097 VTE 2357, *Vibrio cholerae* 01 hybrid 02459.

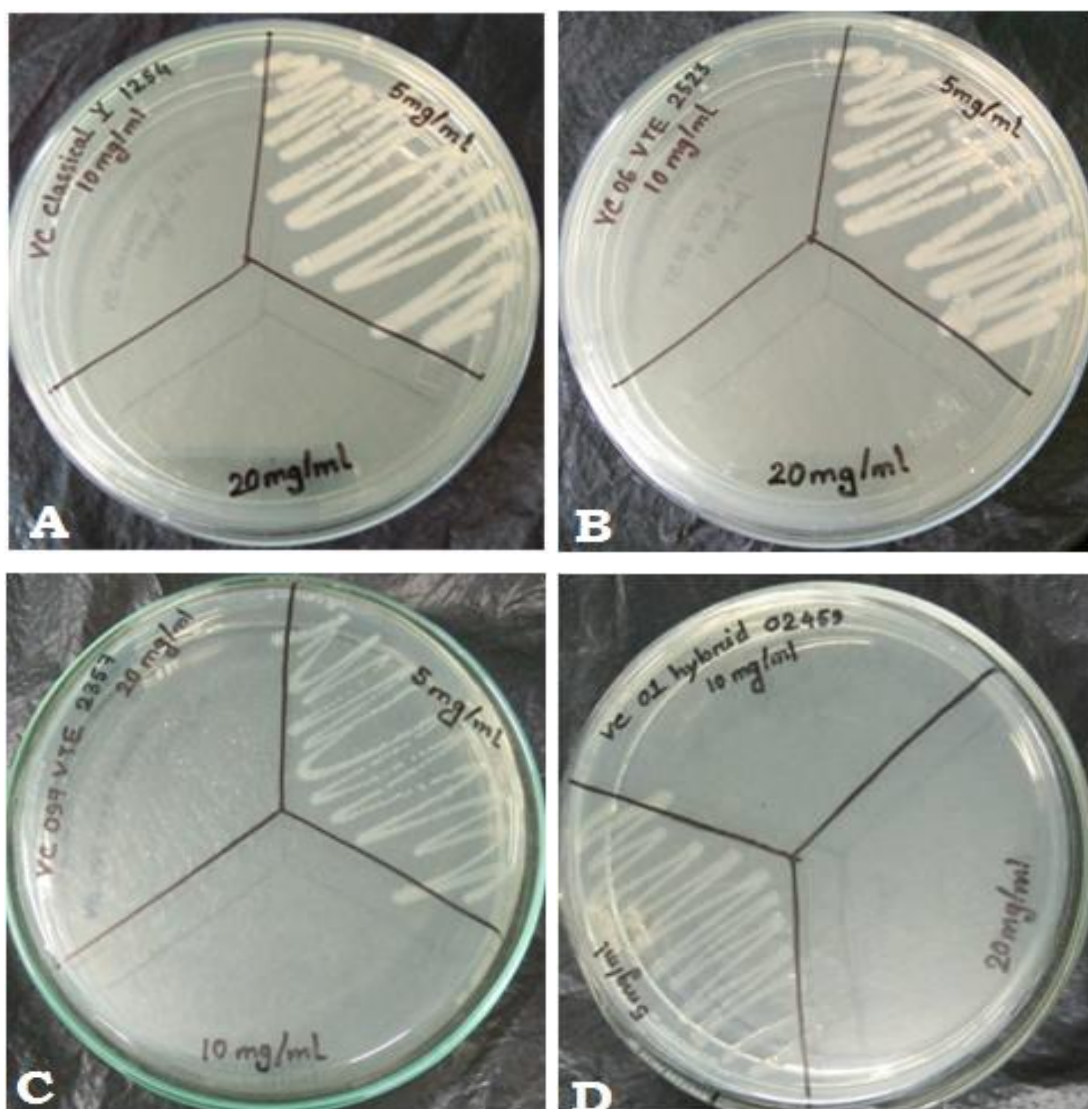


Fig.2 Growth curve of *Vibrio cholerae* strains in presence and absence of MEPJ. This data is significant at a level of $p < 0.05$

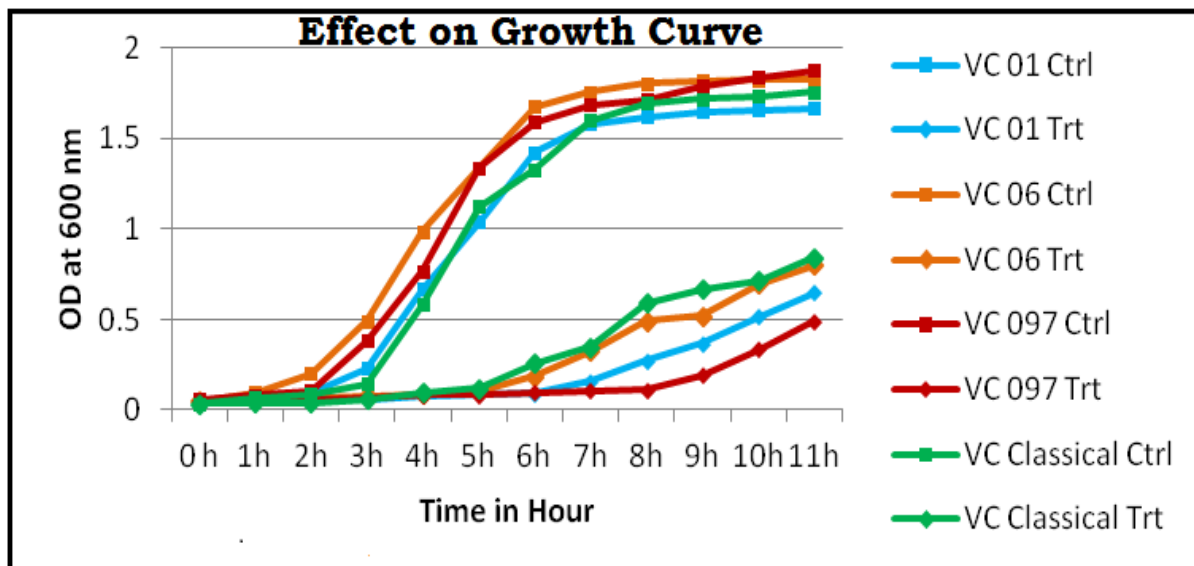
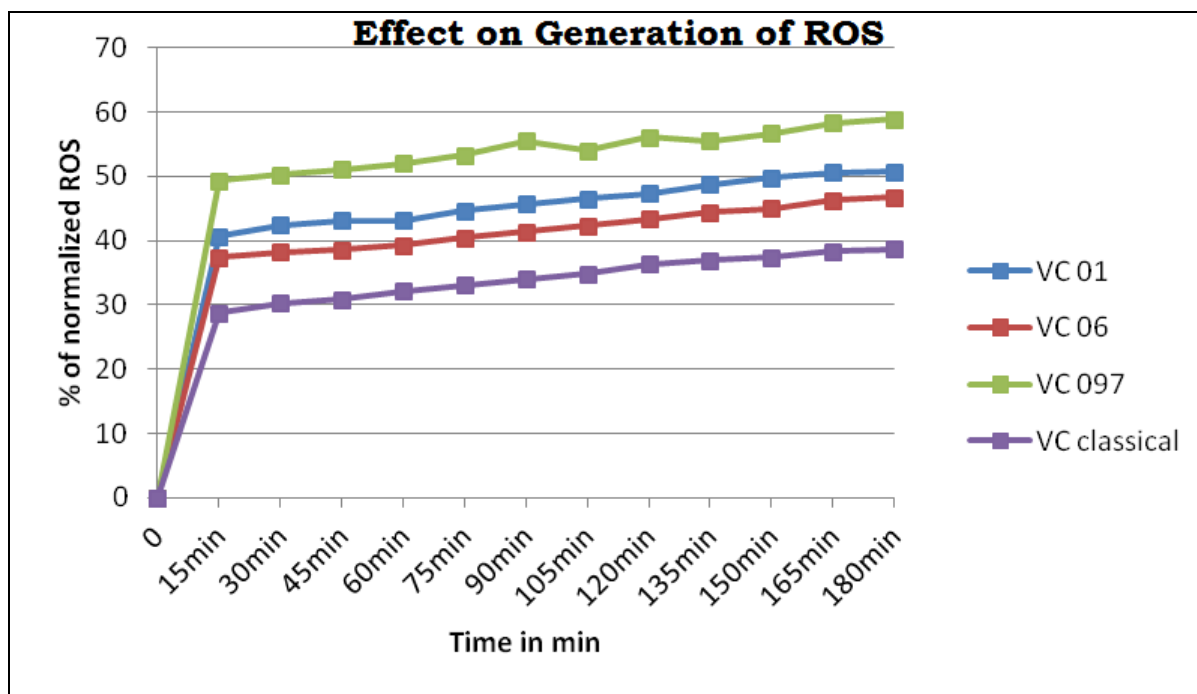


Fig.3 Normalized % of ROS produced by *Vibrio cholerae* strains in presence of MEPJ. This data is significant at a level of $p < 0.05$.



Generation of Reactive Oxygen Species (ROS)

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

reactive oxygen species (ROS) was estimated after treatment with MEPJ at IC_{50} dose. As shown in Fig 3, after treatment of MEPJ, the production of ROS was increased drastically with time. It was highest in VC

097 VTE 2357, in which ROS production increased about 60% in 3 hours compared to control, whereas in *VC classical Y 1254*, ROS production increased about 35%. The order of observed ROS production on 4 *Vibrio cholerae* bacterial strains were, *VC 097 VTE 2357* > *VC 01 hybrid 02459* > *VC 06 VTE 2357* > *VC classical Y 1254*.

Several studies reported that, different edible and medicinal plants had anti-vibrio and vibriocidal activities (Sanchez *et al.*, 2010; Fakruddin *et al.*, 2011). In this study we have reported the anti-vibrio activity of crude methanol extract of *P. javanica* against 4 *Vibrio cholerae* strains namely *Vibrio cholerae 097 VTE 2357*, *Vibrio cholerae 06 VTE 2523*, *Vibrio cholerae classical Y 1254*, *Vibrio cholerae 01 hybrid 02459*. From this study it was observed that, the extract possessed both bacteriostatic and bactericidal activity. At 10mg/ml dose, all the strains were got killed. From growth kinetics study, we found that, the lag phase of all extract treated bacteria is extended compared to untreated cells. The normalized % of ROS was also increased in almost every strain after extract treatment. Therefore, it could be conclude that, the crude methanol extract of *Parkia javanica* possesses anti-vibrio activity and ROS induced bacterial cell damage is the possible mechanism of this anti-vibrio activity.

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