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INVESTIGATION OF THE ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACT OF *PHYLLANTHUS ACIDUS* IN COMBINATION WITH DIFFERENT CLASSES OF ANTIBIOTICS AGAINST SINGLE AND MULTIDRUG RESISTANCE STRAINS

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ABSTRACT
The aim of this project work was to evaluate the Antimicrobial Activities of *phyllanthus acidus* (Family: Phyllanthaceae). Antimicrobial activity of chloroform and ethyl acetate extract leaves of *phyllanthus acidus* was tested by Disc Diffusion Method. Standard antibiotic discs of Kanamycin (30µg/disc) for bacterial species were used as standard and crude extracts were used at a concentration of 400µg/disc and 500 µg/disc. The combination effect of *phyllanthus acidus* extract and drugs are more than single drug concentration. The combination therapy is extract+ciprofloxacin, extract+levofloxacin, extract+ceptriaxone, extract+cefuroxime, extract+azithromycin and extract+nystatin. The Antibiotics showed good result against gram positive, gram negative and resistant bacteria. Some antibiotics showed no result against bacteria such as Cefuroxime and Nystatin. But when they are combinedly used with extract of *phyllanthus acidus* they showed result against most of the gram positive, gram negative and resistant pathogen. When Nystatin with extract of *phyllanthus acidus* is given combinedly showed no result for the pathogenklebsiella spp(Resistant pathogen). But this combination therapy showed good result for the resistant strain of klebsiella spp and here the zone of inhibition is 22mm. Combination therapy of extract of *phyllanthus acidus* and cefuroxime showed no result for the pathogen salmonella typhi. But these combination therapy also give result for the resistant strain of Escherichia coli, and the zone of inhibition is 22mm. The effect of combination of *phyllanthus acidus* and azithromycin is more than azithromycin to against Shigella dysenteriae and Vibrio cholera.

KEYWORDS: Antibiotics, Antibiotics resistant, *phyllanthus acidus*.

INTRODUCTION
Recently the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the available antibiotics has led authors to investigate the antimicrobial activity of medicinal plants (Bisignano et al., 1996; Lis-Balchin and Deans 1996; Moaz and Neeman, 1998; Hammer et al., 1999). With the introduction of a variety of antimicrobials it became necessary to perform the antimicrobial susceptibility test as a routine. For this, the antimicrobial contained in a reservoir was allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. Even now a variety of antimicrobial containing reservoirs are used but the antimicrobial impregnated absorbent paper disc is by far the commonest type used. The disc diffusion method of AST is the most practical method and is still the method of choice for the average laboratory. Automation may force the method out of the diagnostic laboratory but in this country as well as in the smaller laboratories of even advanced countries, it will certainly be the most commonly carried out microbiological test for many years to come. It is, therefore, imperative that microbiologists understand the principles of the test well and keep updating the information as and when necessary. All techniques involve either diffusion of antimicrobial agent in agar or dilution of antibiotic in agar or broth. Even automated techniques are variations of the above methods.

Resistance to antimicrobial agents (AMR) has resulted in morbidity and mortality from treatment failures and increased health care costs. Although defining the precise public health risk and estimating the increase in costs is not a simple undertaking, there is little doubt that emergent antibiotic resistance is a serious global problem.

Appropriate antimicrobial drug use has unquestionable benefit, but physicians and the public frequently use these agents inappropriately. Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance.

The results of in-vitro antibiotic susceptibility testing, guide clinicians in the appropriate selection of initial empiric regimens and, drugs used for individual patients in specific situations. The selection of an antibiotic panel for susceptibility testing is based on the commonly observed susceptibility patterns, and is revised periodically. Ciprofloxacin, Levofloxacin, Ceftriaxone, Cefuroxime, Azithromycin, Nystatin These antibiotics are used for combination with plant extract intests.

METHODS AND MATERIALS

Collection of the plant parts

The leaves of plant *Phyllanthus acidus* were selected for the study. The leaves of these plants *Phyllanthus acidus* were collected during month of October, 2016 from the area of Raipura, Narsingdi, Bangladesh and were identified by the experts of Bangladesh National Herbarium, Dhaka, where voucher specimen were retained.

Drying, Pulverization and Preservation of plant parts

The leaves were first washed with water to remove the adhering dirt and then cut into small pieces, sun dried for 12-15days. After complete drying, the entire portions were pulverized into coarse powder with the help of grinding machine and were stored in an air tight container for further use.

Extraction of Plant Material

The each ground leaves 100gm were extracted with 3 times methanol of their weight in a round bottom flask container with 1:2 sample and solvent ratio at room temperature through occasional shaking and stirring for 7 days. After 7 days, the extracts were filtered through filter paper. The filtrates were concentrated at 50ºC under reduce pressure in a rotary evaporator to afford a greenish mass of biological investigation. Then the crude extract ready for assaying of antimicrobial, antioxidant, total phenolic content.

Extraction procedure

Chemical constituents from crude plant can be extracted by following two extraction procedures-Cold extraction & Hot extraction.

In our current study we used cold extraction method.

Cold Extraction for the four plant parts

Preparation of ethanolic extracts

For each plant the dried and powdered materials (500 g for) were soaked in 2500 ml of 90% ethanol for about 15 days at room temperature with occasional stirring. After 15 days the solution was filtered using filter cloth and Whatman's filter paper. The filtrates (Ethanolic extract) obtained were evaporated under rotary evaporator and in a water- bath until dried. It rendered a gummy concentrates and were designated as crude extracts of Ethanol.

Antimicrobial testing methods

The following three methods have been shown to consistently provide reproducible and repeatable results when followed correctly. They are- Disk diffusion, Broth dilution & Agar dilution.

Disc diffusion method

Principle

In this method, measured amount of the test samples are dissolved in definite volumes of solvent to give solutions of known concentrations (µg/ml). Then sterile filter paper discs (5mm in diameters) are transfered in the petridish andapplied test sample in the disc by micropipette with known amounts of the test substances and dried. These plates are kept at low temperature in the refrigerator for 1-2 hours to allow maximum diffusion. The plates are then kept in an incubator (37ºc) for 12-18 hours to allow the growth of the organisms. If the test material has antimicrobial activity, it will inhibit the growth of microorganisms, giving a clear, distinct zone called "zone of inhibition".

The principal factors which determine the size of the zone of inhibition are.

Intrinsic antimicrobial susceptibility of the test sample. Growth rate of the test organisms. Diffusion rate of the test sample which is related to its water solubility. Concentration of the test organisms inoculated in the medium. Concentration of the test sample per disc.Thickness of the test medium in the petridishes.

MATERIALS

In our present study, the antimicrobial activity of distil water extract of *phyllanthus leaves* was investigated in comparison with standard Kanamycin (30µg/disc) antibiotic against a number of pathogenic gram-positive and gram-negative bacteria.

Apparatus and Reagents

Filter paper discs (5mm in diameter), Petri dishes, Refrigerator, Test tubes, Sterile forceps, Sterile cotton, Incubating loop, Bunsen burner, Micropipette(10-100µl), Laminar air flow unit (Biocraft's Scientific Industries, India), Autoclave, Incubator(OSK-9636, Japan), Nutrient agar media(DIFCD), Ethanol or Methanol, Standard disc(Kanamycin 30 µg/disc).

Agar media

Agar is a gelatinous substance, obtained from algae. Agar is derived from the polysaccharide agarose, which forms the supporting structure in the cell walls of certain species of algae, and which is released on boiling. Throughout history into modern times, agar has been chiefly used as an ingredient in desserts throughout Asia and also as a solid substrate to contain culture media for microbiological work.

Composition of Agar media
0.5% Peptone,0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl, distilled water, pH adjusted to neutral (6.8) at 25 °C.

Procedure
Preparation of Medium
To prepare agar media solution 28grams of agar has to be mixed with 1L of water. In case of preparation of 250ml agar media solution 7grams of agar has to be mixed with 250ml distil water. Then it is transfer to autoclave for sterilization for 20 minutes at 121ºC.

Preparation of subculture
With the help of an inoculating loop, the test organisms were transferred from the pure culture to the agar slants under a laminar air flow unit. The inoculated slants were then incubated at 37ºc for 18-24 hours to ensure the growth of the test organisms.

Preparation of test plates
The agar media is taken in test tube after autoclaving and the organism was transferred from the subculture to the test tube containing autoclaved medium with the help of an inoculating loop in an aseptic area. The test tube was shaken by rotation to get a uniform suspension of the organism.

Preparation of Discs
There are three types of disc available. They are
Sample discs, Standard discs & Blank/control discs.

Sample discs: - Sterilized filters paper discs (5 mm in diameter) were taken in a blank petridish. Sample solution of the desired concentration was applied on the discs with the help of a micropipette in an aseptic condition.

Standard discs: - These were used to compare the antibacterial activity of test material. In our investigation, Kanamycin (30µg/disc) standard disc was used as a reference.

Blank/control discs: - Only solvent was applied to the discs to determine the antibacterial effects of the solvent used. But in these study we use water as a solvent so that blank or control disc is not required.

Preparation of Discs Containing Sample
Prepare the disc and then it transferred to the petridish by the help of a forcep. Then drug or sample is transferred in the disc by micropipette.

Sample preparation: Take 0.1 g of *phyllanthus acidus* and mixed it with 5 ml of distil water.

Transfer of drug to the disc: Two concentration of drug is applied two the disc. And it is reffered here as 1 and 2.
1=400 µl/disc & 2=500 µl/disc.

Diffusion of dug and Incubation
After incorporation of drug into the disc then petridish is transferred to the refrigerator for 1-2 hours at inverted position for the diffusion of drug. After 1-2 hours the petridish is transferred to the incubator at 37 ºC for 12-18 hours.

Measurement of Zone of Inhibition
After 18 hours of incubation, the antibacterial activity of the test samples was determined by measuring the diameter of inhibitory zones in term of millimeter.

The drug used in the combination therapy
Here we used six drug for combination therapy. They are.

Ciprofloxacin, Levofloxacin, Ceftriaxone, Cefuroxime, Azithromycin, Nystatin.

Bacteria used for combination therapy
Table1: Test organism.

Gram-positive bacteria	Gram-negetive bacteria	Resistant bacteria
Sarcina lutea	*Salmonella paratyphi*	*Escherichia coli*
Staphylococcus aureus	*Klebsiella pneumoniae*	*Acinetobacter baumannii*
	Shigella dysenteriae	*Shigella sonnei*
	Vibrio cholerae	*Klebsiella* spp.
	Escherichia coli	*Pseudomonus* spp.
	Salmonella typhi	
	Proteus vulgaris	

Procedure of disc diffusion method for combination therapy
Prepare the preparation medium, subculture & test plates according to previous procedure.

Preparation of Discs
In case of combination therapy we used two disc. They are.

Drug disc: Contain the antibiotic.

Combination disc: Combination of antibiotic and test sample of *phyllanthus acidus.*

Preparation of Discs Containing Sample
Prepare the disc and then it transferred to the petridish by the help of a forcep. Then drug or sample is transferred in the disc by micropipette.

Sample preparation: Take 0.1 g of *phyllanthus acidus* and mixed it with 5 ml of distil water.

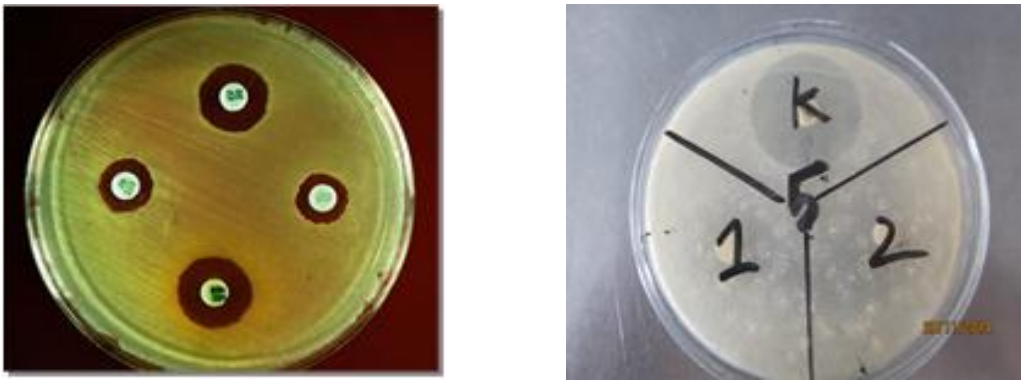
Antibiotic sample preparation: Take 0.1 g of Antibiotic and mixed it with 5 ml of distil water.

Transfer of drug to the disc
Here, Drug disc=300 µl/disc & Combination disc=400µl/disc of test sample+300 µl/disc of antibiotic.

Diffusion of dug and Incubation: According to previous procedure.

Measurement of Zone of Inhibition
After 18 hours of incubation, the antibacterial activity of the test samples was determined by measuring the diameter of inhibitory zones in term of millimeter.

Determination of antimicrobial activity by measuring zone of inhibition
The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition.



(A) **(B)**

Figure 1: (A) Clear zone of inhibition and(B) Determination of clear zone of inhibition.

RESULTS AND DISCUSSION

Test for antimicrobial activity
In the antimicrobial study it was observed that the extract of *phyllanthus acidus* showed high activity against all 13 gram-positive, gram negative and resistant bacteria. *Phyllanthus acidus* extract showed good activity against all organism but it has no effect against 8 or *Salmonella typhi.*

Table 2: Antimicrobial activity of *phyllanthus acidus.*

Bacteria	Type of Bacteria	Diameter of zone of inhibition of 1(mm) (1=400 µl/disc)	Diameter of zone of inhibition of 2(mm) (2=500 µl/disc)	Diameter of zone of inhibition ofkanamycin
1=*Sarcina lutea*	Gram-Positive (Gram +ve)	12	17	18
2=Salmonella paratyphi	Gram-Negaitive (Gram -ve)	15	18	34
3=Klebsiella pneumonia	Gram-Negaitive (Gram -ve)	17	23	32
4.Staphylococcus aureus	Gram-Positive (Gram +ve)	14	18	37
5.Shigella dysenteriae	Gram-Negaitive (Gram -ve)	16	21	36
6.Vibrio cholera	Gram-Negaitive (Gram -ve)	18	19	20
7.Escherichia coli	Gram-Negaitive (Gram -ve)	14	16	30
9.Proteus vulgaris	Gram-Negaitive (Gram -ve)	18	21	22
R1= *Escherichia coli*	Resistant (R1)	20	24	25
R2=*Acinetobacter baumannii*	Resistant (R2)	18	21	23
R3= *Shigella sonnei*	Resistant (R3)	23	29	26
R4= *Klebsiella* spp.	Resistant (R4)	19	25	33
R5= *Pseudomonus* spp.	Resistant (R5)	22	24	32

Result for combination therapy: In case of, combination therapy we used antibiotics and extract of *phyllanthus acidus* combinedly.

Table 3: Antimicrobial activity of *phyllanthus acidus* combination with Ciprofloxacin and Levofloxacin.

Bacteria	Type of Bacteria	Diameter of zone of inhibitionof Ciprofloxacin(mm) (300 µl/disc)	Diameter of zone of inhibitionof Levofloxacin(mm) (300 µl/disc)	Diameter of zone of inhibitionof Extract+Ciprofloxacin(mm),(400µl/disc+300 µl/disc)	Diameter of zone of inhibition of Extract+Levofloxacin(mm), (400µl/disc+300 µl/disc)
1.Sarcina lutea	Gram-Positive	27	30	28	32
2.Salmonella paratyphi	Gram-Negaitive	35	37	30	42
3.Klebsiella pneumonia	Gram-Negaitive	34	39	32	40
4.Staphylococcus aureus	Gram-Positive	35	40	34	42
5.Shigella dysenteriae	Gram-Negaitive	40	34	30	38
6.Vibrio cholera	Gram-Negaitive	30	30	36	38
7.Escherichia coli	Gram-Negaitive	40	35	34	35
8.Salmonella typhi	Gram-Negaitive	45	35	40	36
9.Proteus vulgaris	Gram-Negaitive	35	40	38	35
R1= *Escherichia coli*	Resistant (R1)	35	36	40	36
R2=*Acinetobacter baumannii*	Resistant (R2)	35	40	38	38
R3= *Shigella sonnei*	Resistant (R3)	31	35	36	35
R4= *Klebsiella* spp.	Resistant (R4)	27	36	32	32
R5= *Pseudomonus* spp.	Resistant (R5)	36	37	35	40

Table 4: Antimicrobial activity of *phyllanthus acidus* combination with Ceftriaxomeand Cefuroxime.

Bacteria	Type of Bacteria	Diameter of zone of inhibition of Ceftriaxone (mm) (300 µl/disc)	Diameter of zone of inhibition of Cefuroxime (mm) (300 µl/disc)	Diameter of zone of inhibition of Extract+ Ceftriaxone(mm) (400µl/disc+300 µl/disc)	Diameter of zone of inhibition of Extract+ cefuroxime(mm) (400µl/disc+ 300 µl/disc)
1.Sarcina lutea	Gram-Positive (Gram +ve)	14	0	18	14
2.Salmonella paratyphi	Gram-Negaitive (Gram -ve)	11	0	17	12
3.Klebsiella pneumonia	Gram-Negaitive (Gram -ve)	11	0	16	12
4.Staphylococcus aureus	Gram-Positive (Gram +ve)	16	0	15	11
5.Shigella dysenteriae	Gram-Negaitive (Gram -ve)	10	0	16	12
6.Vibrio cholera	Gram-Negaitive (Gram -ve)	11	0	22	14
7.Escherichia coli	Gram-Negaitive (Gram -ve)	17	0	20	0
8.Salmonella typhi	Gram-Negaitive (Gram -ve)	14	0	22	0
9.Proteus vulgaris	Gram-Negaitive (Gram -ve)	23	0	24	14
R1= *Escherichia coli*	Resistant (R1)	13	20	17	22
R2=*Acinetobacter baumannii*	Resistant (R2)	18	0	27	17
R3= *Shigella sonnei*	Resistant (R3)	14	0	30	25
R4= *Klebsiella* spp.	Resistant (R4)	0	0	25	14
R5= *Pseudomonus* spp.	Resistant (R5)	21	0	23	16

Table 5: Antimicrobial activity of *phyllanthus acidus* combination with Azithromycin and Nystatin.

Bacteria	Type of Bacteria	Diameter of zone of inhibition of Azithromycin (mm) (300 µl/disc)	Diameter of zone of inhibition of Nystatin (mm) (300 µl/disc)	Diameter of zone of inhibition of Extract+ Azithromycin(mm) (400µl/disc+300 µl/disc)	Diameter of zone of inhibition of Extract+ Nystatin(mm) (400µl/disc+300 µl/disc)
1.Sarcina lutea	Gram-Positive (Gram +ve)	35	0	32	16
2.Salmonella paratyphi	Gram-Negaitive (Gram -ve)	32	0	28	12
3.Klebsiella pneumonia	Gram-Negaitive (Gram -ve)	38	0	32	14
4.Staphylococcus aureus	Gram-Positive (Gram +ve)	35	0	30	16
5.Shigella dysenteriae	Gram-Negaitive (Gram -ve)	28	0	32	13
6.Vibrio cholera	Gram-Negaitive (Gram -ve)	34	0	40	20
7.Escherichia coli	Gram-Negaitive (Gram -ve)	36	0	38	0
8.Salmonella typhi	Gram-Negaitive (Gram -ve)	37	0	36	14
9.Proteus vulgaris	Gram-Negaitive (Gram -ve)	25	0	32	18
R1= *Escherichia coli*	Resistant (R1)	30.5	19	36	24
R2=*Acinetobacter baumannii*	Resistant (R2)	35	17	28	15
R3= *Shigella sonnei*	Resistant (R3)	34	19	32	21
R4= *Klebsiella* spp.	Resistant (R4)	15	0	26	22
R5= *Pseudomonus* spp.	Resistant (R5)	26	0	28	16

DISCUSSION

The standard drug, Kanamycin, showedzone of inhibitionagainst 13 pathogen. And extract showed different zone of inhibition at different concentration. *Phyllanthus acidus* extract has no effect against 8 or *Salmonella typhi.*

The Antibiotics showed good result against 14 gram positive, gram negative and resistant bacteria. Some antibiotics showed no result against bacteria such as Cefuroxime and Nystatin. But when they are combinedly used with extract of *phyllanthus acidus* they showed result against most of the gram positive, gram negative and resistant pathogen. When Nystatin with extract of *phyllanthus acidus* is given combinedly showed no result for the pathogen*klebsiella spp(Resistant pathogen).* But this combination therapy showed good result for the resistant strain of *klebsiella spp* and here the zone of inhibition is 22mm.

Combination therapy of extract of *phyllanthus acidus* and cefuroxime showed no result for the pathogen *salmonella typhi*. But these combination therapy also give result for the resistant strain of *Escherichia coli*, and the zone of inhibition is 22mm. The effect of combination of phyllanthus acidus and azithromycin is more than azithromycin to against *Shigella dysenteriae and Vibrio cholera.*

CONCLUSION

The plant *phyllanthus acidus* is found everywhere in Bangladesh. Research demonstrates that the edible parts of *phyllanthus acidus* have anti-microbial activity. This study was conducted to evaluate the antimicrobial activity with Minimum Inhibitory concentration and total phenolic content and antioxidant activity of the whole plant.

For chemical investigation the plant were cut into small pieces, washed well with water and dried in the sun for 7 days. Later the dried pieces of plant were ground into coarse powders and extracted with chloroform and ethyl acetate.

The present study revealed that The chloroform extract of leaves (CEL) and ethyl acetate extract of leaves (EEL), of *phyllanthus acidus* has got profoundtotal phenolic content, antibacterial may have potential use in medicine.

The *phyllanthus acidus* has got profound antibacterial activity in different concentration.it has also combine effect with different classes drugs.

The comparison with the drug, the combination effect has more than single drug effect. chloroform extract of leaves (CEL) and ethyl acetate extract of leaves (EEL showed mild to moderate antimicrobial activity against most of the tested bacteria and fungi.

The activity of extract has on some resistant pathogens, that means when it will used combined then we can got beneficial result & protect resistant of pathogens.

In future, we can get more beneficial result to study about this plant in standard procedure of microbiology.

Even we can get drug product from this plant by more study.

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