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Antibacterial effects of *Phyllantus amarus* on urinary tract pathogens

Abstract

Antibiotic resistance has become a global concern as the clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug resistance pathogens. Phyllantusamarus has been showing to possess various properties including antibacterial property by many authors. This study was therefore designed to investigate the antibacterial effects of aqueous and methanolic extract of Phyllantusamarus on urinary tract pathogens. 10g of the concentrated extract from methanol and aqueous was simultaneously dissolved in 100ml of water and used to make two-fold dilutions, to give100, 50, 25, 12.5, 6.3, 3.2, 1.6 and 0.8mg/ml as extract concentrations. 10 different concentrations, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05mg/ml and 5µg/ml, of the control drug ciprofloxacin were also prepared. The phytochemical constituents of both methanolic and aqueous extract were also investigated. Fourteen isolates from urine sample were identified. These isolates, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klesiella pneumonia*, Enteroccocusfeacalis, *Proteus mirabillis*, *Staphylococcus saprophytic* sand Control standard *Escherichia coli* ATCC 25922 were subjected to antibacterial effect of varying concentration of these methanolic and aqueous extract of phyllantusamarus and standard drug ciprofloxacin using agar well diffusion method. The diameter in millmiters of the inhibition zones was measured, the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration were also determined. The data obtained were analysed using SPSS version 17. The extract was found to containsaponins, flavonoids, alkaloids, tannins, phlobatanins and terpenoids. Both aqueous and methanolic extract of P. amarus caused a concentration dependent increase in zones of inhibition of the growth of the isolates. The MIC and MBC values were however found to be high compare to the standard. There was no significant different between Agar well diffusion method and disc diffusion method. The study clearly showed that P. amarus has appreciable antibacterial properties. However, there is need for improvement in approaches for natural product isolation and characterization so that the bioactivity and potency of the drug can be improved upon.

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Introduction

A urinary tract infection (UTI) (also known as acute cystitis or bladder infection) is an infection that affects part of the urinary tract, also can be defined as a significant bacteriuria in the presence of symptoms, also UTI is said to exist when a significant number of microorganisms, usually greater than 10^5 cells per milliliter of urine, are detected in properly collected mid-stream " clean catch" urine.[1] When it affects the lower urinary tract it is known as a simple cystitis (a bladder infection) and when it affects the upper urinary tract it is known as pyelonephritis (a kidney infection). UTI is said to exist when a bacterial colony count of greater than or equal to 10^5colony-forming units per ml of a typical urinary tract organism in properly collected mid-stream "clean catch" urine.[2] Among the most common infectious diseases, urinary tract infections (UTIs) are a commonly encountered diseases by clinicians in developing countries with an estimated annual global incidence of at least 250 million.[3] Each year urinary tract infections account for about 8 million doctor visits. It has been reported that, 10% hospital patients acquire this infection while staying in hospital Gokulakrishnan et al.,[4] In most of the studies, the common bacterial uropathogens are, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klesiella pneumonia, Enteroccocus feacalis, Proteus mirabillis, Proteus vulgaris, Staphylococcus saprophyticus*.[5–7]

Treatment with available antibiotics is becoming a great threat and difficult due to increase rate of resistance development among these pathogenic bacteria.[8–10] The significant of drugs derived from plants cannot be over emphasized with the recent trend of high percentage of resistance of microorganisms to the present-day antibiotics.[11–13] The progressive shift of chemotherapy from first line to second and even to last line due to failure of the earlier ones can be cumbersome and expensive. The effect is pronounced in third world as the costly replacement drugs for treating the highly resistant infectious diseases are unaffordable (WHO, 2002).[14] This brought about the need for developing new novel antimicrobials. Effort has been intensified by researcher towards a search for more source of antimicrobial agents. Some plants have been identified to contain medicinal constituents which have potentially significant therapeutic applications against human pathogens including bacteria, fungi and viruses.[15] Antimicrobial activities of plant constituents such as phenol, quines, flavones, flavonoids, tannins, terpenoids, essential oils and alkaloids have been reported.[16] There is a continuous and urgent need to discover new antimicrobials with diverse chemical structures and novel mechanism of action for new and reemerging infectious diseases.[16]

Phyllanthus amarus (Eyinolobe in Yoruba) which belong to the family Euphorbiaceae is a small, erect, annual herb having large number of phytochemicals that are attributed to its leaves, stem and roots. The herb is a distinguished plant worldwide which has been used over the years because of its rich medicinal component.[17] *P.amarusis* traditionally used among different ethnic groups in Nigeria for the treatment of jaundice, diarrhea, dysentery, diabetes, fevers, uro-genital diseases, ulcers, sores, boils and wounds.[18] Juice from the roots and leaves are taken internally to stimulate the kidney. Previous findings have revealed that extracts from different parts of *P.amarus* demonstrated anti-oxidant, anti-inflammatory, hypocholesterolemic anti-carcinogenic and anti-HIV potential.[19–21] Antibiotic resistance

has become a global concern Westh et al.,[22] as the clinical efficacy of many existing antibiotics is being threatened by the emergence of multi-drug-resistant pathogens.[23] Though, more synthetic drugs were being produced to replace the resistance ones but the cost of its production is becoming alarming in which the patients or end users of these drugs may not be able to afford its purchase. Also variety of side effects of these drugs has placed a concern on the clinicians to consider the side effect first notwithstanding the therapeutic outcome. Therefore, researchers are increasingly turning their attention to ethno-medicine, looking for new leads to develop more effective drugs against microbial infections Benkeblia[24] and this has led to the screening of several medicinal plants for potential antimicrobial activity.[25,26]

P.amarus is used ethno-medically in the treatment of infections arising from bacterial, viral and fungi infestation. Recently there is an increase in resistance of these uro-pathogens thereby making urinary tract infection more severe. Hence this study was designed to investigate the antibacterial effect of *P. amarus* against these common urinary tract pathogens and the objectives are to; Screen *P.amarus* extracts (aqueous and methanol) alongside with standard antibiotics for antimicrobial activities against Urinary tract pathogens using agar well diffusion method, to determine the MIC and MBC of these extracts on Urinary tract pathogens, to compare the effectiveness of the methods used for this screening (Agar Well diffusion and Disk diffusion method) and to carry out preliminary test for the presence of phytochemical constituents in *P.amarus*.

Materials and methods

Collection and preparation of plant

The samples of whole *P.amarus* were collected from their natural habitat within the compound of Lagos University Teaching Hospital Idi Araba. They were collected in a proper season and condition as recommended by W.H.O., 2003. The plants were identified and authenticated by a technologist at Pharmacognosy department, of University of Lagos, Idi araba with voucher number; PCGH 453. *P. amarus* leaves and stem were thoroughly washed and rinsed with distilled water and were air dried at room temperature (25°C) for two weeks, they were further dried using oven until constant weight was ensured. The dried plant material was grounded to fine powder with a domestic electric grinder, packaged in glass jars and store until required for use.

Plant extraction

174gram from the grounded plant materials was separately mixed with 1.5litre of methanol and distilled water. The mixtures were independently loaded into soxhlet extractor and extractions were completely done at 67°C and 92°C for methanol and aqueous extraction respectively. The extracts were then concentrated using rotary evaporator. The percentage yield of these extracts was approximately calculated to be 32% and 31% for methanol and aqueous extract respectively (see appendix). A sample of 10 g of the concentrated extract (from methanol and aqueous extract) was simultaneously dissolved in 100ml of water and used to make two-fold serial dilutions, to give 8 extract concentrations (100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0.8mg/ml). These concentrations were used as the extracts in this study. Alongside with these extracts preparation, 10 different concentrations of the control drug, ciprofloxacin were prepared, these concentrations were 12.5, 6.3, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05mg/ml and 5µg/ml. They were aseptically kept refrigerator at 2 - 4^0c till when needed.

Phytochemical screening

The methanolic and aqueous extracts of *P.amarus* were subjected to standard phytochemical analysis for different constituents such as Saponins, tanins, alkaloids, flavonoside, phlobatannins, terpenoids as described by Sofowora, Trease and Evan.[27,28]

Detection of saponins (Froth test)

0.5g of the extracts were diluted with distilled water to 20ml which was shaken in a graduated cylinder for 15 minutes. Formation of 1cm layer of foam indicated the presence of saponins.[27]

Detection of cardiac glycosides (Keller Killian's test)

0.5g of the extracts were dissolved in 2ml of glacial acetic acid containing one drop of 3.5% ferric chloride solution, then 1.5ml of sulphuric acid. For methanolic extract, a brownish ring was obtained at the interface and greenish ring at interphase in water extract, this indicate the presence of a de-oxy sugar characteristic of cardenolides.[27]

Detection of flavonoids (Ferric Chloride test)

0.2ml of the 10% $FeCl_3$ was added to each extract, the mixture was shaken together to observe the colour. A bluish green colour was observed both in methanolic extract and water extract indicating the presence of phenolic nucleus.

Detection of alkaloids

0.5g of each extract was stirred with 5ml of 1% aqHCl on a steam bath; 1ml of the filtrate was treated with a few drops of Mayers' reagent and a second 1ml portion was treated similarly with Dragendorff's reagent and finally another 1ml portion with Wagner's reagents.[27] With Mayers' reagent, slightly yellow colour is observed in methanolic extract and yellowish brown is observed in water extract. Also with Dragendorff's reagent, orange brown colour is observed in methanolic extract and dark orange colour is observed in water extract. Orange yellow colour is seen in methanolic extract and dark greenish brown colour observed in water extract when treated with Wagner's reagents. These observations indicated that Alkaloid is presence.

Detection of tannins

5g portion of methanolic and water extract were stirred with 10ml of methanolic and distilled water respectively then filtered. Ferric chloride reagent was added to 1ml of each filtrate and 1ml portion from each filtrate was also treated with bromine water.[28] Bluish green colouration was observed in both extracts when treated with ferric chloride, indicating the presence of tannins. Also, when treated bromine water, reddish green colour was observed in methanolic extraction and brownish green observed in water extract, this indicate that there is presence of condensing tannins.

Detection of phlobatannins

Little portion of aqueous and methanolic extract were boiled with 1% aqueous hydrochloride acid. Deposition of red precipitate indicate the presence of phlobatinnins.[27]

Test for terpenoids

5.0ml solution of the powder was mixed in 2ml of chloroform and 3ml concentrated H_2SO_4 was carefully added to form a layer. A

reddish-brown coloration at the interface show positive result for the presence of terpenoids.

Collection of the isolates

The test bacteria used were *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klesiella pneumonia, Enteroccocusfeacalis, Proteus mirabillis, Staphylococcus saprophyticus* and Control standard *Escherichia coli* ATCC 25922. These organisms were chosen because they are commonly pathogens known to be responsible for urinary tract infections (Ronald, 2002; Mansour *et al.,* 2009; Iregbu and Nwajiobi-Princewill, 2013). They were all collected from the Urine bench in the Department of Microbiology, Lagos University Teaching Hospital, except the Control standard *Escherichia coli* ATCC 25922, which was collected from Department of Microbiology, Nigeria Institute of Medical Research, Yaba, Lagos state.

Isolates identification

Isolates were collected from the Department of Microbiology, Lagos University Teaching Hospital. Gram staining was done for all the isolates, the gram-negative ones were manually identified using Oxoid Microbact identification kit while catalase, coagulase test were carried out on the gram-positive ones for identification. These isolates were appropriately labeled and maintained on nutrient agar slant at 4°C for further use.

Media used and its preparation

Muller Hinton broth was used as the primary medium for the tube dilution to determine the minimal inhibitory concentration (MIC) for each test microorganism (NCCLS, 2000).[29] Muller Hinton agar plates were used to plate out agar for the well diffusion assay, Nutrient and MacConkey agar were also selectively used during sub-culturing of the isolates. Mueller-Hilton Agar, its broth and MacConkey agar were prepared according to their manufacturer's instruction and sterilized by autoclaving at 121°C for 15minutes. The sterilized media of about 15ml was aseptically poured into sterile plates at the angle of 45^{o} and were allowed to be solidified before use. Sterility of the prepared broth and media were checked by incubating the media and broth at 37°C for 18 hours. The contaminated ones were discarded while non-contaminated ones were sealed and kept in the refrigerator at 2-8°C to prevent moisture loss and contamination.

Standardization of inoculums (McFarland's standard)

The inocula were prepared from the stock cultures, which were maintained on nutrient agar slant at 4°C and sub cultured onto Muller Hinton broth using a sterilized wire loop. The density of the suspension inoculated onto the media for susceptibility test was determined by comparison with 0.5 McFarland standard of barium sulphate solution.[30]

Antibacterial screening

Antibacterial activity of the methanolic and aqueous extracts of *Phyllanthus amarus* was individually tested against the studied organisms. The modified agar well-diffusion method was used to determine the antibacterial activity in-vitro.[31-33] Muller Hinton Agar (MHA) plates were inoculated using sterile cotton swabs which was deepen into the test tubes containing eight-hour-old broth cultures of the isolate to be tested. After 15minutes, sterile cork borers were used to make wells of 6mm diameter and 3cm apart from each other. About 200µl of each formerly prepared concentration of the extracts and the standard antibacterial drug 'Ciprofloxacin' were dispensed into the wells and then allowed to stand for about 15minutes for pre-diffusion to occur. These were then incubated at 37°C for 24hours. At the end of the period, inhibition zones formed on the agar were evaluated in mm.[34] The (Minimum Inhibitory Concentration) MICs of the extracts on the isolates were determined by CLSI method (2006).[35] 5ml of nutrient broth were measured into empty sterile tubes, 1ml of the different concentrations of the extracts, (methanolic and aqueous) were then added and 2 drops from the standardized inoculum of each isolate were added. This was incubated for 24hours at 37°C and the tubes were then observed for visible growth with the help of a spectrophotometer. The tube with the least concentration of the extract that showed no growth were determined as the Minimum inhibitory concentration (MIC) while aliquots of 50µl from broths showing no growth were plated onto Nutrient Agar and incubated for 24hours at 37°C and the minimum bactericidal concentration (MBC) were determined. MBC was determined as the highest dilution from which no bacterial growth was recorded. In all tests, sterile phosphate buffer saline and broth with only plant extracts were used as controls.

Data analysis

The data gotten from this research work were analyzed using three different software. They are;

SPSS version 17, Excel 2007 version, ANOVA and Sample t-test were used to analyze the data. *P*-value < 0.05 was considered to indicate statistically significant difference.

Results

Sorting phytochemical aqueous and methanolic extracts revealed the presence of different phytochemical compounds. Saponins, flavonoids, alkaloids, tannins, phlobatannins and terpenoids were present while cardiac glycosides were absent in both the aqueous and methanolic extract of *P. amarus*. This shows that the composition is essentially the same using these methods of extractions. The composition is illustrated in the Table 1.

Table 1 Phytochemical constituents of Methanolic and Aqueous extract of *Phyllanthusamarus*

Phytochemical composition	Methanolicic extracts	Aqueous extracts
Saponins	+	+
Cardiac glycosides	-	-
Flavonoids	+	+
Alkaloids	+	+
Tannins	+	+
Phlobatannins	+	+
Terpenoids	+	+

Key: - signifies absent, + signifies present

Table 2 shows the diameter of zones of inhibition of *Phyllanthus amarus* against the isolates at different concentrations of the aqueous extract. The zones of inhibition range from 1mm to 21mm in diameter. The table also shows that for most of the isolates, there is a concentration dependent response to the degree of inhibition which is indicated by the diameter of each inhibition zone. Table 3 shows

the diameter of zones of inhibition of *Phyllanthus amarus* against the isolates at different concentrations of the methanolic extract (Table 4). The zone of inhibition ranges from 1mm to 21mm in diameter as well. Just like in the aqueous extract, there is also a concentration dependent response to the degree of inhibition which is indicated by the diameter of each inhibition zone. At very high concentrations, most of the isolates are inhibited though at varying degrees while most isolates are resistant to inhibition at low concentrations. The ciprofloxacin produced an obvious inhibition of the growth of the isolates. This inhibition was observed at all concentrations (5µg/ml–12.5mg/ml). However, the degree of inhibition was low for PA I, PA II, SS I and SS II. Table 5 shows the susceptibility pattern of the various isolates on different antibiotics using disc diffusion methods.

Table 2 *Phyllanthusamarus (*Aqueous extract) Inhibition zones diameter (mm) on the isolates

Isolates	Concentration of aqueous extract	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.3 mg/ml	3.2 mg/ml	1.6 mg/ml	0.8 mg/ml
	Inhibition zone (mm)								
PA1		7	5	0	0	0	0	0	0
PAII		5	3	0	0	0	0	0	0
SSI		5	3	1	0	0	0	0	0
SSII		7	5	3	0	0	0	0	0
SAI		17	13	9	6	3	0	0	0
SAII		18	15	12	9	5	3	0	0
KPI		9	6	3	0	0	0	0	0
KPII		8	6	4	2	0	0	0	0
ECI		11	7	4	2	0	0	0	0
ECII		11	8	5	3	0	0	0	0
PMI		18	14	10	7	4	2	0	0
PMII		20	16	12	9	6	3	0	0
EI		20	17	15	12	9	7	5	4
EII		21	19	16	13	10	8	5	3
ECA		15	10	7	4	2	0	0	0

Keys: PAI, *Pseudomonas aeruginosa* I ; PAII, *Pseudomonas aeruginosa* II; SAI, *Staphylococcus aureus* I; SAII, *Staphylococcus aureus* II ; KPI, *Klesiellapneumonia* I; KPII, *Klesiella pneumonia* II; EI, *Enteroccocusfeacalis* I; EII, *Enteroccocusfeacalis* II; PMI, *Proteus mirabilis* I; PMII, *Proteus mirabilis* II; ECI, *Escherichia coli* I; ECII, *Escherichia coli* II; SSI, *Staphylococcus saprophyticus* I; SSII, *Staphylococcus saprophyticus* II; Control standard *Escherichia coli* ATCC 25922 (ECA)

Table 3 *Phyllanthusamarus (*Methanolicic extract) Inhibition zones diameter (mm) on the isolates

Isolates	Concentration of methanolic extract	100 mg/ml	50 mg/ml	25 mg/ml	12.5 mg/ml	6.3 mg/ml	3.2 mg/ml	1.6 mg/ml	0.8 mg/ml
	Inhibition zone (mm)								
PA1		7	4	0	0	0	0	0	0
PAII		7	5	0	0	0	0	0	0
SSI		7	4	2	0	0	0	0	0
SSII		9	6	4	2	0	0	0	0
SAI		18	14	11	9	6	4	0	0
SAII		20	17	15	12	10	7	4	0
KPI		15	12	9	6	3	0	0	0
KPII		14	11	8	4	2	0	0	0
ECI		9	6	3	0	0	0	0	0
ECII		10	7	5	2	0	0	0	0
PMI		19	17	10	8	6	5	0	0
PMII		21	18	14	11	9	6	3	0
EI		21	18	14	13	10	8	6	5
EII		20	18	15	12	10	8	5	3
ECA		11	8	5	2	0	0	0	0

Table 4 Inhibition zones of Standard antibiotic 'Ciprofloxacin' on the isolates

Isolates	Concentration of Ciprofloxacin	2.5 mg/ml	1.3 mg/ml	0.6 mg/ml	0.3 mg/ml	0.2 mg/ml	0.08 mg/ml	0.04 mg/ml	0.02 mg/ml	0.011 mg/ml	1 µg/ml
	Inhibition Zone (mm)										
PA1		8	4	2	0	0	0	0	0	0	0
PAII		7	38	1	0	0	0	0	0	0	0
SSI		12	7	5	0	0	0	0	0	0	0
SSII		13	9	6	3	0	0	0	0	0	0
SAI		35	33	32	30	28	25	23	20	16	14
SAII		36	34	32	29	27	25	22	20	17	15
KPI		39	37	33	30	28	26	25	24	22	20
KPII		38	35	32	30	29	27	26	23	21	19
ECI		45	42	40	37	36	34	32	30	27	23
ECII		45	43	41	38	36	33	31	29	27	23
PMI		44	43	41	40	38	34	32	30	26	22
PMII		45	43	40	38	36	33	31	29	27	24
EI		42	40	38	34	33	32	30	28	26	24
EII		43	41	39	37	34	32	29	27	25	23
ECA		40	38	35	33	31	30	28	27	25	21

Table 5 Inhibition zones (mm) of Multiple Antibiotic sensitivity disc on the isolates

Isolates	Ceftazidime CAZ 30µg	Cefuroxime CRX 30µg	Gentamicin GEN 10µg	Ciprofloxacin CPR 5µg	Ofloxacin OFL 5µg	Amoxycilin / Clavulanate AUG 30µg	Nitrofurantoin NIT 300µg	Ampicilin AMP 10µg
	Zone of inhibition (mm)							
PA1	15	0	10	0	0	0	0	0
PAII	13	0	9	0	0	0	0	0
SSI	0	0	0	0	0	14	29	0
SSII	0	0	0	10	0	12	24	0
SAI	11	22	0	24	18	20	24	0
SAII	15	23	11	20	20	17	21	0
KPI	20	17	17	19	18	17	14	0
KPII	21	20	16	17	18	19	15	0
ECI	21	18	15	22	17	0	25	0
ECII	18	18	19	25	20	0	23	0
PMI	21	17	15	22	20	0	10	0
PMII	22	19	14	20	19	0	13	0
EI	22	18	15	24	20	19	20	0
EII	21	14	17	25	18	21	21	0
ECA	20	20	15	24	15	0	24	0

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Figure 1 compares the inhibition zones of isolates at 12.5mg/ml of aqueous extract, methanolic extract of *P. amarus* and Standard antibiotic 'Ciprofloxacin'**.** The zones of inhibition follow essentially the same pattern at the other concentrations used under this study. Table 6 shows the comparism of the means of diameters of zones of inhibition of the isolates. For all the isolates at a concentration of 12.5mg/ml, there was no statistical significant difference between the means obtained for aqueous and methanolic extract of *P. amarus*. However, comparism between the means of the aqueous extract and ciprofloxacin showed a marked and statistical significant difference in the means. There was also a statistical significant difference between the means of inhibition of the methanolic group and ciprofloxacin of the isolates. The results show that there is no statistical significant difference between the antibacterial effect of the methanolic and aqueous extracts of *P. amarus* whereas antibacterial effect of ciprofloxacin differed significantly from the antibacterial effect of the two extracts. While the MIC and MBC were not determined for PA I and PA II, both extracts showed the lowest MIC (12.5mg/ml) as well as MBC (25mg/ml) against PM II, E I and E II isolates. Both extracts with MIC of 50 mg/ml and MBC of 100 mg/ml showed highest concentrations on SS II, SA I, KP I, KP II, EC II and ECA isolates, as seen in Table 7.

Tables 6 Comparing the zones of inhibition at concentrations 12.5 mg/ml.

	Aqueous extract	**Methanolic extract**	**Ciprofloxacin**		**P values**	
Isolates	Mean ± SEM (mg/ml)	Mean ± SEM (mg/ml)	Mean ± SEM (mg/ml)	(Aqueous vs Methanolic)	(Aqueous vs Ciprofloxacin)	(Methanolic Vs Ciprofloxacin)
PA1 & PAII	6.00 ± 1.00	7.00 ± 0.00	7.50 ± 0.50	0.25*	0.05#	0.13
SSI & SSII	6.00 ± 1.00	8.00 ± 1.00	12.50 ± 0.50	0.49*	0.07#	0.08
SAI & SAII	17.50 ± 0.50	19.00 ± 1.00	35.50 ± 0.50	0.205*	0.019	0.016
KPI & KPII	8.50 ± 0.50	14.5 ± 0.50	38.50 ± 0.50	0.25*	0.005	0.018
ECI & ECII	11.00 ± 0.00	9.50 ± 0.50	45.00 ± 0.00	0.12*	0.009	0.008
PMI & PMII	19.00 ± 1.00	20.00 ± 1.00	44.50 ± 0.5	1.0*	0.012	0.011
EI & EII	20.50 ± 0.5	20.50 ± 0.5	42.50 ± 0.5	1.2*	0.029	0.9

Key; # signify Significant*signifyNot significant, At 0.05 level

Table 7 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Aqueous and Methanolic Extract of the *Phyllanthusamarus*on the Isolates

Isolates	**Concentration of methanolic extract**		**Concentration of aqueous extract**	
	MIC mg/ml	MBC mg/ml	MIC mg/ml	MBC mg/ml
PA1	ND	ND	ND	ND
PAII	ND	ND	ND	ND
SSI	100	ND	ND	ND
SSII	50	100	50	100
SAI	50	100	50	100
SAII	25	50	25	50
KPI	50	100	50	100
KPII	50	100	50	100
ECI	50	100	50	100
ECII	50	100	50	100
PMI	25	50	25	50
PMII	12.5	25	12.5	25
EI	12.5	25	12.5	25
EII	12.5	25	12.5	25
ECA	50	100	50	100

Key: ND -Not Determined

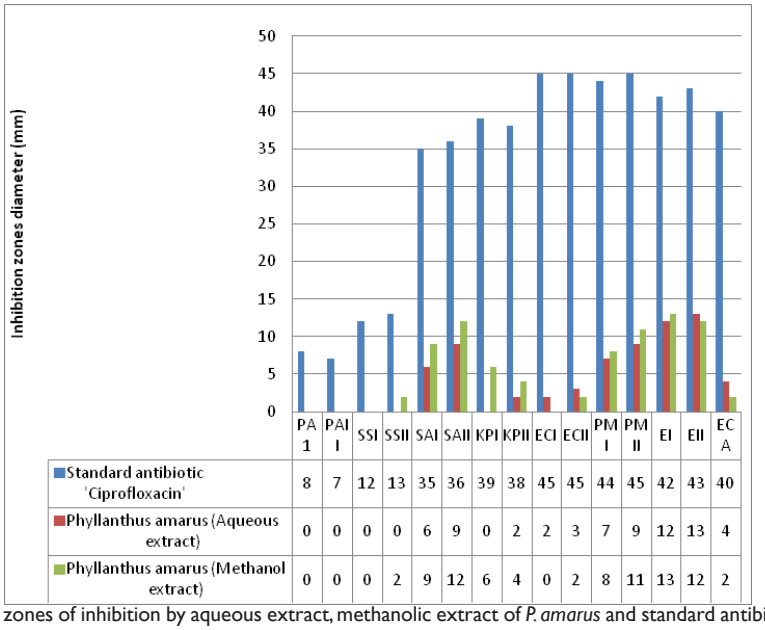

Figure 1 Comparison between the zones of inhibition by aqueous extract, methanolic extract of *P. amarus* and standard antibiotic 'ciprofloxacin' on the isolates.

Table 8 shows the susceptibility rate of the isolates to different standard antibiotic used. It was observed that none of the isolates were susceptible to Ampicilin. Also, susceptibility rates of Gentamicin and Ciprofloxacin, Ceftazine and Nitrofurantoin were the same in this course of studies. Similarly, Table 9 shows the susceptibility rate of the isolates to the extracts of *P amarus*. of particular significance is the 100% sensitivity of both extracts to the isolates a concentration of 50 and 100 mg/ml. the susceptibility tends to be concentration dependent in both extract. An independent sample test that was conducted to test the hypothesis above at 0.05% level of significance. Inhibitory zones generated on agar well method used and disc diffusion method on ciprofloxacin at 5µg/ml were subjected to the comparison test, the result was summarized and presented in the Table 10. Finally, Table 10 shows the two methods (Agar well and disc diffusion) used for the study. Agar well method has a mean of 15.2±9.929 while disc diffusion has a mean of 16.8±9.495, $p<0.725$. from the values in the table, there is no statistically significant difference between the means. This shows that there is no statistically significant difference between the methods used for this study. Antimicrobial sensitivity pattern shows zones of inhibition at different concentration of ciprofloxacin on the isolate tested using Agar well diffusion method and the susceptibility tends to be concentration dependent. Also, zones of inhibition at different concentration (100mg/ml, 50mg/ml, 25mg/ml and 15.5mg/ml) of aqueous and methanolic extracts on the isolate tested using Agar well diffusion method was revealed which tends to be concentration dependent in both extract.

Table 8 Susceptibility rate of the isolates to Standard Antibiotics used

	Sensitive No. of isolates (%)	**Resistance No. of isolates (%)**
Ceftazidine	13 (86.67%)	2 (13.33%)
Cefuroxime	11 (77.33%)	4 (26.67%)
Gentamicin	12 (80%)	3 (20%)
Ciprofloxacin	12 (80%)	3 (20%)
Ofloxacin	11 (77.33%)	4 (26.67%)
Amoxycilin / Clavulanate	8 (53.33%)	7 (46.67%)
Nitrofurantoin	13 (86.67%)	2 (13.33%)
Ampicilin	0 (0%)	15 (100%)

Table 9 Susceptibility rate of the isolates to the extracts of *Phyllatusamarus*

Concentration of the extracts (mg/ml)	**Aqueous extracts**		**Methanolic extracts**	
	Sensitivity	**Resistance**	**Sensitivity**	**Resistance**
	No. of Isolates (%)		**No. of Isolates (%)**	
100	15 (100%)	0 (0%)	15 (100%)	0 (0%)
50	15 (100%)	0 (0%)	15 (100%)	0 (0%)
25	13 (86.67%)	2 (13.33%)	13 (86.67%)	2 (13.33%)
12.5	11 (73.33%)	4 (26.67%)	10 (66.67%)	5 (33.33%)
6.3	11 (73.33%)	7 (46.67%)	7 (46.67%)	8 (53.33%)
3.2	6 (40%)	9 (60%)	5 (33.33%)	10 (66.67%)
1.6	11 (73.33%)	4 (26.67%)	2 (13.33%)	13 (86.67%)
0.8	13 (86.67%)	2 (13.33%)	2 (13.33%)	13 (86.67%

Table 10 Summary table of independent t-test table comparing agar well method used and disc diffusion method on ciprofloxacin

Variable	N		S. D	Df	T	P
Ciprofloxacin on Agar well	15	15.2	9.929			
				28	1.798	0.725
Ciprofloxacin on disc diffusion	15	16.8	9.495			

Discussion

Several investigations had reported that plants contain antimicrobial substances.[36] The results of the present study agree essentially with the reports of these previous workers. The results show that both aqueous and methanolic extracts have the same bioactive agents consisting of saponins, flavonoids, alkaloids, tannins, phlobatannins and terpenoids. Cardiac glycosides were however absent. This shows that the method of extraction does not really affect the composition of the extracts. Saponins, terpenoids, flavonoides, steroids, phlobatannins and tannins are the effective agents of medicinal plant which have medicinal values as shown in some other studies.[37] Saponins have been reported as natural antibiotics helping the body to fight infections as well as knocking out some tumors especially blood and lung cancers.[38] Flavonoids are antioxidants and free radical scavengers which prevent oxidative cell damage, have strong anti-cancer activity and protect the cell against all stages of carcinogenesis. Terpenoids are known to have analgesic properties. Phlobatannins are diuretic. The presence of terpenoids in the aqueous and methanolic extract of *P. amarus* indicated it is a natural agent for antibacterial and antifungal botanicals.[39] Steroids have been shown to have anti-inflammatory properties.[40] The zones of inhibition of aqueous and methanolic extracts of *P. amarus* range from 1mm to 21mm in diameter. It was observed that for most of the isolates, there is a concentration dependent response to the degree of inhibition which is indicated by the diameter of each inhibition zone. At very high concentrations, most of the isolates are inhibited though at varying degrees while most isolates are resistant to inhibition at low concentrations.

As expected, the standard antibiotic being a combination of pure antibiotic agents produced a better concentration dependent inhibition of the growth of the isolates. Using ANOVA to compare the means of the zones of inhibition of these isolates at different concentration of aqueous, methanolic extract of *P. amarus* and standard antibiotic, the degree of inhibition follows essentially the same pattern at the concentration under study. At the concentrations of 12.5, 6.3, 3.2, 1.6 and 0.8 mg/ml of all these antimicrobial agents, the degree of inhibition of the ciprofloxacin differed significantly from the two groups of *P. amarus* extract, F (44.817), $p < 0.05$. at concentration of 12.5 mg/ml for example, ciprofloxacin has the highest degree of inhibition and least in both the aqueous and methanolic extracts of *P. amarus*. Thus, all the plant extracts inhibited the growth of all the test isolates at the tested concentrations. This might be due to the resultant effect of the bio-active agents in the plant materials. The results were in agreement with the work of Akinjogunla et al.[41] With few exceptions, it was observed that the higher the concentration, the more the activity of the extract and as the concentration decreases, the lower the anti-bacterial activity against the urinary tract pathogens.[42]

According to Daniyan et al.,[43] agents with low activity against a particular organism usually gives high MIC and MBC values, while a highly reactive agent gives low MIC and MBC values. The MIC and MBC techniques are used to evaluate the efficacies of antimicrobial agents. In this study, MIC values obtained for most pathogens under study were high ranging between 50 and 100mg/ml (Table 7) when compared to MIC values of 10 to 30 µg/ml frequently recorded for convectional antibiotics. The observed differences could be due to the fact that while synthetic antibiotics are in a pure form, crude plant extracts contain some impure substances that may be inert and do not have any antimicrobial activities. Worthy of note is the fact that the resistance pattern and susceptibility rate of the isolates differ. Some isolates are more resistant to antibiotics than others. Maximum zones of clearance by *P. amarus* were observed in gram negative bacteria. Similar results were obtained by Mazumder et al.,[32] where the extract showed significant concentration dependent antibacterial activity particularly against gram negative microbes. Mazumder et al.,[32] reported that bacteria causing diarrhea and dysentery were effectively inhibited by extract of *P. amarus*. The reason for the difference in activities in both of the findings is supposed to be dependent on plant habitat Rajeshkumar et al.[44] The results obtained in antimicrobial activity are similar to those of Lin et al.,[45] Finally, there is need to test the methods used in other to ascertain their suitability. Table 4 showed that there is no statistically significant difference between the Agar well method and the Disk diffusion method. This shows that results obtained from this study are reliable.

Conclusion and recommendation

The results of the antimicrobial screening show that *P. amarus* aqueous and methanolic extract exhibited appreciable antibacterial properties inhibiting growth of all bacteria. This study therefore, has provided bases to the folkloric use of this plant as a remedy for urinary tract infections caused by the pathogens studied as practiced ethno-medically in Nigeria. It also justifies the folklore medicinal uses and claims about the therapeutic values of these plants as curative agent. However, if the plant extracts are to be used for medicinal purpose, there should be improvement in approaches for natural product isolation, characterization and synthesis. This could be opening door to a new era in the investigation of natural products in academia and industry. I therefore recommend that; Herbal medicine should be incorporated and encouraged in the treatment of infections and diseases and there should be establishment of a comprehensive medicinal plants information and trained personnel.

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Conflicts of interest

The author declare no conflicts of interest.

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