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Research Article

REACTIVE OXYGEN SPECIES AS POSSIBLE MEDIATOR OF ANTIBACTERIAL ACTIVITY OF *PARKIA JAVANICA*, AGAINST BACTERIAL SPECIES PREDOMINANTLY FOUND IN CHRONIC WOUND

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ABSTRACT

The crude methanol extract of *Parkia javanica* was screened for antibacterial activity. against bacterial species predominantly found in chronic wound, by serial dilution technique. Growth kinetics study was performed and percentage of ROS production was measured by NBT reduction assay. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were obtained with a range of IC₁₀₀ 5-40 mg/ml in case of standard bacterial strains. The lag phase of all extract treated bacteria is extended compared to untreated cells. The normalized % of ROS is increased in presence of crude extract. This study suggests that the crude methanol extract of *Parkia javanica* possesses promising antimicrobial substances which are having activity against Standard ATCC bacterial species and ROS induced DNA damage could be the possible mediator of its antimicrobial activity.

Keywords: *Parkia javanica*, antibacterial activity, standard ATCC bacterial strains, growth curve, ROS, DNA damage

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INTRODUCTION

Skin is the largest organ of our body, which protects us from various environmental factors¹. Skin infections have become more common worldwide due to changes in the environmental conditions on the globe, as a hazardous result of industrialization, pollution, deforestation, etc². So, we should concern ourselves, to protect this organ, as it is easily subjected to microbial attacks³. Certain bacterial species, such as, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Escherichia coli* commonly occur in skin infection, even in the chronic skin wound of diabetic patients⁴. Different antibiotics produced by various pharmaceutical companies gradually becoming

ineffective due to the emergence of resistance to these drugs^{5,6} and, as a result, the rate of morbidity and mortality has been increased due to bacterial infections⁷. So, it is an urge to identify new substances with effective antimicrobial activity, which can act as a source and template for the synthesis of new antimicrobial drugs. Plants have always been valuable source of antimicrobial agents⁸.

The plant, *Parkia javanica* is traditionally used as a food and ethno medicine by tribal population of Northeast India^{9, 10}. In spite of having long ethno medicinal history, this plant has not been fully explored on scientific basis regarding its medicinal activities. The present work has been designed to understand

antibacterial properties of this plant as well as possible mechanism of action using standard ATCC bacterial strains.

MATERIALS AND METHODS

Plant collection & Authentication

Fresh stem barks of *Parkia javanica* were collected from Tripura, India. The plant was initially identified by Dr. B. K. Dutta, Taxonomist, Department of Botany, Tripura University and finally authenticated by Dr. H. J. Chowdhery, Joint Director, Central National Herbarium, Botanical Survey of India, Shibpur, Howrah, West Bengal and respective voucher specimen No. #BD-01/06 has been deposited in the Herbarium.

Preparation Plant Extract

Barks were cut into small pieces and allowed to dry in shade. Then 500 gm of powdered bark was soaked in 2000 ml of methanol to prepare the crude methanol extract of *Parkia javanica* (Crude MEPJ) and then kept in a shaker for 48 hours. After that the solution was filtered through Whatman filter paper no. 1 for 3 times. Then the solution was dried in rotary evaporator at 70°C. Finally the solution was freeze-dried and stored at -20°C¹¹.

Bacterial Culture and Growth Conditions

Both standard gram negative bacterial species: *Escherichia coli* (ATCC 11229), *E. coli DH5α* (MTCC 1652 India) artificially transformed with pUC19 plasmid 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. For long time storage 15% glycerol solution was used and vial was stored at -80°C¹².

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC was determined by serial dilution technique, with an inoculum of 10⁶ CFU/ml of both Gram positive and Gram negative standard bacterial strains in separate 96 well plates, in presence of increasing concentrations of crude 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, extract with media, used as blank; extract, media 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. Then, % of Inhibition was calculated by following formula,

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

Then MBC for each bacterial species were determined by treating the bacterial strains 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 species¹³.

Measurement of Bacterial growth Kinetics

To determine the bacterial growth kinetics, in presence of crude MEPJ, each bacterial species were grown in Muller Hinton Broth in presence and absence of extracts 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 extract were determined by measuring the OD at 600 nm in every 1 hour interval. Bacterial growth kinetics was plotted graphically with time versus OD₆₀₀¹².

Estimation of Reactive Oxygen Species (ROS)

0.1ml of each bacterial suspension (where OD₆₀₀ = 1.0) in Hank's balanced salt solution (HBSS) was incubated with respective IC₅₀ dose of crude MEPJ for 2 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)¹⁴.

DNA damage Assay

To examine the effect of crude MEPJ on DNA inside bacterial cell, reporter (β -galactoside) gene expression assay was performed. In this assay, pUC19 transformed DH5α cells were incubated for 3 hours at 37°C in presence or absence of IC₅₀ dose of crude MEPJ. Then these bacterial cells were inoculated on Muller Hinton agar plate (*amp*⁺) containing X-gal and IPTG in medium and incubated for 12 hours at 37°C to observe the blue colour forming colonies¹⁴.

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 MIC:

Antibacterial activity of crude MEPJ on Standard gram positive and gram negative bacterial species, was obtained by determining the minimum inhibitory concentrations. As shown in table 1, the growth of standard gram positive bacterial species, like *B. subtilis* and *S. pyrogenes* were inhibited completely at lower concentrations of MEPJ (5 mg/ml), but growth of standard gram negative bacterial species *E. coli* and pUC19 plasmid transformed *E. coli DH5α* were completely inhibited at too higher concentration of MEPJ (20 mg/ml and 40 mg/ml respectively).

Table 1: MIC and MBC values of Standard bacterial Strains

	IC ₁₀₀ *	MBC*	MBC/MIC
<i>E. coli</i>	20 ± 0.01	20 ± 0.05	1.00
<i>DH5α</i>	40 ± 0.03	40 ± 0.01	1.00
<i>B. subtilis</i>	5 ± 0.02	5 ± 0.08	1.00
<i>S. aureus</i>	10 ± 0.05	10 ± 0.01	1.00
<i>S. pyogenes</i>	5 ± 0.04	5 ± 0.03	1.00

*Concentration of extracts in mg/ml. MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; Experiments were performed in triplicate and all the MIC and MBC values are significant at the level of $p < 0.05$.

Minimum bactericidal concentration of crude MEPJ on each bacterial strain were also determined. According to Table 1 and Fig 1, the ratio between MBC and MIC for each bacterium is same (~1, for all bacteria). This result indicated that, crude MEPJ is a bactericidal agent rather than bacteriostatic agent.

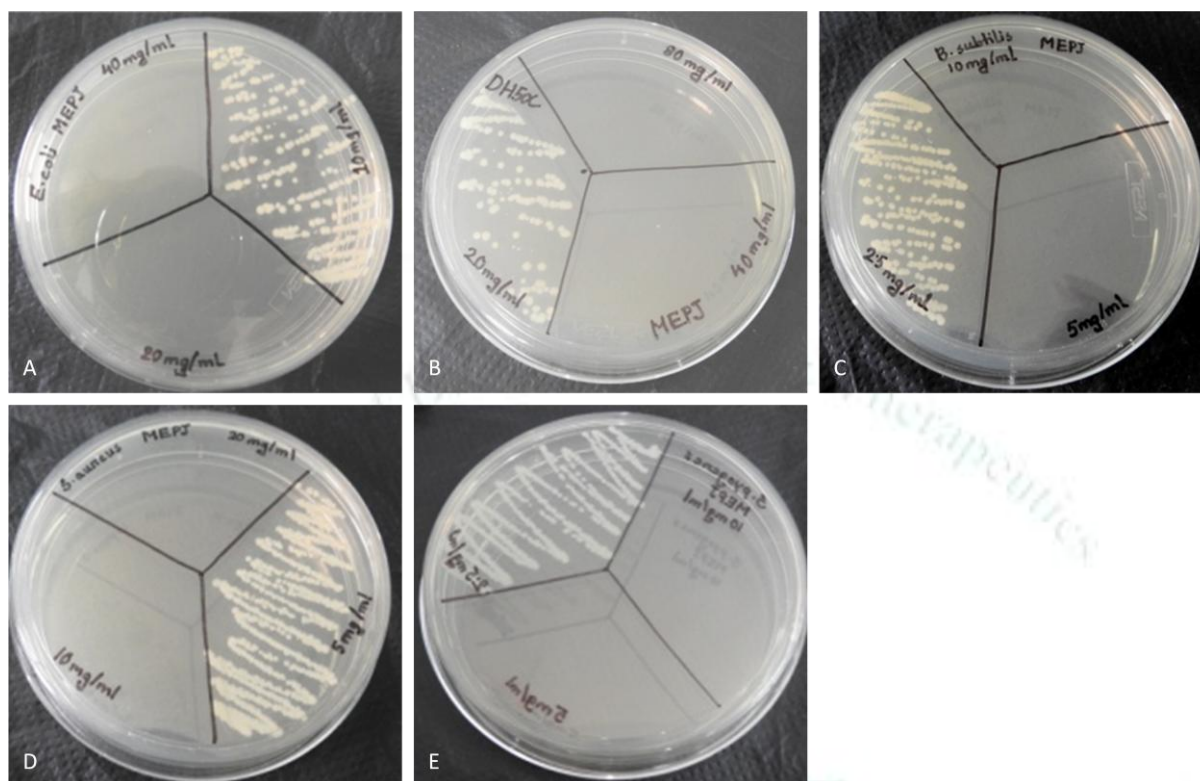


Figure 1: Muller Hinton agar plate showing the minimum bactericidal concentration (MBC) of MEPJ on standard ATCC strains of gram positive and gram negative bacterial species; (A) *E. coli*; (B) *E. coli DH5α*; (C) *B. subtilis*; (D) *S. aureus*; (E) *S. pyogenes*.

Measurement of Bacterial Growth Kinetics:

As crude MEPJ possess killing potential of different the bacterial species, so, we next measured the growth curve of both gram negative and gram positive standard bacterial strains to examine the pattern of the growth curve in presence and absence of crude MEPJ. All the bacterial strains were exposed to *P. javanica* extracts separately, at a concentration of IC₅₀ dose for each bacterium. As shown in Fig 2, the lag phase of all crude MEPJ treated bacteria were extended compared to control. The growth of *S. pyogenes* is mostly affected by the *P. javanica* extract.

Estimation of ROS:

Finally, to understand the mechanism of antibacterial activity of *P. javanica*, intracellular reactive oxygen species (ROS) were estimated after treatment with crude MEPJ at IC₅₀ dose. As shown in Fig 3, after treatment of crude MEPJ, the production of ROS was increased drastically with time. It was highest in *B. subtilis*, in which ROS production increased about 70% in 3 hours compared to control, whereas in *DH5α*, ROS production increased about 35%. The order of observed ROS production on 5 different bacterial strains were, *B. Subtilis* > *S. pyogenes* > *S. aureus* > *E. coli* > *DH5α*.

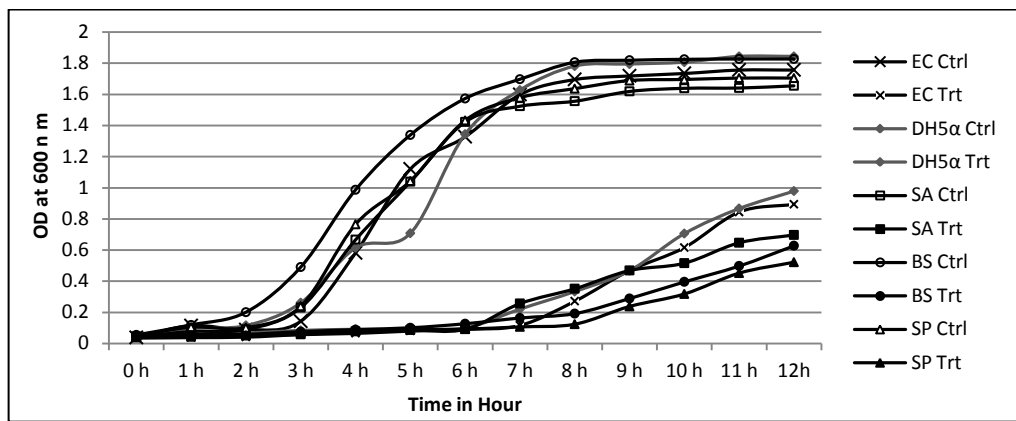


Figure 2: Effect of crude MEPJ at respective IC₅₀ dose on pattern of growth curve of standard gram positive and gram negative bacterial strains; Ctrl: Control; Trt: treated with respective IC₅₀ dose of MEPJ of each bacterium; EC: *E. coli*; DH5α: *E. coli DH5α* (Transformed bacteria); SA: *Staphylococcus aureus*; BS: *Bacillus subtilis*; SP: *Streptococcus pyogenes*.

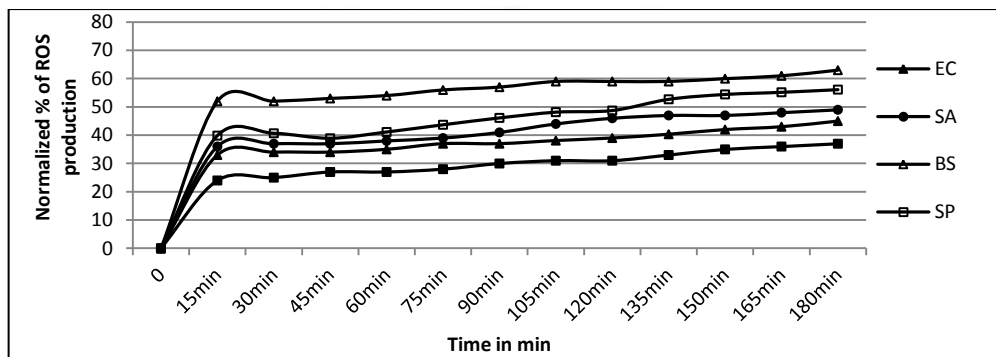


Figure 3: Effect of crude MEPJ at respective IC₅₀ dose of each bacterium, on normalized % of ROS production in standard gram positive and gram negative bacterial strains; EC: *E. coli*; DH5α: *E. coli DH5α* (Transformed bacteria); SA: *Staphylococcus aureus*; BS: *Bacillus subtilis*; SP: *Streptococcus pyogenes*.

DNA Damage assay:

As shown in Fig 3, ROS production was increased 35% compared to control in *E. coli DH5α*. ROS usually targets the cellular DNA, so, to observe the effect of ROS inside bacterial cells, we used plasmid based reporter gene assay.

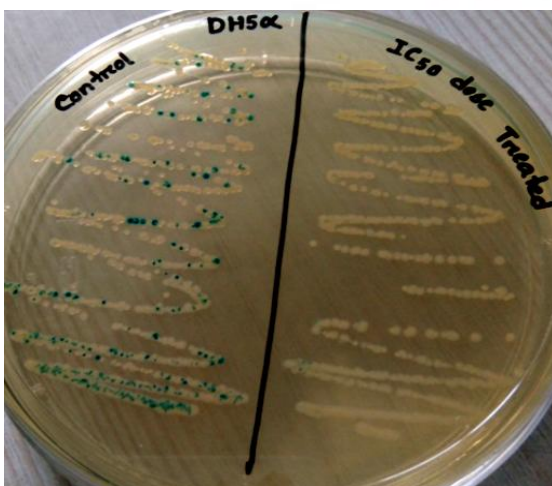


Figure 4: Reporter gene (β -galactosidase) assay on pUC19 Transformed *E. coli DH5α* treated with IC₅₀ dose of MEPJ; Absence of blue colour colonies in treated cells indicates the DNA damage in tested bacteria.

In Fig 4, reporter gene β -galactosidase was assayed by transforming the bacteria with the pUC19 plasmid and then the bacterial cells were treated with crude MEPJ and chloroform fraction. The blue colonies, formed due to hydrolysis of X-gal by β -galactosidase enzyme, were completely absent in case of bacterial cells treated with crude MEPJ.

DISCUSSION

Plants have been a cornerstone in traditional folk medicine to treat microbial infections and they also constitute sources of conventional antimicrobials¹⁵. In vitro evaluation of plants for antimicrobial properties is the first step towards achieving the goal for developing eco friendly management of infectious diseases¹⁶. In this study, *Parkia javanica*, a plant possessing an age old history of use as traditional folk medicine in North-eastern region of India, has been screened in vitro for antibacterial activity against five human bacterial species known to occur and aggravate the skin wound, even the chronic skin wound of diabetic patients.

Crude methanol extract showed antimicrobial activity against all the tested standard ATCC strains of gram positive (*Staphylococcus aureus*, *Bacillus subtilis*, *Sreptococcus pyrogenes*) and gram negative bacteria (*Escherichia coli*, *E. coli DH5α*) with a range of MIC (IC₁₀₀) values. The two-fold serial dilution technique

was used to determine the MIC values and it was observed that, the IC₁₀₀ dose and MBC, obtained using this technique are too less on some bacterial species. From growth kinetics study, it is found that, the lag phase of all extract treated bacteria is extended compared to untreated cells.

The same condition also observed in ROS production. The normalized % of ROS is increased in presence of crude extract. Reactive by products of oxygen, such as superoxide anion radical (O₂), hydrogen peroxide (H₂O₂), and the highly reactive hydroxyl radicals (•OH), are generated continuously in cells grown aerobically because these aerobic bacteria use molecular oxygen of nutrients to obtain energy¹⁷. These species cause damage to proteins, lipids, and nucleotides, negatively impacting the organism¹⁸. Living organisms operate mechanisms to protect themselves against oxidative stress, with enzymes such as catalase and superoxide dismutase, small proteins like thioredoxin and glutaredoxin, and molecules such as glutathione. However the damage ensues when the concentration of active oxygen increases to a level that exceeds the cell's defense capacity.

Absence of blue colour colonies after treatment in reporter gene assay indicates reporter pUC19 plasmid

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CONCLUSION

In this study, we reported the antibacterial activity of crude methanol extract of *P. javanica* with their mode of action. The studies showed that, the crude extract was effective on variety of Gram negative and Gram positive bacterial standard strains. The ROS induced DNA damage is the possible mechanism of antibacterial activity of methanol extract of *Parkia javanica*.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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