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Research Article

ACTIVITY OF THE MEDICINAL PLANT *PARKIA JAVANICA* AGAINST MULTIDRUG-RESISTANT *NEISSERIA GONORRHOEAE* AND OTHER CLINICAL ISOLATES

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ABSTRACT

Objective: The objective of this study was to look into the *in vitro* activity of *Parkia javanica* against isolates of *Neisseria gonorrhoeae*.

Methods: Methanolic extract of *P. javanica* bark (MEPJ) and organic fractionation were tested against one standard strain and 10 clinical isolates including one multidrug-resistant (MDR) isolate of *N. gonorrhoeae* through minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) tests.

Results: The MDR isolate, standard strain, as well as all the clinical isolates were inhibited by MEPJ as well as all the fractions except water fraction. Chloroform fraction showed the best activity with MIC and MBC values, both being 0.2 mg/ml. Ethyl acetate fraction also showed MIC value at 0.2 mg/ml; however, MBC value was at 0.3 mg/ml. MIC and MBC values of MEPJ were both 0.3 mg/ml.

Conclusion: Chloroform fraction, ethyl acetate fraction, and MEPJ bark showed the lowest MIC values and can be considered as prospective candidates for the development of antigonococcal topical drugs.

Keywords: Medicinal plants, *Parkia javanica* (Lam.) Merr., *Neisseria gonorrhoeae*, Multidrug resistance, Organic fractions, Minimum inhibitory concentrations, Minimum bactericidal concentrations.

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INTRODUCTION

All living organisms including plants have innate mechanisms to defend themselves against invading pathogens, which may be targeted to particular attackers, or generalized for diverse pathogens from insects to bacteria and fungi [1]. Before the invention of synthetic drugs and antibiotics, man had to rely on these active natural products mostly from plants, microbes, and other animals [2]. As per the World Health Organization estimation, even today 65–80% of the world population relies on herbal medicines [3] which are eco-friendly, natural and have lesser chances of giving adverse drug reactions and resistance [4].

Gonorrhea is a sexually transmitted infection (STI) causing urethral or cervical discharge and ocular, pharyngeal, and anorectal infections [5]. Worldwide, an estimated 62 million new infections of gonorrhea occur annually [6], making it the second most common STI. The infectious agent, *Neisseria gonorrhoeae* remains asymptomatic in as high as 55% of men and 86% of women [7]. Untreated gonorrhea is a problem, causing pelvic inflammatory disease and infertility in women [8], and prostate cancer in men [9]. *N. gonorrhoeae* can also increase HIV-1 transmission by 500% [10]. Once an easily curable disease, gonorrhea has become a major problem due to drug resistance and cross-resistance.

Parkia javanica (Lamk.) Merr. (Synonyms: *Parkia roxburghii* G. Don, *Parkia timoriana* (DC.) Merr.) belonging to the family Mimosaceae is a medicinal plant well distributed in India and Southeast Asia [11]. It is a multipurpose tree being used as an insecticide, pesticide, human food, and cosmetic purposes [11]. Medicinally, it is used for stomach problems such as stomach ache and cholera [12], diarrhea, dysentery, and food poisoning [13]. Tribal people of Tripura use its extract to cure wounds and scabies [14]. With its widely suited applications and activity against standard Gram-positive and Gram-negative bacteria [15], we wanted to look into the *in vitro* activity of *P. javanica* on locally circulating clinical isolates of *N. gonorrhoeae*.

MATERIALS AND METHODS

Plant material

Fresh barks of *P. javanica* were collected from the Tripura University Campus at Suryamaninagar, Agartala, Tripura, during December 2015. For ensuring correct botanical identification, the plant was cross-verified by Prof. B. K. Datta, Taxonomist, Department of Botany, Tribhuvan University, and a herbarium specimen (#BD-01/06) was deposited in the Central National Herbarium, Botanical Garden, Shibpur, Howrah, West Bengal, India.

Preparation of extract

Barks of *P. javanica* were washed and air-dried for 3 weeks. They were then chopped into smaller pieces and partially ground with mortar and pestle. Around 200 g of ground bark was macerated with 600 ml of methanol at room temperature for 48 h. Next, the decanted extract was filtered through Whatman filter paper 4 and evaporated in a rotary evaporator (Hahn Shin Scientific Corporation, South Korea). At the end of this, methanolic extract of *P. javanica* bark (MEPJ) was left to air dry for a week at 4°C to remove traces of the solvent. Similarly, organic fractionation was carried out by extracting successively with 600 ml of different polarity-based solvents, which were chloroform, ethyl acetate, *n*-butanol, methanol, and water. Percentage recovery was calculated as per the method of Anokwuru *et al*. [16] using the formulae:

$$\% \text{ recovery} = \frac{\text{Amount of pure product recovered (g)}}{\text{Amount of crude material used (g)}} \times 100$$

The final products were dissolved in 25% DMSO in water to give a stock of 100 mg/ml. Concentrations used in the study were 0.05, 0.1, 0.2, 0.3, 0.6, and 1.2 mg/ml.

Identification, isolation, culturing, and antimicrobial susceptibility testing (AST) of clinical isolates of *N. gonorrhoeae*

The institutional ethical committee clearance was obtained before collection of urethral discharge from consented male symptomatic subjects. Of each specimen, direct Gram stain was observed and cultured on chocolate agar as previously described [17]. Confirmatory tests were colony characteristics, Grams staining reactions, biochemical tests, and sugar assimilation tests, which were done as per standard protocol. AST through E test was tested against penicillin G, tetracycline, ciprofloxacin, nalidixic acid, azithromycin, spectinomycin, and ceftriaxone on CA without supplements as per Clinical and Laboratory Standards Institute guidelines [18]. E-strips were obtained from HiMedia Laboratories, Mumbai, India. One quality control (ATCC®49226™) strain and 10 clinical isolates including one multidrug-resistant (MDR) strain were tested against MEPJ and *P. javanica* fractions. The isolates were stored at −80°C for long-term storage.

Determination of the minimum inhibitory concentration (MIC)

MIC of MEPJ and *P. javanica* fractions was determined against the isolates of *N. gonorrhoeae* as per the previous protocol [19]. Briefly, double dilutions of the extract in deionized water in 100 µl aliquots were placed in 96-well plates. To this, 140 µl of Mueller-Hinton broth and 10 µl of *N. gonorrhoeae* isolate were added and incubated at 37°C for 16–18 h in a candle jar. Organism growth was assayed by the measurement of OD600 on a Synergy H1 microplate reader (BioTek, USA). The MIC value (mg/ml) was expressed as the lowest concentration that inhibited 99.99% of bacterial growth.

Determination of minimum bactericidal concentration (MBC)

MBC was determined as per the protocol of Oliveira *et al*. [20] the extract at the MIC and two above and one below concentrations were subcultured onto chocolate agar plates and incubated for 48 h. MBC (mg/ml) was determined as the lowest concentration of the extract that prevented more than 99.99% microbial growth.

RESULTS

Table 1 shows the final yields which were obtained when initial 200 g of ground bark was macerated with 600 ml of respective solvent. Based on the yield, the percentage recovery was calculated. Crude methanolic extract (MEPJ) had the highest percentage recovery. Percentage recovery among the fractions was found to increase with increase in polarity of the solvent. Physical characteristic of the crude and each fraction is also noted in Table 1.

Antibacterial activity of *P. javanica* was tested against 10 clinical isolates of *N. gonorrhoeae* and one standard ATCC strain. Susceptibility of each strain to standard drugs is shown in Table 2. An MDR isolate as per the criteria previously described [21] was also included in the study.

The MDR strain which was resistant to spectinomycin, azithromycin, fluoroquinolones drugs, and tetracycline was found to be inhibited by MEPJ as well as all the fractions except water fraction. The other strains as well as the standard strain ATCC® 49226™ were also inhibited. Activities of MEPJ as well as the fractions against all the 11 isolates were comparable. Fig. 1 shows the antibacterial activity of MEPJ and organic fractions against the isolates. As the percentage inhibition curves show, MIC concentrations ranged between 0.2 and 0.6 mg/ml.

MEPJ, chloroform fraction, and ethyl acetate fractions showed good activity against all the isolates with chloroform fraction showing the best activity with MIC (Fig. 1) and MBC (Table 3) values both being 0.2 mg/ml. Ethyl acetate fraction also showed MIC value at 0.2 mg/ml; however, MBC value was a dilution higher, at 0.3 mg/ml. MIC and MBC values of MEPJ were both 0.3 mg/ml. Although n-butanol fraction inhibited isolates at 0.3 mg/ml, its MBC concentration was found to be 0.6 mg/ml. Inhibitory activity of methanol fraction was also strong but not stronger than that of the previously mentioned extracts. Water fraction showed very high MIC values of >1.2 mg/ml, indicating limited antibacterial activity.

DISCUSSION

P. javanica was selected based on ethnobotanical data on the use of the plant among local tribes and people of Northeast India for medicinal purposes. The previous work from our laboratory showed its activity

Table 1: Yield of *Parkia javanica* crude methanolic extract of stem bark (MEPJ) and various organic fractions

Initial dry weight used: 200 g					
Solvent	**Solvent volume**	**Rotary evaporator temperature**	**Yield (g)**	**Recovery (%)**	**Physical appearance**
MEPJ	600 ml	70°C	30	15	Deep brown powdery mass
Chloroform		65°C	0.64	0.32	Deep yellow sticky mass
Ethyl acetate		80°C	0.29	0.145	Light brown powdery mass
n–Butanol		120°C	1.82	0.91	Light golden powdery mass
Methanol		70°C	5.68	2.84	Deep brown powdery mass
Water		100°C	18	9	Deep brown muddy mass

MEPJ: Methanolic extract of *Parkia javanica*

Table 2: Antibiotic susceptibility profile of the clinical isolates toward recommended drugs

***Neisseria gonorrhoeae* clinical isolates**	**Ceftriaxone**	**Spectinomycin**	**Azithromycin**	**Penicillins**	**Fluoroquinolones**	**Tetracyclines**
Isolate 1	S	S	S	S	S	S
Isolate 2 (MDR)	S	R	R	S	R	R
Isolate 3	S	S	S	R	R	R
Isolate 4	S	S	S	S	R	S
Isolate 5	S	S	S	S	S	S
Isolate 6	S	S	S	R	R	R
Isolate 7	S	S	R	R	R	R
Isolate 8	S	S	S	R	R	R
Isolate 9	S	S	S	R	R	S
Isolate 10	S	S	S	R	S	R
ATCC® 49226™	S	S	S	S	S	S

*A single isolate (Isolate 2) showed multidrug resistance (MDR). †S: Susceptible, R: Resistant

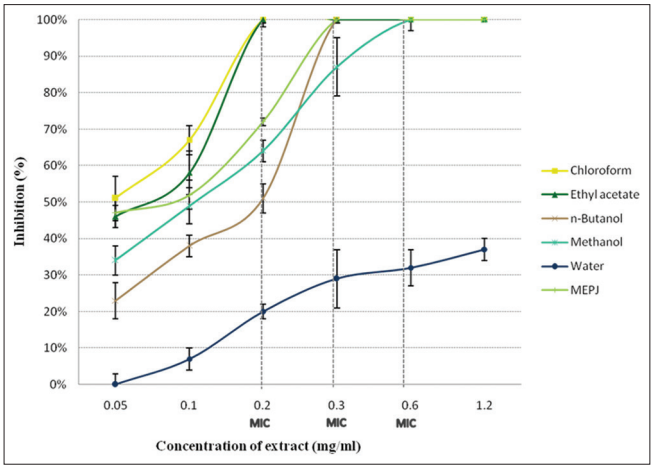

Fig. 1. Concentration-dependent inhibition of *Neisseria gonorrhoeae* by methanolic extract of *Parkia javanica* and various organic fractions. The activity was expressed as percentage inhibition. Each point represents mean ± standard deviation values obtained from 10 clinical isolates and one standard strain

Table 3: Antimicrobial potential of MEPJ and various organic fractions by MIC and MBC assays on *Neisseria gonorrhoeae* isolates

Crude extract	MEPJ	MIC (mg/ml)	MBC (mg/ml)
		0.3	**0.3**
Organic fractions	Chloroform	0.2	0.2
	Ethyl acetate	0.2	0.3
	n-Butanol	0.3	0.6
	Methanol	0.6	0.6
	Water	>1.2	>1.2

*MIC and MBC (mg/ml) were determined as the lowest concentration of extract that prevented more than 99.99% bacterial growth. The data were derived from representative values of 10 clinical isolates and one standard strain. MEPJ: Methanolic extract of *Parkia javanica*, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

against standard Gram-positive and Gram-negative organisms [15] and the parasite *Leishmania donovani* [22].

The phonological age of a plant plays a major role in determining the distribution of secondary metabolites [23]. The plant from which barks in this study were collected was luckily producing rich amount of active principles. It can also be that *P. javanica* produces active principles all through its different stages of life. *P. javanica* being a tree, the chances of the presence of secondary metabolites were higher since active principles are found in higher concentrations in woody plants contributing to herbivore avoidance strategy [24].

Chloroform fraction, ethyl acetate fraction, and MEPJ showed the lowest MIC values of 200 µg, 300 µg, and 300 µg, respectively, and can be considered as prospective candidates for microbicide development. These MIC values were much higher than those reported in similar studies [25,26]. The MIC concentrations observed in our study were, however, lower than those reported by Kuete *et al.*[27].

P. javanica is one of the costliest vegetables in Northeast India and its application in traditional medicine, face wash and shampoo, and to heal wounds [11] implies that the plant possibly has little to no toxicity, though this is a subject of future research.

In our study, the organic fractions of *P. javanica* showed reduction in activity in the order that they were fractionated. The best antibacterial activity against *N. gonorrhoeae* isolates was observed in the chloroform fraction, followed by the ethyl acetate fraction, n-butanol fraction, methanol fraction, and water fraction. Similar studies of antimicrobial activity by other plant extracts have also shown decrease in activity with increase in polarity [28,29]. The order in which the organic solvents were used for extraction could explain that the active ingredients were more lipophilic. The free radical scavenging potential of other plant extract studies [30] has been found in the order of chloroform >ethyl acetate >n-butanol and could explain the antimicrobial potential observed in the current study. The possibility of the presence of impurities with increase in polarity could also be a reason which has diluted the concentration of active ingredients in more polar organic fractions.

The standard drugs recommended for treatment, which are ceftriaxone and spectinomycin, however, exhibited better MIC values against *N. gonorrhoeae*, both clinical isolates and the laboratory standard. This was expected due to the presence of various impurities in MEPJ extract and organic fractions. The MDR strain, however, could not be inhibited by spectinomycin; where as MEPJ and most of the fractions inhibited the strain at low concentrations. This shows that if further purification and isolation can be done to obtain the active antimicrobial ingredients present in *P. javanica*, it is to be expected that the inhibitory and bactericidal values will be in much lower range and comparable to purified molecules present in synthetic drugs.

Drawbacks of the study and all studies related to plant extracts, in general, are the season of collection, phenological age of the plant, climatic, and abiotic factors since determining the ideal conditions and controlling external factors can be difficult. Since batch-to-batch variations affect the presence of secondary metabolites, in the present study, we collected the raw material in one batch sufficient to prepare the crude extract as well as the fractions. Future studies on *P. javanica* should be focused on methods to identify antimicrobial molecules, which can avoid such problems as batch-to-batch variations and also pure compounds can be expected to be effective at much lower concentrations.

CONCLUSION

P. javanica was effective in inhibiting *N. gonorrhoeae*. The plant was able to inhibit MDR clinical isolate as well as other clinical isolates and standard strain of *N. gonorrhoeae*. MEPJ, the crude methanolic extract of the bark of *P. javanica*, and most of the fractions obtained through successive extraction with the various solvents were effective in inhibiting the bacteria, except for the water fraction. Chloroform fraction, ethyl acetate fraction, and MEPJ were the most effective with the lowest MIC values being 200 µg, 300 µg, and 300 µg, respectively. These extracts can, therefore, be considered to be developed into antigonococcal topical drugs. The dosage may be further reduced if the active antimicrobial compounds can be identified and isolated in future.

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AUTHORS' CONTRIBUTIONS

The concept of the study was of Prof. Samir Kumar Sil, Dr. KVR Reddy, Dr. Tapan Majumdar, and Prof. Sumita Mukherjee. Jhinuk Basu Mullick was involved in clinical and experimental studies and data acquisition. All the authors contributed equally to study design, literature search, data analysis, manuscript preparation, and review.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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