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CHLOROFORM FRACTION OF PARKIA JAVANICA BARK IS THE MOST POTENT SOLVENT FRACTION REGARDING ANTIBACTERIAL ACTIVITY AGAINST STANDARD BACTERIAL SPECIES COMMONLY FOUND IS SKIN WOUND

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ABSTRACT

Aim: *To evaluate potent solvent fraction of Parkia javanica having antibacterial activity against standard bacterial strains which are commonly found in skin wound.* ***Methods:*** *The different solvent fractions of Parkia javanica were 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 and finally reporter gene assay was performed to understand the mode of action of the active fraction of this study plant.* ***Results:*** *The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were obtained with a range of IC_{100} dose of 0.1563 to 0.625 mg/ml in case of standard bacterial strains. Chloroform fraction of Parkia javanica was comparatively more potent than the other fractions. The lag phase of all treated bacteria is extended compared to untreated cells. The normalized % of ROS is increased in presence of chloroform fraction and this fraction is also responsible for breakdown of the plasmid DNA.* ***Conclusion:*** *This study suggests that, the chloroform fraction 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.*

KEY WORDS

Parkia javanica, standard ATCC bacterial strains, growth curve, ROS, DNA damage

INTRODUCTION

The therapeutic properties of various medicinal plants have been used to treat human diseases. It has been estimated that between 90% of the populations of developing countries used traditional and botanical medicines almost exclusively and consider them to be a normal part of primary healthcare (WHO, 2002). Consumers are interested in complementary and alternative medicines, including herbal medicine, as they perceive these forms of healing as being both safe and effective [1]. The expanding bacterial resistance to antibiotics has become a growing concern worldwide [2]. Intensive care physicians consider antibiotic-resistant bacteria, a significant or major problem in the treatment of patients [3]. Certain bacterial species, such as, *Staphylococcus aureus*, *Streptococcus pyogens*,

Bacillus subtilis, *Escherichia coli* commonly occur in skin infection, even in the chronic skin wound of diabetic patients [4]. 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 infections [7]. So, there is a continuous and urgent need to discover plants with anti-microbial activities with diverse chemical structures and novel mechanisms of actions. The wide acceptance of traditional medicine as an alternative form of healthcare and the alarming increase in the incidence of new and re-emerging infectious diseases bring about the necessity to investigate these medicinal plants. The plant extracts have great potential as antimicrobial activities and the medicinal values of a plant lies in the bioactive compounds such as alkaloids, flavonoids, tannins and phenolic compounds that produce a definite physiological action on the human body.

The plant, *Parkia javanica* is traditionally used as a food and ethno medicine by tribal population of Northeast India [8, 9, 10]. In spite of having long ethno medicinal history, this plant has not been fully explored on scientific basis regarding its medicinal activities. 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 [11,12,13]. Therefore, the present work has been designed to identify the active solvent fraction and to understand mode of action of this fraction as antibacterial agent using standard ATCC bacterial strains, which are predominantly found in skin wound.

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 of Plant Extract

Fresh stem barks of *Parkia javanica* 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 [14].

Bacterial Culture and Growth Conditions

Both standard gram-negative bacterial species: *Escherichia coli*. (ATCC 11229) 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° C [15].

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 bacterial strains in separate 96 well plates, in presence of increasing concentrations of 5 solvent fractions. 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,

% 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° 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 [16].

Measurement of Bacterial growth Kinetics

To determine the bacterial growth kinetics, in presence of chloroform fraction, each bacterial species were grown in Muller Hinton Broth in presence and absence of extract 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 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} [15].

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 *Parkia Javanica* chloroform fraction (PJCF) 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) [17].

DNA damage Assay

To examine the effect of PJCF 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 IC_{50} dose of PJCF. 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 colonies [17].

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 and MBC:

Antibacterial activity of fractions of *P. javanica* on standard gram positive and gram-negative bacterial strains, were obtained by determining the minimum inhibitory concentrations. As shown in table 1, among 5 different solvent fractions, PJCF is most effective at 0.1563 mg/ml concentration on *B. subtilis* and *S. pyogenes,* compared to other bacterial strains and other solvent fractions. The order of observed sensitivity to chloroform fraction, of standard bacterial strains were, *B. Subtilis ≈ S. pyogenes > S. aureus > E. coli.*

Table 1: MIC values of Standard bacterial Strains.

	E. coli	***B. subtilis***	***S. aureus***	***S. pyogens***
	IC_{100}*	***IC_{100}****	***IC_{100}****	***IC_{100}****
n-Haxane	*0.62 ± 0.03*	*0.31 ± 0.05*	*0.62 ± 0.02*	*0.31 ± 0.06*
Chloroform	*0.31 ± 0.02*	*0.15 ± 0.06*	*0.31 ± 0.02*	*0.15 ± 0.02*
Ethylacetate	*0.62 ± 0.07*	*0.31 ± 0.03*	*0.62 ± 0.07*	*0.31 ± 0.06*
n-Butanol	*0.62 ± 0.04*	*0.31 ± 0.04*	*0.62 ± 0.04*	*0.31 ± 0.09*
Methanol	*0.62 ± 0.03*	*0.31 ± 0.07*	*0.62 ± 0.03*	*0.31 ±0.01*

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

Table 2: MBC values and ratio of MBC/MIC of Standard bacterial Strains.

	E. coli		*B. subtilis*		*S. aureus*		*S. pyogens*	
	MBC*	***MBC/MIC***	***MBC****	***MBC/MIC***	***MBC****	***MBC/MIC***	***MBC****	***MBC/MIC***
n-Haxane	0.62 ± 0.08	1.00	0.31 ± 0.03	1.00	0.62 ± 0.04	1.00	0.31 ± 0.02	1.00
Chloroform	0.31 ± 0.04	1.00	0.15 ± 0.08	1.00	0.31 ± 0.07	1.00	0.15 ± 0.08	1.00
Ethylacetate	0.62 ± 0.02	1.00	0.31 ± 0.02	1.00	0.62 ± 0.02	1.00	0.31 ± 0.05	1.00
n-Butanol	0.62 ± 0.09	1.00	0.31 ± 0.01	1.00	0.62 ± 0.06	1.00	0.31 ± 0.07	1.00
Methanol	0.62 ± 0.06	1.00	0.31 ± 0.05	1.00	0.62 ± 0.03	1.00	0.31 ±0.06	1.00

****Concentration of extracts in mg/ml. 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 different fractions of *P. javanica* on each bacterial strain was also determined. According to Table 1 and Table 2, the ratio between MBC and MIC for each bacterium is same (~1, for all bacteria). This result indicated that, fractions of *P. javanica* possess bactericidal activity rather than bacteriostatic.

Measurement of Bacterial Growth Kinetics:

As shown in Table I, PJCF is most active fraction that kills the bacterial species at too lower concentration. So, we next measured the growth curve of both gram negative and gram positive standard bacterial strains and MDR strains to examine the pattern of the growth curve in presence and absence of PJCF. All the bacterial strains were exposed to PJCF separately, at a concentration of IC_{50} dose for each bacterium. As shown in Fig 2, the lag phase of PJCF treated all bacterial strains were extended compared to control. The growth of *E. aerugenes* is mostly affected by the PJCF extract.

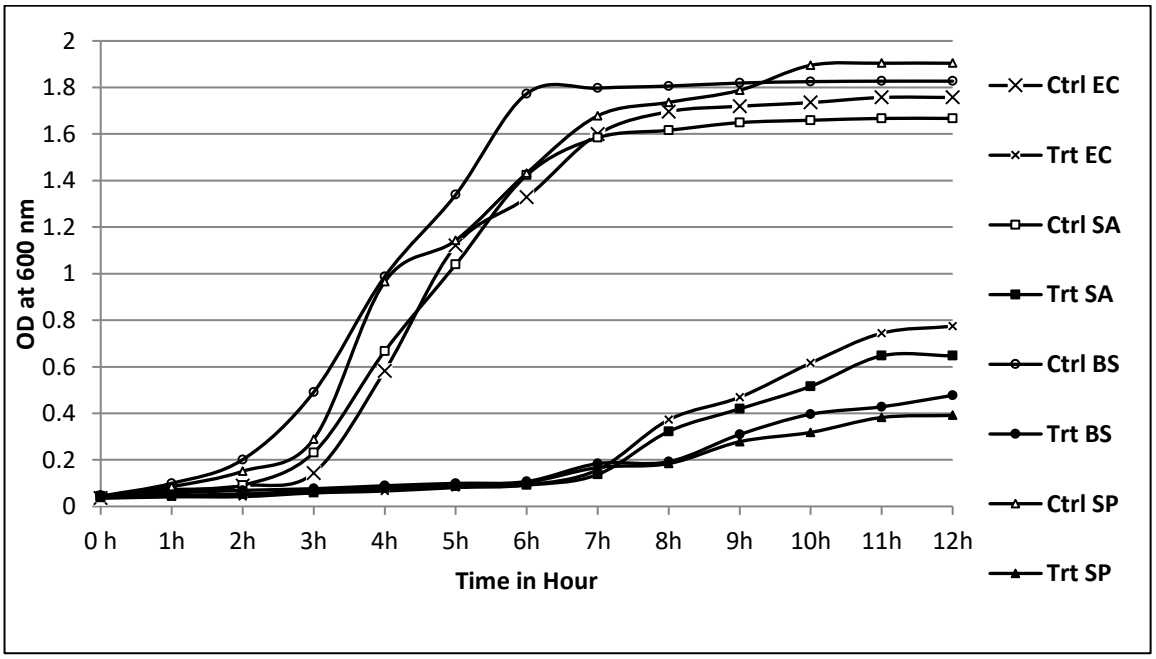

Fig. 1: Effect of chloroform fraction of PJ on growth pattern of standard gram positive and gram negative bacterial strains. Ctrl: Control; Trt: Treated with respective IC50 dose of PJCF; EC: *E. coli*; SA: *S. aureus*; BS: *B. subtilis*; SP: *S. pyogens*; DH: *E. coli* DH5α.

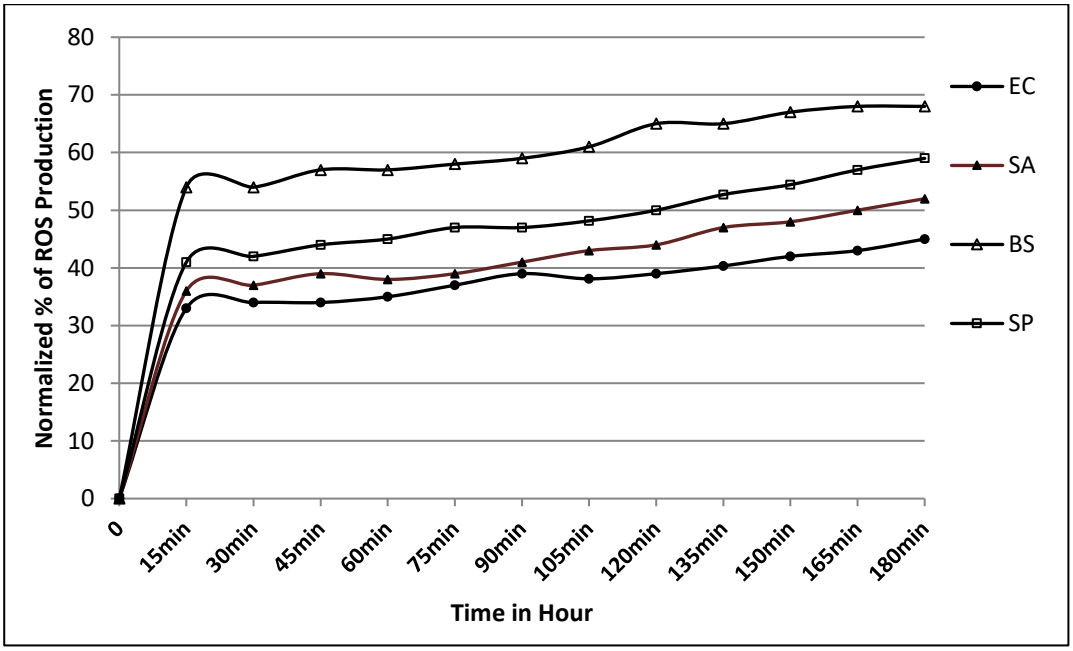

Fig. 2: Effect of PJCF on % of normalized ROS production of standard gram positive and gram negative bacterial strains.; EC: *E. coli*; SA: *S. aureus*; BS: *B. subtilis*; SP: *S. pyogens*.

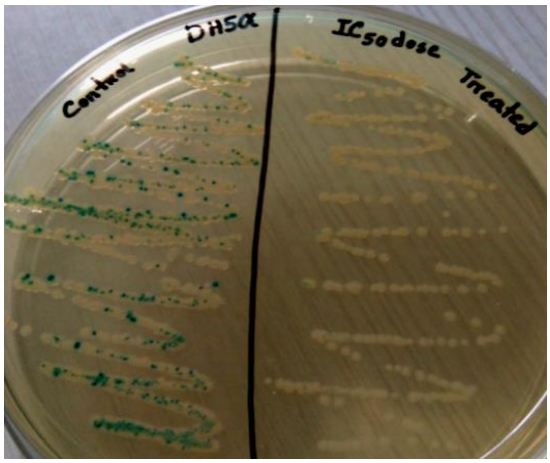

Fig .3: Reporter gene (*β*-galactosidase) assay in PJCF treated pUC19 Transformed *E. coli DH5α*.

Estimation of ROS:

Finally, to understand the mechanism of antibacterial activity of PJCF, intracellular reactive oxygen species (ROS) were estimated after treatment with PJCF at IC_{50} dose. As shown in Fig 3, after treatment, 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 *E. coli*, ROS production increased about 35%. The order of observed ROS production on different bacterial strains were, *B. Subtilis* > *S. pyogenes* > *S. aureus* > *E. coli.*

DNA Damage assay:

As shown in Fig 2, ROS production was increased 35 - 70% compared to control and as ROS usually targets the cellular DNA, so, to observe the effect of ROS inside bacterial cells, we used plasmid-based reporter gene assay. In Fig 4, reporter gene *β*-galactosidase was assayed by transforming the bacteria with the *pUC*19 plasmid and then the bacterial cells were treated with PJCF. The blue colour colonies, formed due to hydrolysis of X-gal by *β*-galactosidase enzyme, were completely absent in case of bacterial cells treated with PJCF.

DISCUSSION

The use of natural products as alternatives or complementary to conventional therapy has gained interest due to the perception that herbal products may be safe. Research on the efficacy of many natural products is currently under way with efforts to validate the reported pharmacological effects and also to identify active constituents that are responsible for many of the reported biological activities (18). *In vitro* evaluation of plants for antimicrobial properties is the first step towards achieving the goal for developing eco-friendly management of infectious diseases (19). 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* to identify the active fraction having antibacterial activity, against bacterial species known to occur and aggravate the skin wound.

All the solvent fractions of *Parkia javanica* (PJHF, PJCF, PJEF, PJBF, PJMF) 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 (IC_{100}) values. The two-fold serial dilution technique was used to determine the MIC values and it was observed that, both the IC_{100} dose and MBC, obtained using this technique are too less on some bacterial species. Although, each solvent fraction of *Parkia javanica* possess antibacterial activity, however, PJCF is more potent compared to other fractions, as it can inhibit the bacterial growth at comparatively lower concentration. As, the ratio between MBC and MIC is equal to one, therefore, it can mention that, PJCF not only inhibit the growth of bacteria but, also can kill the bacterial strains as well as it is a bacteriocidal agent. From growth kinetics study, it was found that, the lag phase of all PJCF 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 PJCF. 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 energy [20]. These species cause damage to proteins, lipids, and nucleotides, negatively impacting the organism [21]. Living organisms have to build up 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. Bacterial genetic responses to oxidative stress are controlled by two major transcriptional regulators (OxyR and SoxRS). ROS damage a variety of cellular macromolecules and thus elicit adaptive oxidative stress responses in bacteria intended to permit survival in the presence of this stressor [22, 23]. The antioxidant mechanisms are recruited in response to antimicrobial exposure, antimicrobials being known to generate ROSs that are key to the often-lethal effects of these agents [24]. However, the damage ensures when the concentration of active oxygen increases to a level that exceeds the cell's defense capacity.

There is a fully absence of blue colour colonies after treatment in reporter pUC19 plasmid DNA damage or mutation. Therefore, the active compound(s) present in PJCF perhaps operated ROS induced DNA and other macro molecular damage to exert antibacterial activity. As the PJCF can kill the variety of bacterial strains at lower concentrations, so, it may be a potent and cost effective antibacterial therapeutic agent(s).

CONCLUSION

In this study, we reported the antibacterial activity of PJCF with their mode of action. The studies showed that, the PJCF was effective on variety of bacterial strains. The ROS induced DNA damage is the possible mechanism of antibacterial activity of chloroform fraction of *Parkia javanica*. As PJCF can kill bacteria at lower concentration and also alters growth pattern of tested bacteria, therefore, in conclusion it can be mentioned that, chloroform fraction of *Parkia javanica* is the most potent solvent fraction regarding antibacterial activity against standard bacterial species commonly found is skin wound.

CONFLICT OF INTERESTS

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

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