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ANTIMICROBIAL ACTIVITY OF *Sida acuta, Phyllanthus amarus* AND *Phyllanthus muellerianus* AGAINST MICROORGANISMS IMPLICATED IN URINARY TRACT INFECTIONS

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ABSTRACT

Increasing level of antimicrobial resistance among bacterial pathogens causing Urinary Tract Infection (UTI) is one of the most significant public health challenges globally. Hence, the search for alternatives from medicinal plants. This study investigated the efficacy of *Phyllanthus amarus* (PA), *Phyllanthus muellerianus* (PM) and *Sida acuta* (SA) leaf extracts on microorganisms implicated in UTI. Mid-stream urine samples collected from 100 patients clinically diagnosed with UTI were cultured. The microorganisms isolated were identified using their morphological and biochemical characteristics. Methanol leaf extracts of the three plants were obtained by cold maceration in 60% methanol. Crude extract of PM was thereafter purified by solvent partitioning. Antibiotic susceptibility test was determined using the Kirby Bauer disc diffusion. Antimicrobial effects of the extracts and oil was ascertained using agar well diffusion. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC) were also determined. Rate of kill and mechanism of action of the purified extract of PM on isolates were investigated. Cytotoxicity of plant extracts were assayed on brine shrimps while synergism of the purified extract with ciprofloxacin was ascertained using overlay inoculum susceptibility disc method. Antioxidant and phytochemical analyses of the extracts were conducted using standard methods. Phytochemical analysis of the leaf extracts showed the presence of alkaloids, tannins, saponins and steroids. Antioxidant assay also indicated SA had the highest total flavonoids and phenol content of 339.86 mgQUE/g and 27.63 mgGAE/g. Microorganisms isolated include: *Escherichia coli* (24%), *Proteus mirabilis* (24%), *Staphylococcus aureus* (19%), *Klebsiella pneumoniae* (13%), *Candida albicans* (11%), *Enterobacter sp.* (5%) and *Citrobacter sp.* (4%). The crude extract of PA had zone of inhibition ranging from 16.7 ± 1.53 mm to 24 ± 1.00 mm while SA crude extract had 14.7 ± 1.53 mm to 27 ± 2.00 mm. PM crude extract had inhibition zones of 17 ± 1.00 mm to 22.3 ± 2.12 mm. The MIC and MBC ranged from 6.25 mg/ml to 50 mg/ml and 12.5 mg/ml to 50 mg/ml respectively. Ethyl acetate fraction of PM showed the highest percentage yield and had a zone diameter range from 13.5 ± 1.00 mm to 28 ± 1.53 mm with MIC and MBC ranges of 6.25 mg/ml – 12.5 mg/ml and 25 mg/ml to 50 mg/ml respectively. Synergism with ciprofloxacin was observed at 25% of the microorganisms, 50% antagonism and 25% additively. Toxicity analysis showed lethal dose concentrations of 19.05 mg/ml, 25.12 mg/ml and 130.11 mg/ml for PM, PA and SA respectively. The findings of this study suggest that the methanol extracts of the medicinal plants used in this study does possess a potent lead molecule in combating microorganisms causing UTI.

Key words: Antimicrobial activity, *Phyllanthus muellerianus,* Phytochemicals, Toxicity, UTI,

INTRODUCTION

Over the years, urinary tract infection (UTI) has been an issue of public health concern in many countries. Maurya and Singh, (2014) stated that it accounts for 8.3 million doctor visits yearly in the United states and it is the second most common type of infection in the body. This form of infection occurs mostly among women but only a few are found among men. Parveen *et al.,* (2011), defined UTI from the microbiological point of view as the presence of at least 10^5 organisms /ml of urine in an asymptomatic patient or as more than 100 organisms/ml of urine in a symptomatic patient.

Gbadamosi (2015) highlighted common symptoms to include burning with frequent urination (or an urge to urinate) in the absence of vaginal discharge and significant pain. These symptoms may vary from mild to severe and in healthy women lasting an average of six days (Nicolle, 2008). UTIs are caused by both Gram-negative and Gram-positive bacteria, as well as by certain fungi. Maurya and Singh (2014) stated that UTI is primarily caused by Gram negative bacteria such as *Escherichia coli, Proteus mirabilis, Klebsiella pneumonia* and *Enterobacter aerogenes.* Other Gram positive bacteria that may be involved in urinary tract infection include *Staphylococcus saprophyticus,*

Enterococci and *Staphylococcus aureus*. The most common causative agent for both uncomplicated and complicated UTIs is uropathogenic *Escherichia coli* (UPEC). For the agents involved in uncomplicated UTIs, UPEC is followed in prevalence by *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, group B Streptococcus (GBS), *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida spp*. (Foxman, 2014).

In the past, the use of antibiotics has proven to be very effective in curtailing the spread of these infections either completely or partially, but in recent times, the uncontrolled use of antibiotics had resulted in bacterial resistance thus limiting their effectiveness and usability (Laxminarayam *et al*., 2013). Flores-Mireles *et al*., (2015) opined that patients suffering from a symptomatic UTI are commonly treated with antibiotics; these treatments can result in long-term alteration of the normal microbiota of the vagina and gastrointestinal tract and in the development of multidrug-resistant microorganisms. Thus, the availability of niches that are no longer filled by the altered microbiota can increase the risk of colonization with multidrug-resistant uropathogens (Kostakioti *et. al.,* 2012; Jasmine *et. al.,* 2014). Rajasekara *et al.,* (2007) also opined that other draw backs such as the high cost of these chemical antibiotics and their undesirable side effects such as hypersensitivity and allergic reactions are serious burning global issues in treating infectious diseases (Kiffer *et. al.,* 2007).

Nature has been a source of medicinal agents for thousands of years and quite a number of modern drugs have been isolated from natural sources (Tcheghebe *et al.* 2017). Sravanthi Pammi *et al.* (2016) noted that *Phyllanthus amarus* has been useful in several health problems such as diarrhoea, dysentery, dropsy, jaundice, intermittent fevers, urinogenital disorders, scabies and wounds (Narismnudhu and Venkata, 2012). The plant *Phyllanthus muellerianus* (Kuntze) Excell, has been commonly used to treat intestinal troubles, severe dysentery, constipation, stomach ache, jaundice and urethral discharges (Katsayal and Lamal, 2009). Arbonnier (2004) opined that throughout West Africa, pastes of the leaves are applied as wound dressing and various extracts of the leaf are used as vapour bath to treat veneral diseases and toothache. *Sida acuta (*Burm F.*)* on the other hand has been used traditionally to treat diseases such as fever, headache, diarrhea, dysentery and lots more through oral route or applied on the skin (Karou *et al.* 2007). Prabhu (2016) stated that the aqueous and acetone extracts of *Sida acuta* has antibacterial activity against Extended Spectrum Beta Lactamase (ESBL) producing *Escherichia coli.*

The prevalence of UTI in our community calls for concern for all considering the fact that most of the people involved are asymptomatic thus making other individuals vulnerable suggesting the need to look inwards at our local medicinal products and their efficacy in the treatment of prevalent infections such as UTI. Hence this study is aimed at determining the efficacy of coconut oil, *Phyllanthus amarus, Phyllanthus muellerianus* and *Sida acuta* extracts on organisms implicated in UTI.

MATERIALS AND METHODS

Ethical Approval

Ethical approval was obtained, from the Health research ethics committee of the State Hospital, Ijaye Abeokuta Ogun State, Nigeria.

Sample Collection

A total of one hundred (100) fresh midstream urine samples were collected in sterile sample bottles from patients clinically diagnosed with UTI at the Medical Microbiology Laboratory of the State Hospital, Abeokuta and transported immediately to the Postgraduate Laboratory, Department of Microbiology, Federal University of Agriculture, Abeokuta, South West Nigeria, for analysis.

Isolation and Characterization of Microorganisms

A loopful of the urine sample was streaked on sterile plates of Cystine Lysine Electrolyte Deficient agar (CLED), Nutrient agar and MacConkey agar to isolate urinary pathogens. The characterization and identification of isolates was based on standard morphological and biochemical features (such as their Gram stain, the shape, size and arrangement of the organisms microscopically as well as biochemical tests such as Catalase, Coagulase, Indole, Voges Proaskeur, Methyl red, Urease, Citrate, Oxidase, Sugar fementation tests). Germ tube test was also used

in the identification of opportunistic yeast pathogens. Also ten typed cultures were collected and maintained on nutrient agar slant as reference.

Plant Collection and Extraction

The three plants used in this study*, Phyllanthus amarus, Phyllanthus muellerianus* and *Sida acuta* were collected, identified and confirmed at Obafemi Awolowo University Ile–Ife Nigeria. The plant extracts were obtained using the method described by Oluwafemi and Debiri (2010). The leaves of *P. amarus, P. muellerianus* and *S. acuta* plants were air – dried, chopped into small pieces and thereafter pulverized using a blender. Two hundred grams (200 g) of the pulverized leaves were macerated with 2 L of 60% methanol and stirred continuously for 72 hours. The mixture was then filtered using Whatman No. 1 filter paper and the filtrate evaporated to dryness under *vacuum* to a solid mass using a rotary evaporator and subsequently stored in a Petri dish in a desiccator.

The plant extracts was later reconstituted using water as solvent to a concentration of 50 mg/ml for antimicrobial activity evaluation.

Purification of the Extracts

The crude extracts were purified by solvent partitioning using N – hexane, dichloromethane (DCM), ethyl acetate and butanol in order of their polarity. The dried crude extract was reconstituted with distilled water and poured into the separating funnel after which N – hexane was added and swirled gently to mix. The mixture was left to settle into layers before collecting the N- hexane fraction. This process was repeated until there was no more change in the colour of the N – hexane. Dichloromethane (DCM) was thereafter added to the remaining solution and its fractions were also collected followed by ethyl acetate and lastly butanol. The remaining solution of the extract was taken as the aqueous fraction. The various fractions of the extracts in solution were concentrated to dryness using the rotary evaporator while the aqueous fraction was lyophilized.

Antimicrobial Studies

Determination of antimicrobial activity: The Agar well diffusion method of Akinpelu and Onakoya (2006) was employed. An overnight plate culture of the organisms was standardized in normal saline to contain approximately 10^8 cfu/ml using a colorimeter and this was then streaked to cover the entire surface of 20 ml of sterile nutrient agar plates using sterile swab sticks. Thereafter, a sterile cork borer (8.0 mm diameter) was used to punch wells in the seeded nutrient agar. The agar plugs were removed with a flamed and cooled wire loop. A 50 mg/ml concentration of the various plants extracts was thereafter dispensed into separate wells in different plates up to the brim of the well. The plates were thereafter incubated at 37 ^{o}C for 24 hours and the zones of inhibition were measured. Also the typed cultures were tested with the crude extracts to determine their susceptibility. The experiment was repeated in triplicates.

Determination of minimum inhibitory concentration (MIC): The MIC of test isolates was performed using the method described by Akinpelu and Onakoya (2006). This assay was done to determine the lowest concentration of extract that will inhibit microbial growth. A two-fold dilution of the extracts was prepared based on the concentration that inhibited growth in the antimicrobial susceptibility test. Ten tubes were obtained and 2 ml of sterile distilled water (reconstituting solvent) was dispensed into each tube except the first tube which contained 4 ml sterile distilled water. A 2.2 g of the crude extract was poured into the first tube containing 4 ml of sterile distilled water and mixed thoroughly, thereafter 2 ml of the mixture was taken and poured into the second tube and mixed properly. Thereafter 2 ml of that mixture was transferred serially up to the tenth tube and 2 ml was removed and kept. These test tubes containing 2 ml of different concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.7825, 0.390, 0.195 mg/ml) respectively were later poured into 18 ml of sterile molten nutrient agar and mixed properly. This agar – extract mixture was thereafter transferred into Petri – dishes and allowed to set. The plates were then inoculated with the various test organisms and incubated at 37 ^{0}C for 72 hours. Controls were set up as inoculated nutrient agar plates without the extract as negative control and un-inoculated nutrient agar plates with extract as positive control.

Determination of minimum bactericidal concentration (MBC): Based on the MIC results

obtained, the concentrations of all extracts that showed no growth were sub-cultured into sterile nutrient agar plates and incubated at same temperature for 48 hours. The MBC was taken as the least concentration that did not show any growth on the agar plates.

Antibiotic Sensitivity Test

Kirby Bauer's disk diffusion method was employed to determine the effect of standard antibiotics against the test microorganism. Ten different standard antibiotics (Oxoid) were used for this study. They include: ciprofloxacin, gentamicin, ampicillin, cefoxitin, chloramphenicol, nalidixic acid, amoxycillin/clavulanic acid, sulphamethoxazol-trimethoprim, amikacin and tetracycline. These antibiotic discs were placed aseptically on the plate already seeded with the test organisms using a pair of sterile forceps. The plates were thereafter incubated at 37° C for 24 hours. After incubation, zones of growth inhibition was measured and recorded. This experiment was carried out in duplicates. The result obtained was thereafter interpreted using the Clinical and Standard Laboratory Institute (CLSI) chart (CLSI, 2013) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST, 2018)

Effect of the combination of the extract with commercial antibiotics

The Overlay Inoculum Susceptibility Disc technique as described by Ofokansi *et al.* (2012) was used. Ciprofloxacin was selected as it shows relative efficacy against all the clinical isolates tested. To prepare the mixture, 2 ml of the MIC value of the ethyl acetate fraction of *P. muellerianus* was dispensed in 18 ml of molten nutrient agar to form an agar – extract mixture. The agar – extract mixture was poured into sterile clean Petri dish and allowed to solidify to form the base agar. About 2 ml of another sterile molten nutrient agar was inoculated with 0.5 ml of the standardized test organism and shaken gently to ensure uniformity of the cells in the medium. The inoculated medium was then poured on the surface of the base agar to form a thin uniform layer, the overlay inoculum agar. When the inoculum agar has solidified; the antibiotics disc (Ciprofloxacin) was placed on the agar medium. Two control plates were prepared. Control A contained only the base agar and the overlay inoculum agar, while control B contained the nutrient agar without the extract, the overlay inoculum and the antibiotic disc. The three plates in the set were kept at room temperature for 1 hour to allow for pre diffusion, and then incubated at 37 °C for 48 hours. The zone of inhibition formed on the test plates was used to determine the combined effect of the antibiotics and the extract by comparing it with the result in control B. Synergism is obtained when the diameter of the zone of inhibition in the test plate is greater than that in control B by at least 19%; lower than 19% indicates additivity, equal to control B indicates indifference; when it is less than control B, there is antagonism.

% increase was calculated using the formula;

$$\%\ Increase = \frac{Diameter\ zone\ of\ inhibition\ of\ test - zone\ of\ inhibition\ of\ Ciprofloxacin}{Diameterzone\ of\ inhibition\ of\ Ciprofloxacin\ only} \times 100$$

Phytochemical Screening of the Extracts

The various extracts were subjected to phytochemical screening using the method as previously described by Kalaivani and Vidhya, (2014). This method was used to test for saponins, tannins, terpenoids, glycosides, alkaloids, flavonoids and reducing sugars.

Determination of Toxicity using the brine-shrimp lethality test

The extracts were evaluated for lethality to brine shrimp larvae (*Artemia salina* Leach) according to the procedure described by Sahely *et al.* (2017) with some modifications. Briefly, dried brine shrimp eggs were bred in saline medium. After 24 hours, a few shrimps were hatched and ready for testing. One-day-old larvae was transferred into 5 ml vials (10 per vial) containing saline solution along with 100, 50, 25, 12.5, 6.25 mg/ml of each extract and diluted serially in saline water. In each case, 3 replicates of each concentration were assayed. After 24 hours, the survivors were counted and the percentage mortality at each dose recorded. A saline solution containing distilled water was used as the negative control while saline water only with the nauplii was used as positive control. After 24

hours of exposure, the median lethal concentration (LC_{50}) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration as described by Hamidi *et al.* (2014). LC_{50} values were estimated using a probit regression analysis.

Antioxidant Assays

The various extracts were also tested for antioxidant properties. The properties assayed include: total phenol content, total flavonoids, total antioxidant capacity and ferric reducing antioxidant power (FRAP). All assays were done in accordance to the reports of Song *et al.* (2010), Ashafa *et al.* (2010), Miliauskas *et al.* (2004), Hanafey (2013) and Nwaehujor *et al.* (2017) respectively.

RESULTS

Socio – demographic Characteristics of Subjects with Urinary tract infection (UTI)

The socio – demographic characteristics of subjects are as shown in table 1. Among the hundred patients clinically diagnosed with UTI, 80% were females, 19% were in the age group 18 – 27 while 46%, 15% and 20% were in the age group 28 – 37, 38 – 47 and > 47 respectively.

Table 1: Socio – demographic Characteristics of Patients Diagnosed with UTI

Socio – Demographic Parameters	Variables	Frequency (Percentage %)
Sex	Male	20 (20)
	Female	80 (80)
Age group	18 – 27	19 (19)
	28 – 37	46 (46)
	38 – 47	15 (15)
	47 - above	20 (20)

Prevalence of Organisms Implicated in UTI

Figure 1 shows the prevalence of the organisms isolated from urinary tract infection (UTI). Of all the organisms, *Escherichia coli* and *Proteus mirabilis* had the highest occurrence of 24% each while *Citrobacter sp.* had the lowest with 4% frequency. *Staphylococcus aureus, Klebsiella pneumoniae, Candida albicans and Enterobacter* specie also had 19%, 13%, 11% and 5% occurrence respectively.

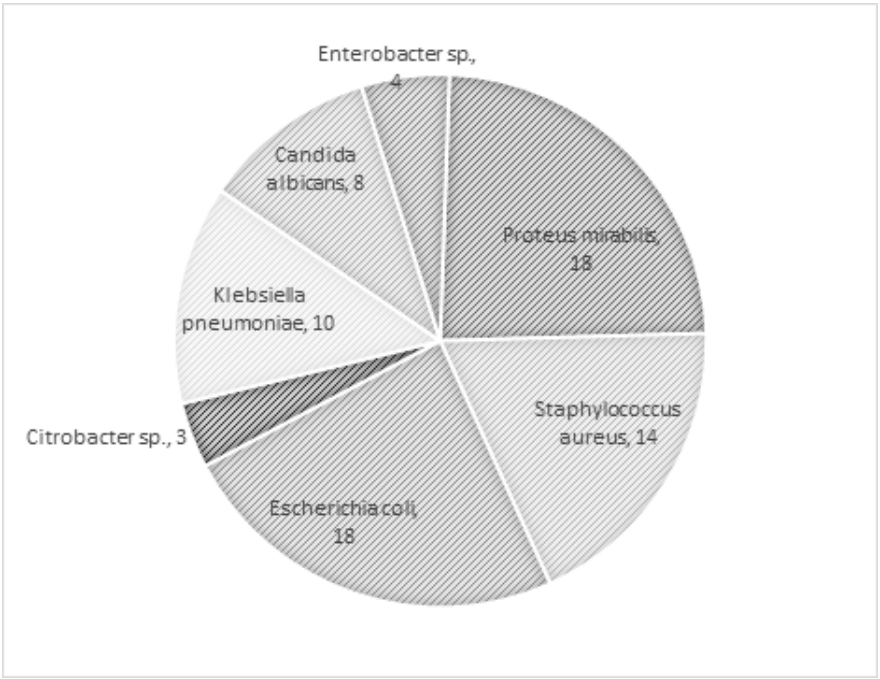

Figure 1: Frequency of Organisms Isolated from UTI Patients.

Antimicrobial Activities Exhibited by the Various Extracts

Table 2 shows the antimicrobial activities of the crude extracts of *P. amarus, S. acuta* and *P. muellerianus* against the organisms isolated in this study. All the isolates were susceptible to the crude extract of *P. amarus* with *Klebsiella pneumoniae* exhibiting the highest zone of 24±1.0 mm while *Proteus mirabilis* had the lowest zone of 16.7±1.5 mm. For *S. acuta* crude extract, the zones of inhibition ranged from 14.7±1.5 mm – 27±2.0 mm with *Candida albicans* showing the highest susceptibility while *Citrobacter sp.* had the lowest zone. However, *P. muellerianus* crude extract also exhibited a great deal of potency against the isolates as *Escherichia coli* had the lowest zone of inhibition of 17±1.0 mm while *Staphylococcus aureus* and *Klebsiella pneumoniae* had the highest zone of 22.3±2.1 mm.

Table 2: Antimicrobial Susceptibility Testing of Different Plant Extracts On Isolates

	Mean diameter zone of inhibition (mm)					
Organisms	PA	SA	PM	PASA	PAPM	SAPM
Proteus mirabilis	16.7 ± 1.5	23.3 ± 2.1	22 ± 2.0	23.3 ± 0.5	22 ± 1.0	14.7 ± 0.6
Escherichia coli	21.7 ± 1.5	15.7 ± 1.2	17 ± 1.0	20.3 ± 0.6	15.3 ± 1.2	13 ± 0.0
Citrobacter sp.	17.7 ± 1.2	14.7± 1.5	19 ± 2.7	14 ± 1.7	16.3 ± 1.2	15.3 ± 1.5
Staphylococcus aureus	19.7 ± 1.2	18.7 ± 1.5	18.7 ± 2.1	13.7 ± 1.2	15.3 ± 1.2	16 ± 2.0
Klebsiella pneumoniae	24 ± 1.0	18.3 ± 1.2	22.3 ± 2.1	23 ± 1.7	21.3 ± 1.5	21.7 ± 1.5
Candida albicans	21.7 ± 1.2	27 ± 2.0	18.3 ± 1.5	16.7 ± 0.6	18 ± 1.0	14.3 ± 0.6
Enterobacter sp.	23 ± 0.6	26 ± 1.7	22 ± 1.73	20.3 ± 1.5	23.3 ± 1.5	17.3 ± 1.5

Key: PA = *Phyllanthus amarus*, SA = *Sida acuta*, PM = *Phyllanthus muellerianus*,

Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) Exhibited by the Extracts against the Test Organisms

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the crude extracts against the microorganisms are as shown in table 3. The crude extract of *P. amarus* had an MIC value of 12.5 mg/ml for *Proteus mirabilis* and 25 mg/ml for all other organisms. The MBC value however showed slight variation as *Staphylococcus aureus* and *Enterobacter sp.* had a MBC value of 25 mg/ml while others had the MBC value of 50 mg/ml. *S. acuta* on the other hand had a lower MIC value of 6.25 mg/ml for about 37.5% of the organisms while the remaining 62.5% had a value of 25 mg/ml. For the MBC, only *Proteus mirabilis* and *Staphylococcus aureus* had the lowest value of 6.25 mg/ml while others had a concentration of 50 mg/ml.

Furthermore, *Candida albicans* had the lowest MIC of 12.5 mg/ml for *Phyllanthus muellerianus* while the highest of 50 mg/ml was observed in *Klebsiella pneumoniae, Citrobacter sp* and *Escherichia coli*. Similarly, MBC results indicated that *Candida albicans* had the lowest value of 12.5 mg/ml while others had a concentration of 50 mg/ml.

Table 3: MIC and MBC Value for the Crude Extracts of *Phyllanthus amarus*, *Sida acuta* and *Phyllanthus muellerianus*

Organisms	*Phyllanthus amarus*		*Sida acuta*		*Phyllanthus muellerianus*	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Proteus mirabilis	12.5	50	6.25	6.25	25	50
Staphylococcus aureus	25	25	6.25	6.25	25	50
Escherichia coli	25	50	25	50	50	50
Citrobacter sp.	25	50	25	50	50	50
Staphylococcus aureus	25	50	25	50	25	50
Klebsiella pneumoniae	25	50	25	50	50	50
Candida albicans	25	50	6.25	50	12.5	12.5
Enterobacter sp.	25	25	25	50	25	50

Percentage Yield of the Purified Fractions of *Phyllanthus mellerianus*

Aqueous fraction showed the highest percentage yield of 40.22% while Ethyl acetate had 34.3% yield and Butanol fraction was 22.64%. Dichloromethane and N – hexane fraction had a very low yield of 1.7% and 1.083% respectively.

Antimicrobial Activities of the Purified Fractions of *Phyllanthus muellerianus*

The four fraction tested showed considerable zones of inhibition on all the test organisms. 50% of the organisms showed the highest susceptibility to ethyl acetate and dichloromethane (DCM) fractions while the aqueous fraction had the lowest zones of inhibition on all the organisms. Figure 2 shows the various zones of inhibition of all the organisms tested in this study. The Minimum Inhibitory Concentration (MIC) of the ethyl acetate fraction was also determined and the result indicated that 37.5% had a MIC of 6.25 mg/ml while others except *Klebsiella pneumoniae* had a concentration of 12.5 mg/ml. Further analysis of the MBC revealed all test organisms except *Klebsiella pneumoniae* had MBC of 25 mg/ml. Table 4 shows the MIC and MBC value for all the organisms by ethyl acetate fraction of *Phyllanthus muellerianus*.

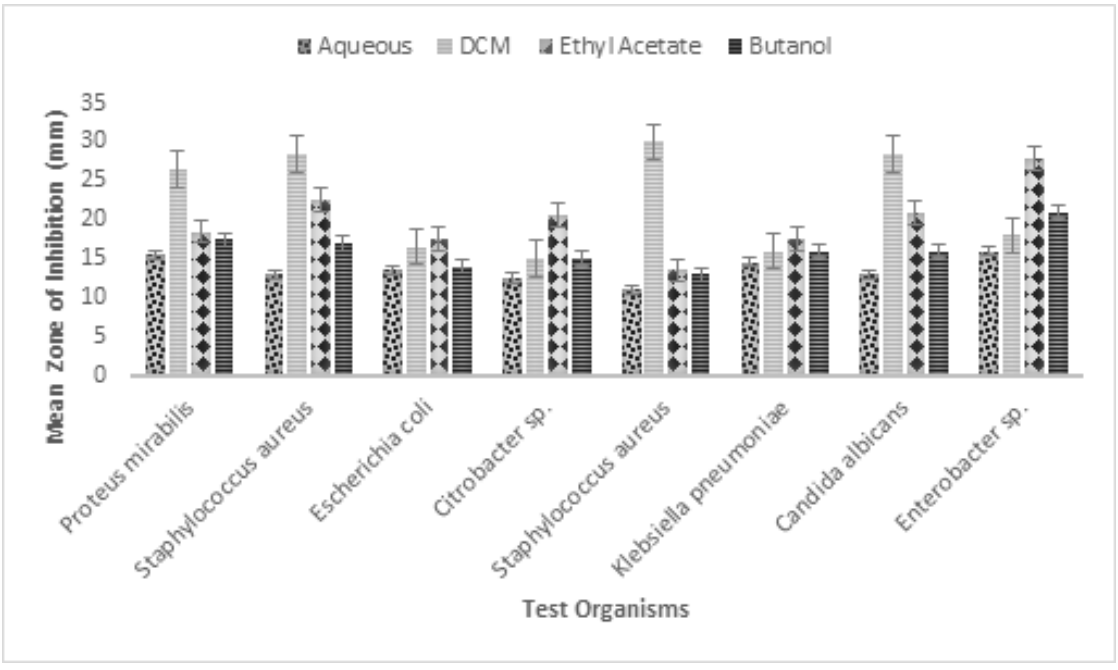

Figure 2: Zones of Inhibition of Partitioned Fractions of *Phyllanthus muellerianus* on Test Organisms (Abbreviation: DCM = Dichloromethane)

Table 4: MIC and MBC for Ethyl Acetate Fraction of *Phyllanthus muellerianus*

Organisms	MIC (mg/ml)	MBC (mg/ml)
Proteus mirabilis	6.25	25
Staphylococcus aureus	12.5	25
Escherichia coli	12.5	25
Citrobacter sp.	12.5	25
Staphylococcus aureus	12.5	25
Klebsiella pneumonia	25	50
Candida albicans	6.25	25
Enterobacter sp.	6.25	25

Effect of the Extracts on Typed Cultures

The ten typed cultures obtained were: *Escherichia coli* (NCIB 86), *Bacillus stereothermophilus* (NCIB 8222), *Micrococcus luteus* (NCIB 196), *Pseudomonas aeruginosa* (NCIB 950), *Clostribium sporogenes* (NCIB 532), *Proteus vulgaris* (NCIB 67), *Bacillus subtilis* (NCIB 3610), *Serratia marscens* (NCIB 1377), *Staphylococcus aureus* (ATCC 43300), *Klebsiella pneumoniae* (NCIB 418). Ethyl acetate fraction of *Phyllanthus muellerianus* showed a promising zone of inhibition for 80% of the test organisms with a zone of inhibition diameter range of 13 mm – 22 mm followed by the crude extract of *Phyllanthus muellerianus* with a range of 8 mm – 18 mm.

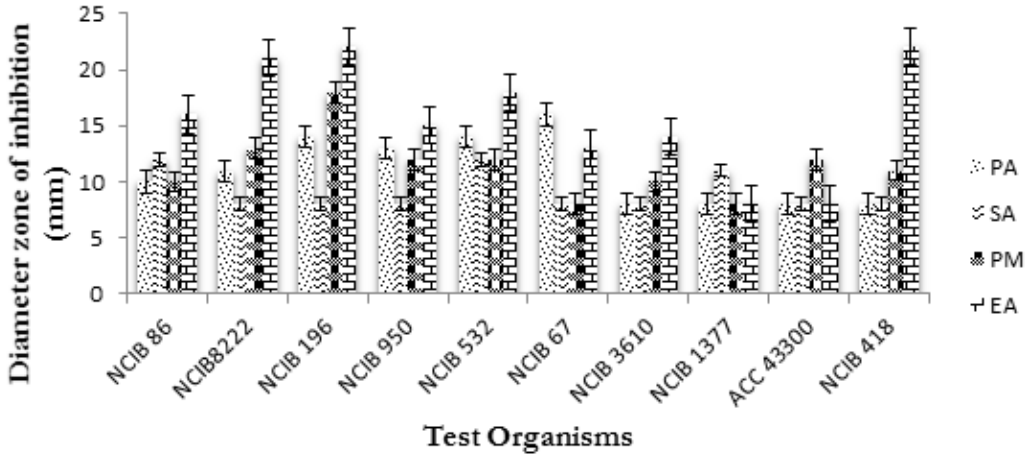

Figure 3: Diameter Zones of Inhibition by the Extracts on Typed Cultures.

Antibiotic Sensitivity of All Test Organisms

The result of the antibiotics sensitivity test as shown in tables 5 and 6 indicates that *Proteus mirabilis* was resistant to 7 of the antibiotics used, *Citrobacter sp*. was resistant to 5 antibiotics, *Staphylococcus aureus, Enterobacter sp*. and *Candida albicans* were resistant to 4 of the antibiotics while *Klebsiella pneumoniae* and *Escherichia coli* was resistant to only 3 antibiotics. However, 75% of the organisms tested were resistant to the penicillin and cephalosporin group of antibiotics, 62.5% were resistant to Chloramphenicol and Sulphamethoxazole Trimetoprim. In addition, the Aminoglycosides were shown to possess great antimicrobial activity against all isolates tested. Also with the exception of *Proteus mirabilis,* all organisms were sensitive to the Fluoroquinolones.

Table 5: Sensitivity of the Organisms from UTI to Antibiotics

Organisms	Antibiotics zones of inhibition (mm)									
	CIP (5 µg)	AK (30 µg)	SXT (25 µg)	NA (30 µg)	FOX (30 µg)	C (30 µg)	AMP (10 µg)	TE (30 µg)	CN (120 µg)	AMC (30 µg)
A	22.5 ±3.5	25±2.8	7 ± 0	8 ±1.4	21.5±2.1	7 ± 0	7.5±0.7	8.5 ± 0.7	25 ± 0	10.5 ± 0.7
C	33 ±4.2	26 ±2.8	7 ± 0	29 ± 4.2	26.5±2.1	8±1.4	7 ± 0	30 ± 2.8	27 ± 1.4	21.5 ±0.7
D	31.5 ±6.4	22 ±1.4	7 ±0	22.5 ±2.1	23± 0	8± 0	10 ± 4.2	9 ± 1.4	23 ± 2.8	8 ±2.8
E	32.5±3.5	10± 1.4	22.5±3.5	6.5 ±0.7	6± 0	23.5±0.7	6 ± 0	25 ± 0	27 ± 1.4	6 ± 0
F	35 ±0	22 ± 1.4	23 ± 1.4	26 ± 0	6± 0	21.5±0.7	6.5 ± 0.7	24.5 ±0.7	26 ± 1.4	6 ± 0
J	27 ±5.7	27 ±2.8	7 ± 0	28 ±2.8	20.5±0.7	26±1.4	10.5 ±4.9	33 ± 2.8	26 ± 0	17.5 ± 0.7
S18	32.5 ± 3.5	25 ±1.4	7.5 ± 0.7	28 ±2.8	8.5±2.1	7.5±0.7	16 ± 1.4	17 ±1.4	27.5 ±3.5	27.5 ± 3.5

Table 6: Antibiotic Sensitivity Interpretation of the Zones of Inhibition

Organisms	Antibiotics									
	CIP (5µg)	AK (30 µg)	SXT (25 µg)	NA (30 µg)	FOX (30 µg)	C (30 µg)	AMP (10 µg)	TE (30 µg)	CN (120µg)	AMC (30 µg)
A	R	S	R	R	S	R	R	R	S	R
C	S	S	R	S	S	R	R	S	S	S
D	S	S	R	S	S	R	R	R	S	R
E	S	R	S	R	R	S	R	S	S	R
F	S	S	S	S	R	S	R	S	S	R
J	S	S	R	S	R	S	R	S	S	R
S18	S	S	R	S	R	R	R	S	S	S

Abbreviations: CIP = Ciprofloxacin, AK = Amikacin, SXT = Sulphamethoxazole/ Trimethoprim, NA = Nalidixic acid, FOX = Cefoxitin;C =Chloramphenicol, AMP = Ampicillin, TE = Tetracycline, CN = Gentamicin, AMC = Amoxycillin/ Clavulanic acid. A = *Proteus mirabilis,* C = *Escherichia coli,* D = *Citrobacter sp,* E = *Staphylococcus aureus,* F = *Klebsiella pneumonia,* J = *Candida albicans,* S18 = *Enterobacter sp.*

Effect of Combination of the Extract with Commercial Antibiotics

Table 7 shows the effect of the combination of the extract with the commercial antibiotics-Ciprofloxacin (Oxoid). Synergism was observed in *Proteus mirabilis* and *Enterobacter sp.* while other organisms with the exception of *Klebsiella pneumoniae* and *Staphylococcus aureus* showed antagonistic relationship as the zone of inhibition diameter was less compared with the combination. Additivity was observed in *Kebsiella pneumoniae.*

Table 7: Effect of the Combination of EA Extract with Ciprofloxacin

Organisms	Diameter zone of inhibition (mm)			
	Test	Ciprofloxacin	% increase	Effect
Proteus mirabilis	25	20	25	Synergism
Escherichia coli	11	20	-	Antagonism
Citrobacter sp.	11	23	-	Antagonism
Staphylococcus aureus	18	30	-	Antagonism
Klebsiella pneumoniae	23	20	15	Additivity
Candida albicans	13	20	-	Antagonism
Enterobacter sp.	14	8	75	Synergism

Key: Test = Combination of Ciprofloxacin and extract;

Phytochemical Analysis of the Extracts

The phytochemical screening of the crude extracts of *P. amarus, S. acuta and P. muellerianus* showed the presence of tannins, resins, phlobatannins, flavonoids, sterols, alkaloids and terpenoids in varying proportion. Table 8 shows the summary of phytochemical screening of the extracts with their varying degree of abundance.

Table 8: Phytochemical Screening of the Extracts

S/N	PHYTOCHEMICALS	PA	SA	PM
1	Tannins	+++	+	++
2	Glycosides	-	-	-
3	Resins	+	+++	+
4	Saponins	-	-	-
5	Phlobatannins	+	++	-
6	Flavonoids	+++	++	+++
7	Sterols	+++	+++	-
8	Phenols	+++	++	-
9	Carbohydrates	+++	-	-
10	Alkaloids	+	+	-
11	Terpenoids	+++	++	+

Abbrviations:

(–): Negative test, (+): Weak Positive test ; (+ +) Positive test ; (+ + +) Test strongly positive

Toxicity of the Extracts on Brine shrimps

The percentage mortality of brine shrimps by *P. amarus* and *P. muellerianus* crude extract at 50 mg/ml was 66.7% which reduced to 33.3% and 50% respectively at 6.25 mg/ml concentration. *S. acuta* on the other hand had a percentage mortality of 50% at 50 mg/ml and 16.67% at 6.25 mg/ml as depicted in table 9. Furthermore, The lethal dose concentration (LD_{50}) results of the extracts revealed that *P. muellerianus* crude extract had an LD_{50} value of 19.05 mg/ml, *P. amarus* had 25.12 mg/ml while *S. acuta* had a value of 130.11 mg/ml.

Table 9: Toxicity of Plant Extracts using Brine Shrimps

	Percentage Mortality (%)		
Concentration (mg/ml)	PA	PM	SA
50	66.7	66.7	50
25	66.7	66.7	50
12.5	66.7	50	33.3
6.25	50	33.3	16.67
Control	0	0	0

Key: PA = *Phyllanthus amarus*; SA = *Sida acuta*; PM = *Phyllanthus muellerianus*

Antioxidant Activity of the Various Extracts

Table 10 shows the antioxidant activity of the extracts. The crude extract of *P. muellerianus* had the highest Total Antioxidant Capacity (TAC) while *S. acuta* had the lowest TAC value of 3.20 mg/g. Conversely, *S. acuta* had the highest Total Flavonoids and Total phenol value of 339.86 mgQUE/g and 27.63 mgGAE/g respectively while *P. amarus* had the lowest with 173 mgQUE/g and 11.14 mgGAE/g. The Ferric Reducing Antioxidant Power (FRAP) of the extracts showed that the ethyl acetate fraction of *P. muellerianus* had the lowest value of 0.22 mgAAE/g while *S. acuta* had the highest FRAP activity of 6.96 mgAAE/g.

Table 10: Antioxidant Analysis of the Extracts

S/N	ANTIOXIDANT ASSAYS	PA	PM	SA	EA
1.	TAC (mg/g)	5.41	9.15	3.20	4.08
2.	Total Flavonoids (mgQUE/g)	173.24	190.36	339.86	229.19
3.	Total Phenol (mgGAE/g)	11.14	23.56	27.63	13.98
4.	FRAP (mgAAE/g)	2.46	6.36	6.96	0.22

DISCUSSION

Urinary tract infection has been known to affect both men and women. However from the result obtained, it is more prevalent among women than men. This supports the report of Salvatore *et al.* (2011) who stated that they are the most common form of bacterial infection in women. Also Hooton (2000) posited that this could be due to the proximity of the urethra of the women to the anus as features of the pelvic anatomy appear to be associated to UTI risk. Patients within the age group 18 – 37 in this study were observed to have the larger percentage of UTI cases which is in consonance with the report of Salvatore *et al.* (2011) who stated that they occur most frequently between the ages of 16 and 35 years because sexual intercourse and spermicidal use are risk factors for recurrent UTI in young women (Hooton, 2012).

Furthermore, the occurrence of the organisms isolated indicated that *Escherichia coli* and *Proteus mirabilis* were the organisms with the highest prevalence which supports the claims of Nicolle (2008) who opined that the main causal agent of cystitis and pyelonephritis is *Escherichia coli*, which causes about 80 to 90% of UTI. In a study conducted by Prabhu (2016), a larger percentage of *Escherichia coli* were observed in the urine samples collected. Similarly, *Proteus mirabilis* which produces the enzyme – urease (which makes the urine less acidic) was also found to be prevalent since its mobile nature helps it to ascend the urinary tract.

In addition, most of the organisms isolated in this study belonged to the Enterobacteriaceae and this could be due to the theory that bacteria are known to be transmitted to the urethra from the gastrointestinal tract (Salvatore *et al.* 2011). Leski *et al.* (2016) also affirms this result as they concluded that Enterobacteriaceae especially *Citrobacter sp* may be becoming an increasingly important emerging urinary tract pathogen.

However, the antimicrobial activity of the crude extracts of *P. amarus* is worthy of note as *Escherichia coli* had the highest zone of inhibition which conforms to the study of Gbadamosi (2015) which showed the potency of the aerial parts of the plant against *Escherichia coli*. Also, Saranraj and Sivasakthivelan (2012) stated that methanol was one of the best solution for extraction of antimicrobial principles from *P. amarus*.

Moreover, *S. acuta* leaf extract also exhibited high antimicrobial property on all the organisms with *Candida albicans* showing the highest susceptibility. This result supports the claims of Akilandeswari *et al.* (2010) who also reported high zones of inhibition against the organisms tested especially against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. The high degree of antibacterial activity and antifungal activity thus seems to confirm the folk therapy of infections and traditional therapeutic claims of this herb.

P. muellerianus methanolic crude extract also exhibited high potency against the organisms used in this study which conforms with the reports of Doughari and Sunday (2008). Furthermore, Assob *et al.* (2011) have shown that the methanol and ethyl acetate stem bark and aqueous leaf extract of *P. muellerianus*, possess antibacterial activity and these support the findings of this study. The demonstration of antibacterial activity against both Gram positive and Gram negative bacteria may be indication of the presence of broad-spectrum antimicrobial compounds (Srinivasan *et al.* 2001).

The antimicrobial activity exhibited by these three plant extracts may likely be due to one or more of phytochemical constituents present in the plants because phytochemical metabolites have been

associated with the antimicrobial activities of several herbs (Akinpelu *et al.,* 2016). The findings from this study revealed the presence of tannins, resins, phlobatannins, flavonoids, sterols, alkaloids and terpenoids in the three plant extracts. This is similar to the reports of Ofokansi *et al.,* (2012), Umoh *et al.,* (2013) and Nwankpa *et al.* (2015) who reported the presence of these phytochemicals in *P. muellerianus, P. amarus* and *S. acuta* respectively. Flavonoids have been found to exhibit antimicrobial activity through various mechanisms such as inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function and energy metabolism (Cushnie and Lamb, 2005). Al – Bayati and Al – Mola (2008) also opined that saponins can increase the permeability of bacterial cell membrane without destroying them and this might facilitate antibiotic influx through bacterial cell wall membrane. Also several reports have stated that phytochemical constituents such as flavonoids (Cushnie and Lamb, 2005), alkaloids (Singh and Kumar, 2012), tannins (Fiori *et al.*, 2013), saponins (Murugan *et al.*, 2013), glycosides (Zearaha *et al.,* 2013) and terpenoids (Irfan *et al.*, 2014) possess antimicrobial activity and exert their effects by affecting the cell membrane integrity of the bacteria.

With further purification of the crude extract of *P. muellerianus,* dichloromethane (DCM) and ethyl acetate fraction had the highest zones of inhibition. This could be because DCM and ethyl acetate are able to leach out more flavonoids from the crude extract and these flavonoids account for the high antimicrobial activity (Cushnie and Lamb, 2005). However, the antibiotic susceptibility test on the isolates in this study showed that the microorganisms were resistant to the penicillins, cephalosporins, chloramphenicols and sulphamethoxazole/ trimetoprim group of antibiotics. This could be due to the modifications on the outer membrane porins (Omps), which are proteins that aid in the passage of hydrophilic solutes across lipid bilayer membranes (Delcour, 2009). Porins provide a path through the outer membrane to small hydrophilic antibiotics and any decrease in the ability or rate of entry of these compounds can lead to resistance (Nikaido, 2003). The lipid and protein compositions of the outer membrane have a strong impact on the sensitivity of Gram negative bacteria to many types of antibiotics. The aminoglycosides used in this study however were shown to possess high antimicrobial activity against all isolates tested.

In addition, with the exception of *Proteus mirabilis,* all organisms were sensitive to the fluoroquinolones, this may probably be due to the fact that fluoroquinolones use both a porin- and a lipid-mediated pathway, (depending on its protonated status) thus, were able to gain access to the cell interior by permeating through both the outer membrane bilayer and the porin pathway (Delcour, 2009).

Antimicrobials are usually regarded as bactericidal if the MBC/ MIC or MFC/MIC ratio is $\leqslant 4$ and bacteriostatic if >4 (Keepers *et al.,* 2014). The ratios obtained for all the test organisms were less than 4 which indicates that the plant extracts used in this study had bactericidal and fungicidal (for *Candida albicans*) effect on all the test isolates.

Furthermore, this study has been able to show that the synergistic interaction between antibiotics and extracts is highly dependent on the species of bacteria against which the combination was tested. This result is in agreement with the reports of Al-Saiym *et al.* (2015) and Sefanovic and Comic (2012) who stated that synergism or antagonism of extracts with antibiotics are organism - specific. Synergism was observed between the extract and antibiotics on *Proteus mirabilis* and *Enterobacter sp.* while all other organisms (with the exception of *Klebsiella pneumonia*) showed antagonistic relationship. Possible explanation to this phenomena may be attributed to phytochemical incompatibility between the combined agents (i.e. ciprofloxacin and *P. muellerianus*) or a competitive inhibition at the site of action. This is in consonance with the findings of Ofokansi *et al.* (2012) who reported synergism in some of the combination ratios but the predominant effects were indifference and antagonism. The ciprofloxacin (disc) might have encountered problems diffusing through the agar since the agar (base agar) contained the plant extract. However, the predominant antagonism observed in the combination on the microorganisms, calls for caution in the use of the standard antibiotics in combination with the plant extract as Adikwu *et al.*

(2010) opined that such interactions could adversely affect therapeutic outcomes.

In addition, the reports of Verma *et al.* (2014) and Karou *et al.* (2007) respectively showed that *P. amarus* and *S. acuta* exhibited a great deal of antioxidant activity as also reported in this study. The high antioxidant activities of *P. muellerianus* obtained in this study is in line with the findings of Boakye *et al.* (2016) who reported high FRAP values similar to this study. Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agents. The reducing power might be due to hydrogen donating ability, and is generally associated with the presence of reductones (Guo *et al.*, 2003). High antioxidant activity of the plant extracts may be due to the high flavonoid content. Khan *et al.* (2012) also reported that many flavonoids and related polyphenols contribute significantly to the antioxidant activity of medicinal plants.

CONCLUSION

The ability of the plants used in this study to exhibit antimicrobial activity supports its previous folklore application. Thus, the potential of the plant as a template for future drugs that could be formulated to combat urinary tract infection has been established. Such drugs would be useful in combating the menace of multidrug resistant microorganisms in human and animal health. However, further research can be carried out in identifying and isolating active ingredient in these extracts so as to reduce the toxic effects of these plant extract as observed in this study.

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