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CHLOROFORM FRACTION OF *PARKIA JAVANICA* BARK POSSESSES ANTIBACTERIAL ACTIVITY AGAINST MULTIDRUG RESISTANT GRAM NEGATIVE BACTERIA PREDOMINANTLY FOUND IN SKIN WOUND

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ABSTRACT

Aim: To evaluate the antibacterial activity of *Parkia javanica* against gram negative MDR bacterial strains which are predominantly found in skin wound. **Methods:** The 5 different solvent fractions of *Parkia javanica* were screened for antibacterial activity against gram negative multi drug resistant bacterial strains namely *Enterobacter aerugenes*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* by serial dilution technique. Growth kinetics study was performed and percentage of ROS production was measured by NBT reduction assay. **Results:** The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were obtained with a range of IC_{100} 0.08-0.31 mg/ml in case of MDR 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 *Parkia javanica* extract. **Conclusions:** This study suggests that, chloroform fraction of *Parkia javanica* possesses promising antimicrobial substances which are having activity against MDR bacterial strains and ROS induced bacterial cell damage could be the possible mediator of its antimicrobial activity.

Keywords: *Parkia javanica*, antibacterial activity, MDR bacterial strains, growth curve, ROS.

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INTRODUCTION

Antibiotic resistance is a major problem of the World that continues to challenge the healthcare sector in both developing and developed countries[1]. The spread of multidrug resistant (MDR) bacteria remains a widely unresolved problem and a heavy burden to health services [2]. In multidrug resistant gram-negative bacteria, the effect of efflux pumps in combination with reduced drug uptake, due to presence of double membrane barrier, is responsible for the antibiotic resistance[3]. Thus, in light of the evidence of rapid global spread of resistant bacteria, there is need to identify new substances with effective antimicrobial activity [4]. The skin infection, especially skin of the foot, is frequently infected by different pathogenic microbes, causing chronic skin wound mainly in diabetic patients[5]. Despite advances in antibiotic therapy, infectious complications remain an important cause of mortality and morbidity among hospitalized patients. Although medical practitioners can resort to second or third-choice drugs

for treating these infectious diseases, the use of these synthetic drugs may produce more harmful side effects[6]. For this reason, researchers are increasingly turning their attention to herbal products, looking for new leads to develop better drugs, against these pathogenic microbes[7]. Plants have been used by several communities to treat a large number of diseases[8]. These plants constitute a potential source for the production of new medicines which should have antimicrobial action and synergism with currently available antimicrobial drugs from medicinal plants[9, 10]. Thus discovery of new antimicrobial agent or formulation from plant resources could be a great advantage to this emergence of MDR strains causing treatment failure. *Parkia javanica*, of leguminece family, has large ethnomedicinal history. This plant is widely used by tribal population of north-east region of India[11, 12, 13]. In our previous study, it was found that, crude methanol extract of *Parkia javanica* possess antibacterial activity against both standard ATCC strains and MDR strains and ROS induced DNA damage may be possible mediator of antibacterial activity of this plant [14,15,16]. Therefore, this work has been undertaken to explore the active fraction of this study plant having antibacterial properties against gram negative multi drug resistant bacterial strains with possible mode of action.

MATERIALS AND METHODS

Plant collection & Authentication

Fresh stem barks of *P. javanica* were collected from Suryamaninagar, 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

After washing with water barks were cut into small pieces. Then 500 gm of powdered bark was soaked in 2000 ml of 5 different solvents from non polar to polar solvents, viz., n-Hexane, Chloroform, Ethylacetate, n-Butanol and Methanol one after another and then 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 5 solvent factions of *Parkia javanica* (PJHF, PJCF, PJEF, PJBF and PJMF for n-Hexane, chloroform, ethylacetate, butanol and methanol fractions, respectively) were freeze- dried and stored at -20°C [17].

Bacterial Culture and Growth Conditions

All the multidrug resistant (MDR) strains *Enterobactor aerugenes* (ATCC 13048), *Pseudomonas aeruginosa* (ATCC 10145) and *Klebsiella aerugenes* (ATCC BAA-1705) were grown, cultured and maintained on Muller Hinton Broth. For long time storage 15% glycerol solution was used and vial was stored at -80 $^{\circ}$C [18].

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

MIC was determined by serial dilution technique, with an inoculum of 10^6 CFU/ml of both Gram positive and Gram negative standard as well as MDR bacterial strains in separate 96 well plate, in presence of increasing concentrations of 5 solvent fractions of *Parkia javanica*. The bacterial cultures were incubated at 37 $^{\circ}$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,

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

Then MBC for each bacterial species were determined by treating the bacterial strains 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 $^{\circ}$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 [6].

Measurement of Bacterial growth Kinetics

To determine the bacterial growth kinetics, in presence of PJCF, each bacterial species were grown in Muller Hinton Broth in presence and absence of extracts separately, at 37 $^{\circ}$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 extract 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} [18].

Estimation of Reactive Oxygen Species (ROS)

0.1ml of each bacterial suspension (where OD_{600} = 1.0) in Hank's balanced salt solution (HBSS) was incubated with respective IC_{50} dose of PJCF for 2 hours with 15 min interval at 37 $^{\circ}$C. Then 500 µl of 1 mg/ml NBT was added and again incubated for 30 min at 37 $^{\circ}$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) [19].

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 5 solvent fractions of *Parkia javanica* on multidrug resistant (MDR) bacterial strains, were obtained by determining the minimum inhibitory concentrations by serial dilution technique. As shown in Table 1, among 5 different solvent fractions, PJCF is most effective at 0.08 mg/ml concentration on *E. aerugenes* compared to other solvent fractions The order of observed sensitivity to PJCF, of 3 MDR strains were, *E. aerugenes* > *P. aeruginosa* > *K. pneumoniae*.

Minimum bactericidal concentration on each bacterial strain was 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, all the solvent fractions of *P. javanica* are a bactericidal agent rather than bacteriostatic agent. However PJCF is more potent than other fractions as it possess the bactericidal activity at comparatively lower concentration than other solvent fractions.

Table 1: MIC values of MDR Strains

	E. aerugenes	***P. aeruginosa***	***K. pneumoniae***
	IC_{100}*	**IC_{100}***	**IC_{100}***
n-Haxane	0.16 ± 0.03	0.31 ± 0.05	0.62 ± 0.02
Chloroform	0.08 ± 0.02	0.15 ± 0.06	0.31 ± 0.02
Ethylacetate	0.16 ± 0.07	0.31 ± 0.03	0.62 ± 0.07
n-Butanol	0.16 ± 0.04	0.31 ± 0.04	0.62 ± 0.04
Methanol	0.16 ± 0.03	0.31 ± 0.07	0.62 ± 0.03

*Concentration of extracts in mg/ml. Experiments were performed in triplicate and all the MIC values are significant at the level of p< 0.05.

Table 2: MBC values of MDR Strains

	E. aerugenes		***P. aeruginosa***		***K. pneumoniae***	
	MBC*	**MBC/MIC**	**MBC***	**MBC/MIC**	**MBC***	**MBC/MIC**
n-Haxane	0.16 ± 0.38	1.00	0.31 ± 0.25	1.00	0.62 ± 0.42	1.00
Chloroform	0.08 ± 0.23	1.00	0.15 ± 0.16	1.00	0.31 ± 0.72	1.00
Ethylacetate	0.16 ± 0.73	1.00	0.31 ± 0.13	1.00	0.62 ± 0.37	1.00
n-Butanol	0.16 ± 0.84	1.00	0.31 ± 0.04	1.00	0.62 ± 0.24	1.00
Methanol	0.16 ± 0.93	1.00	0.31 ± 0.37	1.00	0.62 ± 0.53	1.00

*Concentration of extracts in mg/ml. Experiments were performed in triplicate and all the MBC values are significant at the level of p< 0.05.

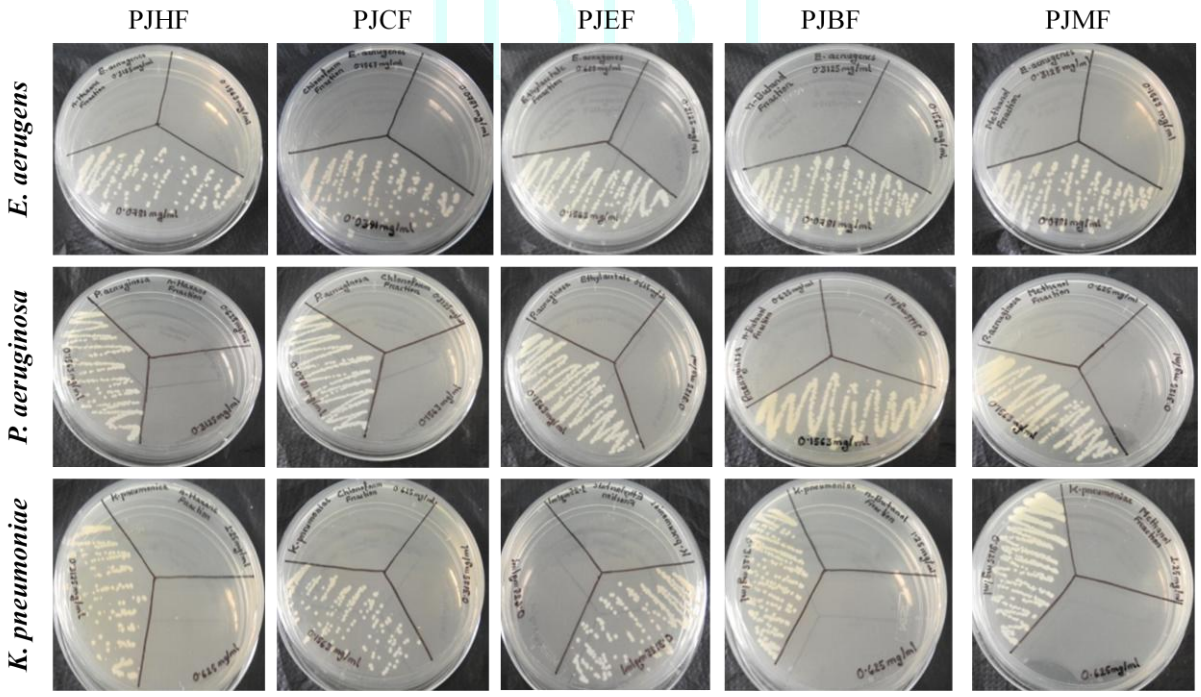

Figure 1: Muller Hinton agar plate showing the minimum bactericidal concentration (MBC) of PJ fractions on gram negative MDR bacterial strains.

Measurement of Bacterial Growth Kinetics:

As shown in Table 1, PJCF can kill the MDR bacterial species, so, we next measured the growth curve of three MDR strains to examine the pattern of the growth with time in presence and absence of PJCF. All the bacterial strains were exposed to *P. javanica* extracts separately, at a concentration of IC_{50} dose for each bacterium. As shown in Fig 2, the lag phase of all PJCF treated bacteria was extended compared to control and the pattern of extension is slightly higher in case of *E. aerugenes*. Among three MDR strains, growth of *E. aerugenes* is mostly affected by the *P. javanica* extract

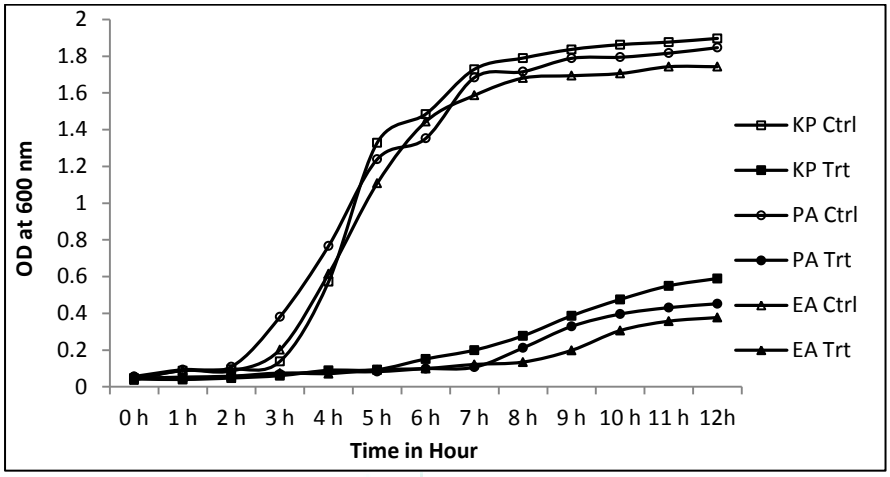

Figure 2: Effect of chloroform fraction of PJ on growth pattern of gram negative MDR bacterial strains. Ctrl: Control; Trt: Treated with respective IC50 dose of PJCF; KP: *K. pneumoniae*; PA: *P. aeruginosa*; EA: *E. aerugenes*.

Estimation of ROS:

Finally, to understand the mechanism of antibacterial activity of *P. javanica*, intracellular reactive oxygen species (ROS) were estimated after treatment with PJCF at IC_{50} dose. As shown in Fig 3, after treatment of PJCF, the production of ROS was increased drastically with time. It was highest in *E. aerugenes*, in which ROS production increased about 70% in 3 hours compared to control. The order of observed ROS production on 3 MDR bacterial strains were, *E. aerugenes* > *P. aeruginosa* > *K. pneumonia*.

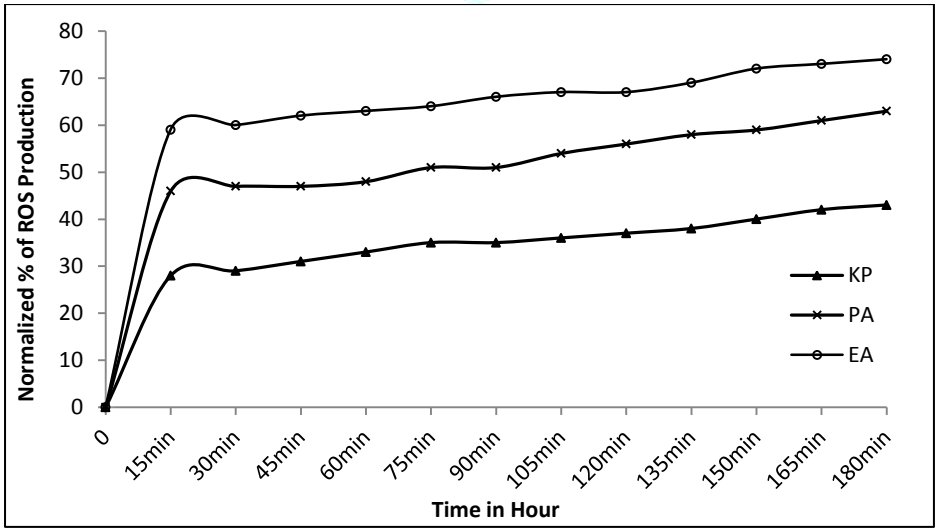

Figure 3: Effect of PJCF on % of normalized ROS production on gram negative MDR bacterial strains. KP: *K. pneumoniae*; PA: *P. aeruginosa*; EA: *E. aerugenes*.

DISCUSSION

The emergence and widespread occurrence of drug resistant bacteria presents a serious global medical crisis, requiring constant surveillance, which continuously challenges the scientific community [20]. The diminishing efficacy and increasing toxicity of synthetic drugs further aggravate this problem. Thus, researchers are directed to seek more natural or organic materials to solve this health problem [14]. Traditional medicine has been used worldwide for centuries, especially the herbal plants for therapeutic purposes against bacterial strains [21]. In this study, *Parkia javanica*, a large plant used as traditional folk medicine

in north-east region of India, has been screened in vitro for antibacterial activity against three multidrug resistant bacterial species known to aggravate the skin wound of diabetic patient.

Five solent fractions of *Parkia javanica* showed antimicrobial activity against all the tested gram negative MDR strains (*E. aerugenes*, *P. aeruginosa*, *K. pneumonia*) with a range of MIC (IC_{100}) values. The two-fold serial dilution technique was used to determine the MIC values and it was observed that, the ratio between IC_{100} dose and MBC of *P. javanica*, for each MDR strains is 1. A sample or any agent is bactericidal when the ratio MBC/MIC ≤ 4 and bacteriostatic when this ratio is > 4 [22]. Therefore, this study plant has bactericidal effect on gram negative MDR strains. However, chloroform fraction of *Parkia javanica* is more potent compared to other fractions, as this fraction can kill the tested MDR bacterial strains at comparatively lower concentration. From growth kinetics study, it is found that, the lag phase of all chloroform fractions of *Parkia javanica* treated bacteria is extended compared to untreated bacterial cells.

The same condition also observed in ROS production. The normalized % of ROS is increased about 70% (in MDR strain), in presence of PJCF. Reactive by products of oxygen, such as superoxide anion radical (O2), hydrogen peroxide (H_2O_2), 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 energy [23]. These species cause damage to proteins, lipids, and nucleotides, negatively impacting the organism [24]. Living organisms own 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[25]. However the damage ensues when the concentration of active oxygen increases to a level that exceeds the cell's defence capacity [26]. Therefore, ROS induced cell damage or macromolecular damage, could be the reason for the death of the microorganism found in this study in response to the *Parkia javanica* chloroform fraction.

CONCLUSION

In this study, we reported the antibacterial activity of chloroform fraction of *Parkia javanica* with their mode of action. The study showed that, chloroform fraction of *Parkia javanica* was effective at lower concentrations compared to other solvent fractions on MDR strains. The increased ROS production may be the possible mechanism of antibacterial activity of of chloroform fraction of *Parkia javanica*.

COFLICT OF INTERESTS

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

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