



Synergism of *Phyllanthus niruri* Extract with Gentamicin against Methicillin Resistant *Staphylococcus aureus*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MAKP, MM and MAR designed the study, author MAR performed most of the laboratory work. Authors MAR and MM wrote the manuscript. Authors MAR, MM and MAKP managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aims: This study aimed to find out any synergism of gentamicin with the solvent extracts of small tropical herb *Phyllanthus niruri* to combat methicillin resistant *Staphylococcus aureus*.

Methodology: Bioactive constituents of *Phyllanthus niruri* were extracted by macerating ground dry powder of the leaves in water, *n*-hexane, chloroform, methanol and ethanol for 48-72 h followed by filtration and evaporation of solvents. Microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of these extracts. The synergistic effects between gentamicin and the extracts were evaluated by the checkerboard assay to calculate the fractional inhibitory concentration index (FICI). In all cases, ten hospital associated MRSA strains were used.

Results: The MIC of aqueous and methanolic extracts of *P. niruri* against different MRSA strains varies from 3.125 mg/ml to 12.5 mg/ml. For the MRSA strain the combination of methanolic extract with gentamicin decreased the MIC of extract from 6.25 mg/ml to 0.2 mg/ml and the MIC of gentamicin from 2048 µg/ml to 256 µg/ml showing a strong synergistic effect with a fractional

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inhibitory concentration index of 0.157. Steroids, flavonoids, phenolic compounds and quinones identified in the extracts may play role in synergistic relation.

Conclusion: The present investigation shows that bioactive constituents from *Phyllanthus niruri* have an excellent synergy with gentamicin against MRSA and can be further explored as an alternative anti-staphylococcal agent.

Keywords: Methicillin-resistant *Staphylococcus aureus*; synergistic effects; checkerboard method; *Phyllanthus niruri*.

1. INTRODUCTION

Methicillin-resistant *S. aureus* (MRSA) is one of the most regular pathogens of hospital and community-associated infections and remains a major public health problem both in developed and developing countries [1]. In Bangladesh, previous studies indicated the presence of MRSA with varied prevalence rate from 15.38% to as high as 43.7%, however, the real picture is obscure due to lack of nation wide data [2,3]. The treatment choices for MRSA are inadequate, based on alternative antibiotics such as doxycycline, minocycline, clindamycin but resistance has been reported [4]. In some cases, the use of synergistic antibiotic drugs combination is the only option for the treatment of MRSA causing infection where the effect of two antibiotics are greater than the sum of the individual effect [5].

At present, the situation of bacterial drug resistance has devolved into critical stage but as it is observed the development of new effective drugs to combat the situation is not satisfactory.

It is worth to notice that constituents of plants are biologically active and some of modern drugs are analogues of these phytochemicals [6]. Currently, the investigation of synergy between commercially available antibiotics with medicinal plant extracts has become a new conduit for overcoming bacterial drug resistance. In fact, there have been positive outcomes for some plants (*Salvadora persica*, *Nymphaea tetragona*, *Syzygium aromaticum*) in this direction [7,8]. Synergistic effect of plant extracts with antibiotics against both Gram positive and Gram negative bacteria are known. For instance, *Camellia sinensis* showed synergistic activity with nalidixic acid (FICI 0.37) against multi-drug resistant (MDR) *Salmonella typhi* strains, and *Juglans regia* was able to reverse oxacillin resistance in MRSA [9]. The essential points of interest in utilising constituents from plants are based on the fact that they are safer than synthetic

alternatives and can provide affordable treatment [10].

Phyllanthus niruri is a branching annual herb of the family Euphorbiaceae, which is usually found in tropical and subtropical countries of the world [11]. This herbaceous plant has set up its traditional usefulness in numerous health issues such as diarrhea, dysentery, influenza, vaginitis, tumours, jaundice, kidney stone, intermittent fevers, urogenital disorders, scabies and wounds [11,12]. Additionally, this plant is effective in the treatment of kidney problems, urinary bladder disturbances, pain, gonorrhoea, diabetes and chronic dysentery [11,12]. It has also shown antibacterial effect against pathogenic bacteria [13]. However, the synergistic potential of this plant extracts with antibiotics are yet to be resolved. In this study, we aimed to explore any antibacterial activity of different solvent extracts of *P. niruri* against MRSA and to identify its synergistic effect with gentamicin.

2. MATERIALS AND METHODS

2.1 Collection, Identification of Plant and Preparation of Extracts

The fresh, healthy and mature leaves of *Phyllanthus niruri* were collected from different zones of Jahangirnagar University (23° 52' 53" N / 90° 16' 1" E) Dhaka, Bangladesh. It was identified by Ms Shayla Sharmin Shetu, taxonomist, Department of Botany, Jahangirnagar University. The sample of the plant was submitted to the herbarium of the Department of Botany, Jahangirnagar University with the accession number 45314. The leaves were rinsed and air dried, then were coarsely powdered.

Five solvents were selected for extraction: *n*-hexane, chloroform, ethanol, methanol and water. Extraction was performed as described in previous studies with slight modification [9,14]. Ten grams (10 g) of the dried powdered leaves were soaked in 100 ml of distilled water at 80°C

and cold-macerated in other organic solvents (*n*-hexane, chloroform, ethanol, methanol) for 48-72 h and then filtered. The filtrates were evaporated on water bath up to a semisolid mass and air dried. Filtrates were weighed and stored in sterile labelled containers and kept in the refrigerator at 4°C until required.

The filtrate of water extract was then mixed with distilled water to prepare an aqueous extract solution, and filtrates of organic extracts were mixed with dimethyl sulfoxide (DMSO) for the organic extract solutions.

2.2 Isolation and Antibiogram of MRSA Strains

Ten MRSA isolates were obtained from a hospital in Dhaka city and the strains were tested against cefoxitin (30 µg/disc), vancomycin (30 µg/disc), ciprofloxacin (5 µg/disc), tetracycline (30 µg/disc), chloramphenicol (30 µg/disc), gentamicin (10 µg/disc), clindamycin (2 µg/disc), oxacillin (30 µg/disc), and ceftazidime (30 µg/disc) according to the disk diffusion method [15].

2.2.1 Antibacterial activity of extracts

To evaluate the antibacterial activity of the extracts against the bacteria, dried and sterilised filter paper discs (6 mm diameter) were soaked with 10 µl of various concentrations of the extracts (10 mg/ml, 50 mg/ml & 100 mg/ml) [14]. Discs were then placed on the Muller Hinton agar medium homogeneously seeded with the test microorganisms (10⁷ CFU/ml). Standard disc of gentamicin was used as positive control and discs impregnated with solvent were used as negative control. Plates were then incubated at 37°C for 18-20 h.

2.2.2 Determination of MIC of extracts

Minimum inhibitory concentration (MIC) of gentamicin and extracts were evaluated by the broth microdilution method in sterile 96-well polystyrene culture plates. 100 µl of Mueller–Hinton broth was dispensed into each well of the 96-well plate. A 100 µl from the stock solution of test extracts (e.g. a concentration of 100 mg/ml) was added into the first row of the plate. Then, serial dilutions were performed to obtain concentration of extracts ranged from 50 to 0.09 mg/ml. A negative control was prepared with plant extract and media as well as a positive control was prepared with the inoculum and media. Then test plates were incubated at 37°C

for 18 hours. The well with the lowest dilution with no recognisable growth by visual assessment was considered as MIC [9].

2.2.3 Determination of MBC

Two fold concentrated test product dilutions were plated to determine the minimum bactericidal concentration (MBC), and enumerated to determine viable CFU/ml. After incubation, the concentration at which no visible growth found was recorded as the MBC [9].

2.3 Determination of Synergistic Activity of Plant Extracts with Gentamicin

The checkerboard method is often combined with the calculation of a fractional inhibitory concentration (FIC) index to test the antimicrobial potencies of drugs. FIC was derived from the lowest concentration of gentamicin and plant extract in combination permitting no visible growth of the test organisms on the 96 wells micro titer plate [16,17]. The FIC value for each agent was calculated using the formula:

FIC (Gentamicin) = MIC of gentamicin in the combination / MIC of gentamicin alone

FIC (extract) = MIC of extract in the combination / MIC extract alone.

FIC index= FIC of extract+ FIC of Gentamicin

Combinations were classified as “synergistic” if the FIC indices were <0.5, “additive” if the FIC indices were = 0.5 to <1 “indifferent” if the FIC indices were between 1 and 2 and “antagonistic” if the FIC indices were ≥ 2.

The stock solutions of gentamicin used ranged from 2 µg/ml to 2048 µg/ml. The stock solutions of aqueous and methanolic extract of *P. niruri* used ranged from 0.4 mg/ml to 12.5 mg/ml. A total of 100 µl of sterile Mueller Hinton broth was distributed aseptically in all the wells of 96 well culture plate. The antibiotic gentamicin was serially diluted along the abscissa, while the extract of *P. niruri* was diluted along the ordinate. Each well was inoculated with 100 µl of the culture and was incubated at 37°C for 18 h. The plates were observed for growth of the test organism. The well where growth was completely inhibited considered as effective MIC for the combination.

2.4 Phytochemical Screening of the Plant Extracts

Preliminary phytochemical screening which consists of performing chemical tests to detect

the presence of steroids, terpenoids, alkaloids, flavonoids, coumarins, saponins, tannins, phenolic compounds and quinones was performed following standard methods [18].

3. RESULTS

3.1 Antibiogram of MRSA

All MRSA strains were completely resistant to oxacillin and gentamicin and showed varied resistance against other antibiotics (Fig. 1).

3.2 Antibacterial Activity of Plant Extract

Out of five types of extract, only methanolic and aqueous extracts showed antibacterial activity. Disk diffusion of the *P. niruri* extracts (aqueous, methanolic) against these MRSA strains showed an increasing zone of inhibition as the concentration of the extracts is increased (Table 1). A highest zone of inhibition was 15 mm with 100 mg/ml aqueous extract. The MIC was 3.125 mg/ml and MBC was 6.25 mg/ml for both extracts (Table 1).

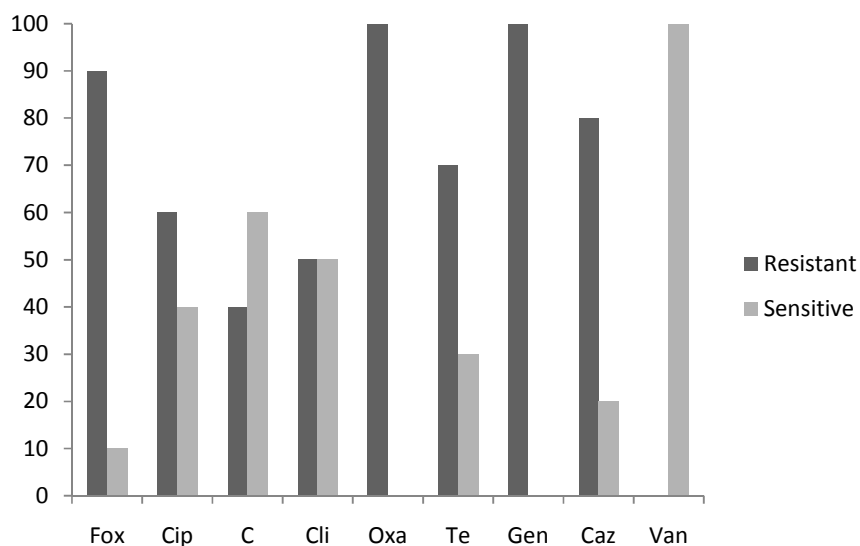


Fig. 1. Level of sensitivity of MRSA strains against different antibiotics. Fox: ceftaxime; Cip: ciprofloxacin; C: chloramphenicol; Cli: clindamycin; Oxa: oxacillin; Te: tetracycline; Gen: gentamicin; Caz: ceftazidime; Van: vancomycin

Table 1. Zone of inhibition of *P. niruri* extracts (aqueous, methanolic) against MRSA strains and respective MIC and MBC values

Organism ID	Zone of inhibition (mm)						MIC (mg/ml)		MBC (mg/ml)	
	Concentration (mg/ml)						(Aqueous)	(Methanol)	(Aqueous)	(Methanol)
	aqueous			Methanol						
100	50	10	100	50	10					
S-7	12	11	7	14	11	7	6.25	3.125	12.5	6.25
S-14	15	12	10	13	11	7	3.125	3.125	6.25	6.25
S-16	10	8	0	12	9	6	12.5	6.25	25	6.25
S-26	13	10	6	14	11	7	3.125	3.125	6.25	3.125
S-29	13	9	7	14	8	7	3.125	3.125	6.25	3.125
S-31	12	9	7	13	9	7	6.25	3.125	12.5	6.25
S-33	10	7	0	13	10	6	12.5	6.25	25	6.25
S-56	12	10	7	10	8	0	12.5	12.5	12.5	25
S-60	12	9	7	11	9	6	6.25	6.25	12.5	12.5
S-61	12	8	0	12	8	7	12.5	6.25	12.5	6.25

3.3 Synergistic Effect of Extracts with Gentamicin

A synergistic relation was found between gentamicin and methanolic extract of *P. niruri* as well as an additive effect between gentamicin and aqueous extract of *P. niruri* in checkerboard method (Table 2). Reduction in the MICs of gentamicin was observed, which explains the strong synergy (FICI 0.157) of the combination of methanolic extracts of *P. niruri* with gentamicin.

3.4 Phytochemical Screening Result

In preliminary phytochemical screening of the aqueous and organic solvent extracts of *P. niruri* leaves the presence of different phytochemicals such as alkaloids, flavonoids, phenolic compounds and quinones were detected. Terpenoid was absent in all types of extracts (Table 3).

4. DISCUSSION

Plants are rich in a wide variety of phytochemicals like flavonoids, tannins, terpenoids, alkaloids, pigments, enzymes and minerals, which have disease preventive properties against plant pathogens as well as some antimicrobial activities [19,20]. *Phyllanthus niruri* has been used in different traditional drugs for centuries in countries where it is endemic

such as India, China and Indonesia [21]. *P. niruri* has found to contain flavonoids, catechin, terpenes, coumarins, lignans, tannins, saponins and alkaloids as its active constituents [21]. Our preliminary phytochemical screening of *P. niruri* extracts revealed the presence of different active phytochemicals such as, flavonoids, phenolics, quinones, saponin etc as previously reported, however, we did not find terpenoid in any types of extracts.

In different investigations, low to moderate antibacterial activity of aqueous, methanolic and ethanolic extract of *P. niruri* was observed against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus cereus* [22,23]. The zone of inhibition against *S. aureus* was reported 15 mm for methanolic extract in a study of Mathur et al. [23]. Similarly, we found antibacterial activity against MRSA with high zone of inhibition 15 mm. In a previous study, the MIC of ethanolic extract against *S. aureus* was 12.5 mg/ml whereas we found the MIC and MBC both was as low as 3.125 mg/ml for methanolic and aqueous extract [22]. However, no antibacterial activity of ethanolic extract was detected in this study, this echoed the result of previous study where methanolic extract was more antibacterial than ethanol and water extract [13]. For most of the MRSA strains MIC and MBC values of the methanolic extract of *P. niruri* were lower

Table 2. Minimum inhibitory concentration of *P. niruri* extracts in combination with gentamicin against an MRSA determined by checkerboard method in a 96 well microtiter plate

Plant extract	MIC of plant extract (mg/ml)		MIC of gentamicin (μ g/ml)		FICI	Outcome
	Alone	Combination	Alone	Combination		
P (Aq)	12.5	1.56	2048	1024	0.625	Additive
P (MeOH)	6.25	0.2	2048	256	0.157	Synergistic

P(Aq) = Aqueous extract of *P. niruri*. *P* (MeOH) = Methanolic extract of *P. niruri*. FICI = Fractional Inhibitory Concentration Index

Table 3. Phytochemical screening of *Phyllanthus niruri* leaves

Phytochemicals	<i>Phyllanthus niruri</i>				
	Aqueous	Chloroform	Methanol	Ethanol	n-Hexane
Steroids	+	+	+	+	-
Terpenoids	-	-	-	-	-
Flavonoids	+	-	+	-	-
Alkaloids	+	-	-	-	-
Saponins	+	-	-	-	+
Phenolics	+	+	+	+	+
Quinones	+	-	+	+	-
Coumarin	-	-	-	+	+

(+) = Present and (-) = Absent

than respective aqueous extract indicating that the methanolic extract contain more active constituents.

All the MRSA in our study were completely resistant to gentamicin and oxacillin and showed varied resistance against commonly used antibiotics, the result coincides with the prevalence of multidrug resistant bacteria from different clinical and environmental specimens regularly reported in Bangladesh [24,25].

Scanty research has been carried out on the antimicrobial property of *P. niruri* in combination with antibiotics against multidrug resistant organisms. We have found synergism between gentamicin and methanolic extract as well as an additive effect of gentamicin with aqueous extract of *P. niruri* in checkerboard method. The significant reduction in the MIC of gentamicin was observed, which explains the strong synergy (FICI 0.157) in the combination of methanolic extract of *P. niruri* with gentamicin. However, the MIC of gentamicin in combination (256 µg/ml) was still too high to reverse the gentamicin resistance of MRSA. This situation may be improved by using purified components of the extracts. This study thus indicates the presence of active chemicals in *P. niruri* which can be very effective against the MRSA in combination with gentamicin. Further study delineating the active compounds would be of interest.

5. CONCLUSION

This study represented that the aqueous and methanolic extracts of the plant have promising antibacterial activity against multidrug resistant MRSA strains. In addition, the combination of extract with gentamicin showed a synergistic effect against MRSA strain.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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