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ANTIBACTERIAL POTENTIAL OF LEAF EXTRACTS FROM *PHYLLANTHUS RETICULATUS* POIR

Md Soriful Islam[1,2]*,
Most. Mauluda Akhtar[2,3], Shamima Akhtar Sharmin[2], M. Atikur Rahman[2], M. Mostafizur Rahman[2], M. Khalekuzzaman[4], M. Anisuzzaman[2] & M. Firoz Alam[2]

[1]Department of Experimental and Clinical medicine, Polytechnic University of Marche, Via Tronto 10/A, 60020 Ancona, Italy
[2]Biotechnology and Microbiology Laboratory, Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh
[3]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, University of Naples Federico II, Via Sergio Pansini 5, 80131 Naples, Italy
[4]Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh

ABSTRACT
Plants are considered as attractive sources for novel antibacterial agents since they are playing fascinating roles in traditional medicine systems as well as in modern pharmaceutical industries from the ancient times. Phyllanthus reticulatus Poir. (family: Euphorbiaceae) is a large climbing medicinal shrub, and commonly found in all over the Bangladesh. Traditionally, this plant parts are used as diuretic and cooling agent, and to treat inflammation, diarrhea, sore in eyes and sores, burns, suppurations and chafing of the skin. In the present study, we investigated the antibacterial activity of ethanol extract, petroleum ether extract and chloroform extract of leaf from P. reticulates using disc diffusion method. We included four Gram negative (Shigella dysenteriae, Salmonella typhi, Pseudomonas aeruginosa, and Shigella sonnei) and six Gram positive bacteria (Sarcina lutea, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus, Bacillus cereus and Streptococcus-β-haemolytica). We found that all the studied leaf extracts produced different degrees of zone of inhibition around the disc against all the tested bacteria however, ethanol extract showed better potency. The highest inhibition zones by ethanol extract: 18.33 and 16.67 mm were found at 250 mg/ml against P. aeruginosa and S. aureus, respectively. For ethanol extract, the least MIC value (15.6 mg/ml) and MBC value (31.3 mg/ml) were recorded against P. aeruginosa, and S. aureus. The results, particularly obtained against P. aeruginosa and S. aureus are interesting, since these two bacteria are known as highly pathogenic for human.

KEYWORDS : *Phyllanthus reticulatus, antibacterial activity, human pathogenic bacteria, MIC, MBC*

Correspondence to Author

MD SORIFUL ISLAM

Department of Experimental and Clinical medicine Polytechnic University of Marche Via Tronto 10/A, 60020 Ancona, Italy

Email: soriful84@gmail.com

INTRODUCTION

Over the last several decades antibiotic resistance of pathogenic bacteria has become a serious threat to public health both in the developing and developed countries [1]. Consequently, the demand for the drugs derived from natural source is rising strikingly. Since therapeutic agents from plant source are known to be more biologically friendly than the totally synthetic drugs, vast diversity of plants has been a fascinating source for the majority of Food and Drug Administration approved drugs [2]. Bangladesh is a country with huge diversity of medicinal plants and there is a rich tradition among the people to exploit plants as source of alternative medicine [3]. *Phyllanthus reticulatus* Poir. (family: Euphorbiaceae) is a large climbing shrub, commonly found in the tropical areas of India, China, the Malay Islands and all over the Bangladesh [4]. Despite the various traditional uses such as diuretic and cooling agent; and to treat inflammation, diarrhea, sore in eyes and sores, burns, suppurations and chafing of the skin [5], relatively less reports are available in the scientific community to explore its biological activities. Up to now, antibacterial [6, 7], antiviral [8], anti-diabetic [9], anti-inflammatory [10], hypocholesterolemic [11], hepatoprotective [12] and anti-plasmodial [13] potential of *P. reticulatus* Poir. have been reported. Considering the use in folk medicine [5], more scientific research should be performed to confirm its biological activities. Therefore, in the present study, we investigated the antibacterial potential of ethanol, petroleum ether and choloroform extracts of *P. reticulatus* against some human pathogenic bacteria.

MATERIALS AND METHODS

Plant collection and identification

The fresh leaves of *Phyllanthus reticulatus* Poir. were collected from Rajshahi University Campus, Rajshahi, Bangladesh. The taxonomic identity of this plant was confirmed by Dr. A.H.M. Mahbubur Rahman, Associate Professor, Department of Botany, Rajshahi University, Bangladesh.

Extract preparation

After collection, leaves were sun dried for 7 days and then coarsely powdered using a mortar and pestle. Fine powder was made by using an electric blender (Nokia, Osaka-Japan). 50 g powder was separately dipped into 200 ml ethanol, petroleum ether and choloroform into a conical flask, closed with rubber corks and left for 7 days with occasional shaking. The extracts were filtered through Teton cloth and Whatman no.1 filter paper, respectively. The resulting filtrates were taken into the glass beaker and concentrated using a rotary evaporator. Finally concentrated residues were used to make the test concentration 250 mg/ml for antibacterial activity screening.

Bacterial samples

Four Gram negative bacteria namely, *Shigella dysenteriae* (BMLRU1011), *Salmonella typhi* (BMLRU1009), *Pseudomonas aeruginosa* (BMLRU1007), *Shigella sonnei* (BMLRU1015) and six Gram positive bacteria namely, *Sarcina lutea* (BMLRU1012), *Bacillus megaterium* (BMLRU1010), *Bacillus subtilis* (BMLRU1008), *Staphylococcus aureus* (BMLRU1002), Bacillus cereus (BMLRU1004), *Streptococcus-β-haemolytica* (BMLRU1006) were used for antibacterial study. All the strains were collected from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B), Mohakhali, Dhaka 1212, Bangladesh.

Determination of antibacterial activity

In vitro antibacterial activity was carried out by Disc diffusion method [14]. Sterilized filter paper discs (6 mm in diameter) were soaked with 10 µl of ethanol, petroleum ether and chloroform extracts (250 mg/ml) and dried under aseptic condition inside the laminar flow. 100 µl of standard bacterial cultures (approximately 10^8 cfu/ml; 0.5 McFarland turbidity standards) were spread on agar plates. Negative controls were prepared using the respective solvents. Tetracycline (30 µg $disc^{-1}$) was used as positive control. After drying in air under aseptic condition discs were placed on seeded agar plates and incubated at 37 °C for 24 h. Antibacterial activity was evaluated by measuring the diameter of zones of inhibition (mm) against the selected

studied bacteria. Three replications were used for each treatment and the experiment was repeated twice.

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) determination

The MIC of the leaf extract was determined using broth microdilution method according to the Clinical and Laboratory Standards Institute [15] with slight modification. For MIC determination, eight serial two-fold dilutions were prepared with the concentration range from 250 to 3.9 mg/ml for all the extracts. From each dilution, 0.5 ml of the extracts was taken out into 2 ml of nutrient broth containing tubes separately. Standardized 100 µl of bacterial suspensions (approximately 10^8 cfu/ml; 0.5 McFarland turbidity standards) were added to each tube. Only nutrient broth containing tube was used as a negative control and the test tube containing nutrient broth with test organism but without extract was used as a positive control. Next, all the tubes were incubated at 37 °C for 24 h. MIC was considered as the least concentration of the extracts which did not show any visible growth after overnight incubation. A loopful nutrient broth was collected from series of test tubes from the MIC position and streaked onto nutrient agar plates and incubated at 37 °C for 24 h. The least concentration of the extracts at which no visible growth was seen on nutrients agar plates was taken as the MBC.

Evaluation of bactericidal and bacteriostatic ability of extracts

After fixing of MIC and MBC value, using these two parameters, it is possible to determine whether the extract is bactericidal and bacteriostatic in nature against the bacterial strains. If the ratio, MBC/MIC = 1 or 2, the action was defined as bactericidal but if the ratio, MBC/MIC = 4 or 16, the effect was considered as bacteriostatic [16].

RESULTS

***In vitro* antibacterial activity**

We found promising antibacterial activity from ethanol leaf extract against all the tested bacteria among the three studied extracts (ethanol, petroleum ether and chloroform) of *P. reticulates* (Figure 1, Figure 2 and Figure 3). The highest inhibition zone (18.33 mm) was found against *S. aureus* produced by ethanol extract. Comparatively slightly lower inhibition zone (16.67 mm) was recorded against *P. aeruginosa* for the same extract. In these both cases, positive control (Tetracycline; a broad-spectrum antibiotic) provided lower inhibition zone than the ethanol extract. However, for ethanol extract lowest inhibition zone (12.33 mm) was recorded against *S. typhi.* All these results are summarized in the Figure 1**.**

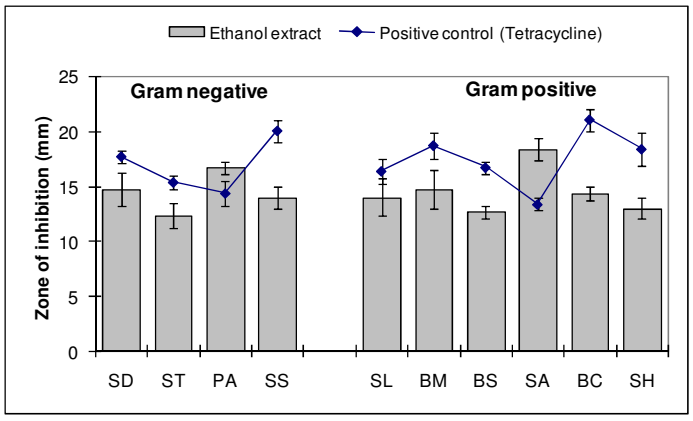

Figure 1: Antibacterial activity of ethanol extract of *Phyllanthus reticulatus*. Data are presented as ± SD of three replications. Here, SD, *Shigella dysenteriae*; ST, *Salmonella typhi*; PA, *Pseudomonas aeruginosa*; SS, *Shigella sonnei*; SL, *Sarcina lutea*; BM, *Bacillus megaterium*; BS, *Bacillus subtilis*; SA, *Staphylococcus aureus*; BC, *Bacillus cereus* and SH, *Streptococcus-β-haemolytica*.

In contrast to ethanol extract, we found less efficient results from petroleum ether and chloroform extracts. These two extracts produced similar results for all the tested bacteria. The inhibition zone ranges were measured 7.33 to 12.33 mm for petroleum ether extract (Figure 2) and 7.33 to 11.67 mm (Figure 3) for chloroform extract. Again highest inhibition zones (12.33 and 11.67mm) were recorded against *S. aureus* by petroleum ether extract (Figure 2) and chloroform extract (Figure 3), respectively.

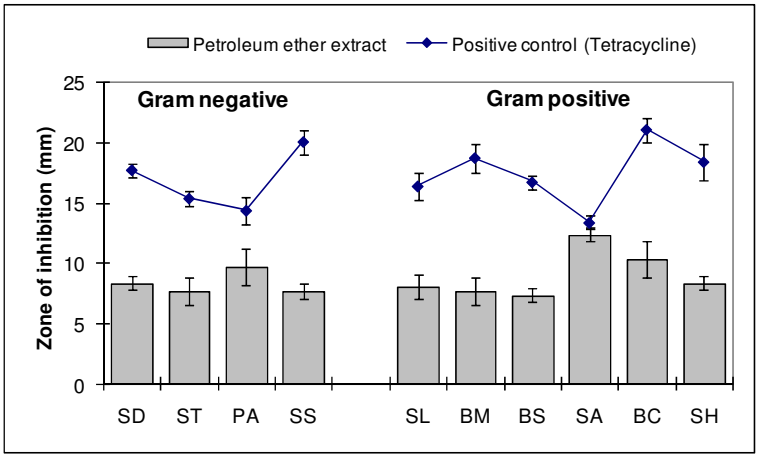

Figure 2: Antibacterial activity of petroleum ether extract of *Phyllanthus reticulatus*. Data are presented as ± SD of three replications. Here, SD, *Shigella dysenteriae*; ST, *Salmonella typhi*; PA, *Pseudomonas aeruginosa*; SS, *Shigella sonnei*; SL, *Sarcina lutea*; BM, *Bacillus megaterium*; BS, *Bacillus subtilis*; SA, *Staphylococcus aureus*; BC, *Bacillus cereus* and SH, *Streptococcus-β-haemolytica*.

Minimum Inhibitory Concentration (MIC) and Minimum bactericidal concentration (MBC)

MIC and corresponding MBC values of ethanol leaf extract were recorded with the range from 15.6 to 62.5 mg/ml and 31.3 to 250 mg/ml, respectively. The least MIC was recorded against *P. aeruginosa* and *S. aureus* for EE. In contrast, we found higher MIC value for both PE and CE which is 62.5 mg/ml against all the tested bacteria. The MBC of these two extracts was ranged from 125 to 250 mg/ml. The results of MIC and MBC are summarized in Table 1.

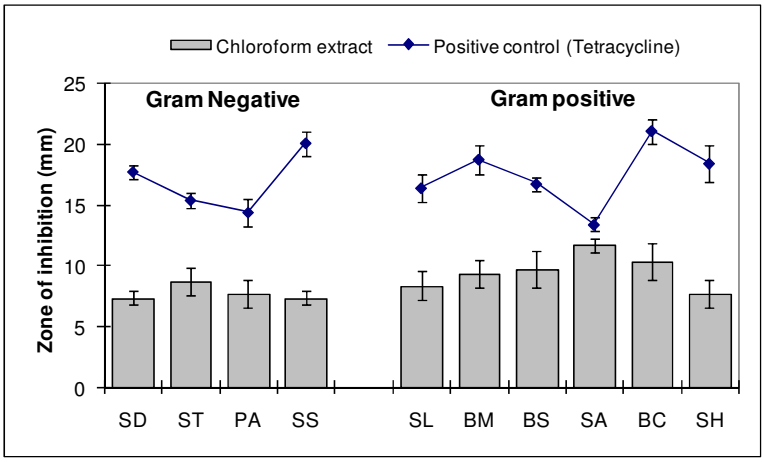

Figure 3: Antibacterial activity of chloroform extract of *Phyllanthus reticulatus*. Data are presented as ± SD of three replications. Here, SD, *Shigella dysenteriae*; ST, *Salmonella typhi*; PA, *Pseudomonas aeruginosa*; SS, *Shigella sonnei*; SL, *Sarcina lutea*; BM, *Bacillus megaterium*; BS, *Bacillus subtilis*; SA, *Staphylococcus aureus*; BC, *Bacillus cereus* and SH, *Streptococcus-β-haemolytica*.

Table 1: Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentrations (MBC) for ethanol extract, petroleum ether extract and chloroform extract of *Phyllanthus reticulatus* against some human pathogenic bacteria.

Name of bacteria	Ethanol extract		Petroleum ether extract		Chloroform extract	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Gram negative						
Shigella dysenteriae	31.3	125	62.5	250	62.5	250
Salmonella typhi	62.5	250	62.5	250	62.5	250
Pseudomonas aeruginosa	15.6	31.3	62.5	250	62.5	250
Shigella sonnei	31.3	62.5	62.5	250	62.5	250
Gram positive						
Sarcina lutea	31.3	62.5	62.5	250	62.5	250
Bacillus megaterium	31.3	125	62.5	250	62.5	250
Bacillus subtilis	62.5	125	62.5	250	62.5	250
Staphylococcus aureus	15.6	31.3	62.5	125	62.5	125
Bacillus cereus	31.3	62.5	62.5	125	62.5	250
Streptococcus-β-haemolytica	62.5	125	62.5	250	62.5	250

The bactericidal and bacteriostatic capacity of extracts was categorized based on the MIC and MBC values which are summarized in the Table 2**.**

Table 2: Bacteriostatic (−) and Bactericidal (+) profile of ethanol extract, petroleum ether extract and chloroform extract of *Phyllanthus reticulatus* against some human pathogenic bacteria.

Bacteria	Ethanol extract	Petroleum ether extract	Chloroform extract
Gram negative			
Shigella dysenteriae	-	-	-
Salmonella typhi	-	-	-
Pseudomonas aeruginosa	+	-	-
Shigella sonnei	+	-	-
Gram positive			
Sarcina lutea	+	-	-
Bacillus megaterium	+	-	-
Bacillus subtilis	+	-	-
Staphylococcus aureus	+	+	+
Bacillus cereus	+	+	-
Streptococcus-β- haemolytica	+	-	-

DISCUSSION

In the present study, our focus was to explore the antibacterial activities of ethanol, petroleum ether and chloroform extract of *P. reticulatus*. Using disc diffusion method, we found promising antibacterial activity at least from ethanol extract regardless to the type of (Gram- and Gram+) bacteria. However, petroleum ether extract and chloroform extract also inhibited the growth of all bacteria but their efficiency was less than ethanol extract. Ethanol extract produced the highest zone of inhibition, consequently, lowest MIC and MBC value against *S. aureus* among all the studied bacteria. This is intriguing since positive control (Tetracycline)

produced much lower inhibition zone than that of ethanol extract. Petroleum ether and chloroform extract also produced highest inhibition zone against *S. aureus* among other bacteria. In addition, two earlier studies reported that methanol and chloroform extracts also effective against *S. aureus* [6, 7]. Our present result confirms previous findings regarding anti *S. aureus* activity of chloroform extracts. Moreover, we provided information regarding two more extracts (ethanol and petroleum ether) that have antibacterial activity against *S. aureus*. The emergence of antibiotic-resistant forms of pathogenic *S. aureus* (e.g. Methicillin-resistant *S. aureus*) has been a serious warning for public health as well as clinical medicine [17]. Therefore, researchers are searching alternative therapeutic agents to treat multi-drug resistant *S. aureus*. In this context, ours findings seem to be promising for future investigation.

Ethanol extract also produced satisfactory inhibition zone with lowest MIC and MBC value against *P. aeruginosa* whereas petroleum ether and chloroform extract failed to show such inhibition. *P. aeruginosa* is also a multidrug resistant bacteria and associated with serious health hazards and mortality [18].

Though we found notable inhibition at least by ethanol extract against *S. aureus* and *P. aeruginosa*, this extract also inhibited the growth of other bacteria potentially. On the basis of MIC and MBC value, we categorized our studied extracts as bactericidal and bacteriostatic; and we found, ethanol extract showed bactericidal activity not only against *S. aureus* and *P. aeruginosa* but also against *S. sonnei, S. lutea*, *B. megaterium, B. subtilis, S. aureu,* B. cereus, *S. -β-haemolytica.* We can not even ignore the results by petroleum ether and chloroform extract since petroleum ether extract was bactericidal against *S. aureus and* B. cereus; CE was bactericidal only against S*. aureus.* It is important to note that our results are preliminary in nature that could be a basis for further investigation to identify the active compounds responsible for bacterial growth inhibition. This could dramatically increase antibacterial efficacy than that of the crude extracts. Plants produce different secondary metabolites as a part of their immune system [19]. These phytochemicals are well known for their various medical uses including antibacterial [20]. Several studies have identified various constituents from *P. reticulatus* such as reticulatusides A and B [21], geraniinic acid derivative [22], triterpenoids, phytosterols, coumarin [6], flavonoids, and other phenols [23], lupeol acetate, stigamasterol and lupeol [24]. Nevertheless, more studies should be performed to isolate and characterize bioactive compound to introduce a precise antibacterial profile which is still significantly lack for *P. reticulatus* considering its traditional use.

CONCLUSION

Although all the studied extracts showed antibacterial activity, specifically ethanol extract could be considered as more promising as this extract showed maximum potency against *P. aeruginosa* and *S. aureus* (highly pathogenic bacteria for human). Therefore, our present findings could be a basis for further study to explore specific compound from crude extracts of *P. reticulatus* that might be useful to develop therapeutic agents to treat the diseases caused by rapidly evolving antibiotic resistant bacteria.

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