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Research Article

ANTIBACTERIAL ACTIVITIES OF ETHANOL LEAVES EXTRACTS OF *PHYLLANTHUS AMARUS* (EUPHOBIACEAE) AND *COSTUS AFER* (COSTACEAE) AND THEIR ACTIVITY IN COMBINATION AGAINST SOME GRAM-NEGATIVE BACTERIA

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ABSTRACT

Phyllanthus amarus and *Costus afer* are medicinal plants which are used traditionally for treatment of stomach disorders, cough and other bacterial infections. The ethanol leaf extracts of each of the plant and their combination was assayed in vitro for antibacterial activities using agar disc diffusion method and agar strip diffusion method respectively. The clinical isolated strains of bacterial used includes: *Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumonia.* The standard antibiotics susceptibility test was also carried out against the test bacterial organisms using the agar disc diffusion method and the result was compared with those of the ethanol leaf extracts. Statistical analysis was carried out using the Anova method; the values were statistically significant at P≤0.05. The ethanol leaf extract of *Phyllanthus amarus* showed antibacterial activity at 50mg/ml and 150mg/ml against *Pseudomonas aeruginosa* with a mean inhibitory zone diameter of 7mm and 9mm respectively. When used against *Escherichia coli,* it had a mean inhibitory zone diameter of 8mm and 9mm respectively and was not effective against *Klebsiella pneumonia.* The ethanolic leaf extract of *Costus afer* showed activity at 150mg/ml with a mean inhibitory zone diameter of 9mm against *Pseudomonas aeruginosa* and there was no activity at 50mg/ml. It also showed activity against *Escherichia* coli with a mean inhibitory zone diameter of 8mm but was not effective against *Klebsiella pneumonia*. Thus, *Phyllanthus amarus* had a more effective antibacterial activity than *Costus afer.* The antibacterial activity of the two plants in combination when assayed had an antagonistic effect. The plants were more effective when used individually than when used in combination.

Keywords: *Costus afer, Phyllanthus amarus,* Gram negative bacteria.

INTRODUCTION

From the beginning of civilization, human survival has been dependent on plant as a source of food, oxygen and medicines for natural remedies[1,2]. Many of the plants used in ethno-medicine have been found to contain useful and important therapeutic substances, and have found a use in orthodox medical practice. The search for new compounds that are useful in the management of diseases that have a defined current therapeutic options focus majorly on plants as a reliable source of lead substances. Phytomedicines are very important as their sources are effective and safer substitutes for synthetically produced antimicrobial agents. Indigenous plants that are being exploited for potential medicines must undergo phytochemical screening and bioassays as a first step to drug development[3].

Due to loss of efficacy of many existing orthodox drugs especially antimicrobial agents, there has been an increasing interest in plants as an alternative source of bioactive principles. Some plants have shown the ability to overcome resistance in some organisms and this has led researchers to investigate their mechanism of action and screening for bioactive compounds. In carrying out preliminary screening or bioassay of a plant, several factors such as the method of antimicrobial assay, extraction solvent, culture media, solvent concentration, extraction method, test organism, their resistant factors can lead to differences in result.

Antimicrobial combination therapy is a frequently used therapeutic approach. The rationale for the use of antimicrobials in combination includes: Empirical treatment of polymicrobial infection in critical ill or immune-compromised patients, prevention of the emergence of antibiotic resistance, utilization of a potential synergism of antibiotics directed against a particular bacteria strain. Nevertheless, antimicrobial combination therapy also harbors some risk for patients, which can be referred to as a potential antagonism between antibiotics, accumulation of side-effects or stimulation of the resistance pathways in bacteria by one drug leading to the destabilization of the other drug. Prospective studies are urgently needed to evaluate the true benefit of combination therapy for the treatment of different infection and another specific microorganism[4].

The use of *Phyllanthus amarus* and *Costus afer* in the treatment of microbial infection, the presence of active phytochemicals that are responsible for their antimicrobial activities and also the need to develop newer drugs of plant origin that are safer and easily affordable to humans in the treatment of different disease infection justify this work. Thus the aim of this work is to evaluate the antibacterial activity of *Phyllanthus amarus* and *Costus afer,*leaf extracts on some gram-negative bacteria, and also to evaluate their effect in combination on the same gram-negative bacteria.

MATERIALS AND METHODS

Materials, chemicals and reagents

Hand blender (corona Nigeria), Buchner funnel, vacuum pump, rotary evaporator, water bath etc. The reagents used includes; Drangendiff Reagent, Mayer`s reagents, Hager`s reagents, 5% ferric chloride, catalase reagent, oxidase reagent (Kovasc`s), sterile normal saline, crystal violet, Lugol`s iodine, Dafranin, Ethanol (kermel limited), Chloroform, (Sigma-Aldrich),dilute suphuric acid (Sigma-Akdrich), concentrated suphuric acid (Sigma-Aldrich), benzene (Sigma-Aldrich), glacial acetic acid (Sigma-Aldrich), acetone (Sigma-Aldrich) 10% ammonia solution, distilled water and purit disinfectant.

Media used

Culture media	Batch No	Expiration date
Nutrient agar (Titan Biotech Limited India)	71859-1	June 2017
Peptone water (Titan Biotech Limited India)	61523-2	June 2019
Mueller-Hinton agar (Lab M Limited England)	40051-3	August 2017
Mac-Conkey agar (Titan Biotech Limited India)	71752-4	May 2017

Media were prepared according to manufacturer's specifications and sterilized by autoclaving at 121^0C for 15 minutes. All media were on non-expiration date as at the time of this research.

Test organism

Test organisms used were clinical human isolates of *Klebsiella pneumonia, Pseudomonas aeruginosa* and *Escherichia coli*.They were obtained from the medical laboratory of the University of Port Harcourt Teaching Hospital, Rivers state, Nigeria. The organisms were collected in sterile agar slants using a sterile wire loop. The organisms were collected from the agar plates containing pure growth of colonies and the loop was gently streaked in a zigzag pattern on the agar slant this was so as to achieve an actively growing culture in their exponential phase of growth. (18 to 24 hours). The bottles were properly labeled with the date, microbe name and coded. The slants were incubated at 37^0C for 24 hours. This was done for the entire organisms collected.

Plant collection and identification

Fresh leaves of *Costus afer* and *Phyllanthus amarus* were collected in the month of November at Alakahia and Choba, University of Port Harcourt, Port Harcourt, Choba River state in Nigeria. They were identified by Suleiman, M and authenticated and deposited in the Herbarium of the department of Pharmacognosy and Phytotherapy of the University, with voucher numbers UPH E300 and UPH C096 for *Phyllanthus amarus* and *Costus afer* respectively. The plants materials were sorted out, washed and dried under shade for *Phyllanthus amarus* and sundried for *Costus afer.*

Extraction of Plant materials

The leaves of *Costus afer* were collected washed and sum dried. The dried leaves were pulverized with the aid of a hand blender (Corona Nigeria). Then five hundred grams (500g) of the dried powder of *Costus afer* was weighed and macerated in 2000ml of 80% ethanol for 72 hours and filtered using a Buchnar funnel and a vacuum pump. The filtrate obtained was then concentrated using a rotary evaporator after which it was placed in an evaporating dish and concentrated to dryness in a water bath at 40^0C[5]. The leaves of *Phyllanthus amarus* was shade dried and pulverized with the aid of a hand blender (Corona Nigeria). A five hundred gram (500g) of the dried powder of *Phyllanthus amarus* was weighed and macerated in 2000ml of 80% ethanol for 72 hours and filtered using a rotary evaporator after which it was placed in an evaporating dish and concentrated to dryness in a water bath at 40^OC [6]. The dried extract of each plant was weighed and placed into sterile universal bottles and kept in the fridge until the time of use.

Phytochemical screening

The presence or absence of alkaloids, tannins, saponins, flavonoids, anthraquinones terpenes, carbohydrates and cardiac glycosides were screened using methods according to (Trease and Evans, 1989 [7] and sofowora, 1998 [3], 2008[8]).

Identification and characterization of test organisms

The organisms from the agar slant were streaked on several media, (Blood agar, MacConkey agar) incubated at 37^OC for 24 hours and the slants were preserved in a refrigerator for future use. The human clinical isolates were identified and characterized by their microscopic and biochemical characteristics using different standards [9].

Biochemical tests

The Catalase test, Oxidase test, Indole test, Citrate utilization test and Gram staining test were carried out according to the method by (Koneman *et al*, 1997) [9]

Standardization of test organisms

A sterile wire loop was used to inoculate the test organism into a universal bottle containing 10ml nutrient broth. The turbidity of the culture was adjusted to 0.5 McFarland standards ($1x10^7$). This procedure was repeated for all the test organisms used.

Preparation of stock solution of crude Extract

Half gram (0.5g), of each of the crude extracts was dissolved in water and made up to 10ml to obtain 50mg/ml stock solution one and half gram (1.5g) of each of the crude extracts was dissolved in water and made up to 10ml to obtain 150mg/ml stock solution. These concentrations of extract obtained were used to carry out this research work. They were kept in the refrigerator till the time of use [3, 6].

Antibiotic sensitivity testing

In vitro susceptibility of the microbes was determined using Bauer-disc-diffusion technique (Bauer, et al, 1996). Half ml of the microbes was inoculated into 20mls of Muller-Hinton agar, swirled and poured aseptically into sterile Petri dishes and allowed to set. Commercial disc containing ceftazidime (30µg), cefuroxime (30µg), gentamicin (10µg), ciprofloxacin (5µg), ofloxacin (5µg), Amoxicillin/clavunate (30µg), Nitrofurantoin (300µg), Ampicillin (10µg) were aseptically placed on the surface of the sensitivity agar plate and were incubated for 18-24 hours at 37^OC. Zone of inhibition after incubation were measured in millimeters [6].

Susceptibility method

Agar disk diffusion method

This involves using a 0.1ml of standardized inoculums of (0.5M McFarland) standard, and was introduced into 20ml of Mueller-Hinton agar, and poured aseptically into sterile Petri dishes. A sterile paper disc previously soaked in varying concentrations of the different plant extracts (50mg/ml, and 150mg/ml), was aseptically placed onto the plate, this was carried out in triplicates. The plates were later incubated at 37^{O}C for 24 hours after which they were observed for zone of inhibition [6].

Antibiotic in combination

The agar strip method.

Strips of sterilized whatman filter (NO 1) absorbent paper were saturated with solutions containing the test plant crude extracts at concentration of 50mg/ml and 150mg/ml. The antibiotic strips were placed on the surface of an agar plate already inoculated with the test microbes (1 x 10^8 cfu/ml), such that the strips touch each other perpendicularly at one end. The plates were incubated at 37^{O}C for 18-24 hours and this was carried out in duplicates. The zones of inhibition appeared along the length of the paper strips and around the point of contact for an effective agent. The combined effect of the test crude extract was determined from the size of the inhibition zone around the individual agents. The effects are inferred as synergistic, additive or antagonistic[10].

RESULT

The result of the phytochemical screening of the two plants showed the presence of alkaloids, tannins, combined anthraquinones, flavonoids, carbohydrates, cardiac glycoside and terpenes in *Phyllanthus amarus*, while alkaloids, flavonoids, carbohydrates, cardiac glycosides and terpenes are found in *Costus afer.*

Table 1: Antimicrobial activity of the ethanol leaf extracts of *Phyllanthus amarus* and *Costus afer*.

Test organisms.	Concentrations (mg/ml).	*Phyllanthus amarus* inhibitory zone diameter in (mm)	*Costusafer* inhibitory zone diameter in (mm)
Pseudomonas aeruginosa	50	7.00	Nil
	150	9.00	9.00
Escherichia coli	50	8.00	Nil
	150	9.00	8.00
Klebsiella pneumonia	50	Nil	Nil
	150	Nil	Nil

Nil = No activity.

From table 1, the antimicrobial activity of the ethanol leaf extract of *Phyllanthus amarus* showed that at 50 mg/ml and 150 mg/ml it was active against *Pseudomonas aeruginosa* with a mean inhibitory zone diameter of 7 mm and 9mm respectively, while *Costus afer* was active at 150mg/ml with a mean inhibitory zone diameter of 9mm. When tested against *Escherichia coli, Phyllanthus amarus* was effective at 50mg/ml and 150mg/ml with a mean inhibitory zone diameter of 8mm and 9mm respectively, while *Costus afer* had a mean inhibitory zone diameter of 9mm. *Klebsiella pneumonia* was resistant to the ethanol extracts of the plants at both concentrations used.

Table 2: Antimicrobial activity of the ethanolic leaf extracts of *Phyllanthus amarus* and *Costus afer* in combination

Test organisms	Concentration (mg/ml)	Ethanolic leaf extracts of plants	Inhibitory zone diameter (mm)
Escherichia coli	50	*Phyllanthus amarus*	Nil
		Costus afer	Nil
		Combination	Nil
Pseudomonas Aeruginosa	50	*Phyllanthus amarus*	8.5
		Costus afer	4
		Combination	7
Klebsiella pneumonia	50	*Phyllanthus amarus*	Nil
		Costus afer	Nil
		Combination	Nil
Escherichia coli	150	*Phyllanthus amarus*	13.5
		Costus afer	9.5
		Combination	11.5
Pseudomonas Aeruginosa	150	*Phyllanthus amarus*	12
		Costus afer	11
		Combination	10
Klebsiella pneumonia	150	*Phyllanthus amarus*	Nil
		Costus afer	Nil
		Combination	Nil

Nil = No activity.

From table 2, the antimicrobial activity in combination of the ethanolic leaf extracts of *Phyllanthus amarus* and *Costus afer* was carried out using the agar strip diffusion and the result was an antagonistic effect. The test was carried out on *Pseudomonas aeruginosa* and *Escherichia Coli* at two concentrations 50 mg/ml and 150 mg/ml. when use against *Pseudomonas aeruginosa* at 150mg/ml, *Phyllanthus amarus* gave an inhibition zone diameter of 12mm while *Costus afer* gave and inhibition zone diameter of 11mm and their combined effect was 10mm which indicates that it is antagonistic effect and antagonistic activity indicate that the effects of the two plants in combination is less than the effect of the most effective component of the combination.

Table 3: Antimicrobial activity of standard antibiotics on the bacterial test organisms.

Test organisms	Standard antibiotics	Inhibitory zone diameter (IZD)mm
Escherichia coli	C eftazidime	8
	Cefuroxime	9
	Gentamicin	10
	Ciprofloxacin	Nil
	Ofloxacin	Nil
	Amoxicillin/Clavulanate	Nil
	Nitrofurantoin	18
	Ampicillin	Nil
Pseudomonas aeruginosa	Ceftazidime	15
	Cefuroxime	Nil
	Gentamicin	9
	Ciprofloxacin	Nil
	Ofloxacin	Nil
	Amoxicillin/Clavulanate	Nil
	Nitrofurantoin	19
	Ampicillin	Nil
Klebsiella pneumonia	Ceftazidime	19
	Cefuroxime	15
	Ampicillin	Nil
	Gentamicin	15
	Ciprofloxacin	26
	Ofloxacin	22
	Amoxicillin/Clavulanate	10
	Nitrofurantoin	19

Nil = No activity

From table 3, the standard antibiotics ceftazidime, cefuroxime and gentamicin showed activity with inhibitory zone diameter of 8mm, 9mm, and 10mm respectively when used against *Escherichia coli*. When used against *Pseudomonas aeruginosa,* ceftazidime, gentamicin and nitrofurantoin showed activity with inhibitory zone diameter of 15mm, 9mm, and 19mm respectively. The standard antibiotics ceftazidime, cefuroxime, gentamicin, ciprofloxacin, ofloxacin, amoxicillin/Clavulanate and nitrofurantoin were effective against *Klebsiella pneumonia* with an inhibitory zone diameter of 19mm, 15mm, 26mm, 22mm, 10mm, and 19mm respectively.

DISCUSSION

From the result of this work, the phytochemical constituents present in *Phyllanthus amarus* include alkaloids, flavonoids, terpenes, combined anthraquinones, tannins, carbohydrates and cardiac glycosides. This conforms to the report by (Akinjogunle *et al,* 2010)[6], Adebayo-Tayo and Adegoke, (2008)[11]. *Custus afer* contained alkaloids, tannins, carbohydrate, terpenes, cardiac glycoside, flavonoids present in trace quantities, and absence of anthraquinones. This was in line with the report of the work by Akpan, *et al* (2012)[3], except that in this work *Costus afer* does not contain anthraquinones and had trace quantities of flavonoids. This variation could be as a result of the seasonal variation of the plant and the stage of maturity proposed by (Buenz *et al* 2007) [12]. These components are called secondary metabolites and could be responsible for their medicinal use traditionally[13, 14]. The antimicrobial effect of these plant extracts could be due to the presence of some of these secondary metabolites [8, 15, 16]. Both alkaloids and flavonoids have antimicrobial activities. Flavonoid possesses some antibacterial and antifungal properties[17].

The antimicrobial activity of ethanol extract of *Phyllanthus amarus* and *Costus afer* against the test bacterial organisms was carried out and the activities varied with the test organisms. The antimicrobial screening was carried out using the disc diffusion method at concentrations of 50mg/ml and 150mg/ml. it was observed that the ethanol extracts of *Phyllanthus amarus* showed activity at 50mg/ml and 150mg/ml with a mean inhibitory zone diameter of 7mm and 9mm respectively when used against *Pseudomonas aeruginosa,* while *Costus afer* was effective at 150mg/ml with an inhibitory zone diameter of 9mm when used against the earlier stated bacterial organisms. Both plant extracts at both concentrations did not show any effect against *Klebsiella pneumonia.* This was represented in table 1

Comparing the antimicrobial activity of the standard antibiotics ceftazidime, cefuroxime and gentamicin on *Escherichia coli* with an inhibitory zone diameter of 8mm, 9mm, and 10mm respectively, with that of ethanol extract of *Phyllanthus amarus* on *Escherichia coli,* with an inhibition zone diameter of 8 mm, and 9mm at 50mg/ml and 150mg/ml respectively, it can be said that *Phyllanthus amarus* is effective against the test bacterial organism stated earlier. *Costus afer* was also as effective as the test standard antibiotics stated earlier, with an inhibitory zone diameter of 9mm. Also comparing the antimicrobial activity of the standard antibiotic gentamicin with inhibition zone diameter of 9mm when used against *Pseudomonas aeruginosa* with those of ethanol leaf extracts of *Phyllanthus amarus* and *Costus afer* at 50mg/ml and 150mg/ml gave an inhibitory zone diameter 7mm and 9mm respectively which were almost as effective as those of gentamicin, particularly that of *Costus afer.*

Several investigations have been reported on the antimicrobial effect of the two plants. According to Akinjogunla *et al*, (2010) [6], that investigated the antibacterial effects of *Phyllanthus amarus* against extended spectrum B-lactamase producing *Escherichia coli*, there was an activity of *Phyllanthus amarus* against B-lactamase producing *Escherichia coli,* and this conforms with this research work that indicated that *Phyllanthus amarus* had an antibacterial activity against *E.coli* at 50mg/ml and 150mg/ml. Babatunde, *et al*, (2014)[18], also reported that *Phyllanthus amarus* had antimicrobial activity against human intestinal facultative anaerobic flora (*Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa*) and was effective at 50mg/ml against *Klebsiella pneumonia*. This is at variance to this study as *Phyllanthus amarus* was not active against *Klebsiella pneumonia,*

at the two different concentrations used (50mg/ml and 150mg/ml), although it was active against the other named bacterial organisms. Ogunjobi *et al,* (2013)[19]also reported that the ethanol extract of *Phyllanthus amarus* had a good antimicrobial property when used against some gram-negative and gram-positive organisms. It was reported to be effective against *Escherichia coli* with an inhibitory zone diameter of 9mm using the agar well diffusion method. This report is also in line with this study that indicates that *Phyllanthus amarus* was active against *Escherichia coli*.

Akpan, *et al* (2012)[3] investigated the antimicrobial activity of the ethanol extract of *Costus afer* at 50mg/ml, 100mg/ml and 150mg/ml and it exhibited antibacterial activity against Gram-positive and Gram-negative organisms. This was carried out using the agar disc diffusion method. It was reported that there was no action against *Klebsiella pneumonia* and in this research work using a concentration of 50mg/ml and 150mg/ml, it was not effective against *Klebsiella pneumonia*. It had activity against *Escherichia coli* and *Pseudomonas aeruginosa,* this result is in line with Akpan, *et al* (2012) [3]

AL-Kattan, et al., (2013) [20] reported that the aqueous extract of Indian *Costus afer* was highly effective against *Klebsiella Pneumonia, Escherichia coli* and *Pseudomonas aeruginosa.* This was not the same outcome in this study report. When *Costus afer* was used against *K. pneumonia,* there was no activity; this could be because of seasonal and geographical variation of the plant.[12] The activity of the two plants in combination was carried out using the agar-strip diffusion method. It was observed in this study that the effect of the two plants in combination was an antagonistic one. Antagonistic activity indicates that the effect of the two plants in combination is less than the effect of the most effective component of the combination. AB is less than A. A is the most active individual plant. There was no activity on *Klebsiella pneumonia* when the extracts were used separately and when used in combination. The combined activity of the two plants when used against *Escherichia coli* and *Pseudomonas aeruginosa* was antagonistic. When used against *Escherichia coli* at 150mg/ml *Costus afer* had an inhibitory zone diameter of 9.5mm and *Phyllanthus amarus* had an inhibitory zone diameter of 13.5mm. Their combined effect was 11.5mm which is less than the activity of the most effective plant extract *Phyllanthus amarus* with an inhibitory zone diameter of 13mm. An antagonistic effect was observed when the combination was used against *Pseudomonas aeruginosa;* the combined effect at 150mg/ml was 10mm which is less than the most effective component of the combination *Phyllanthus amarus,* with an inhibitory zone diameter of 12mm.

CONCLUSION

It has been established in this study that *Phyllanthus amarus* and *Costus afer* both have antibacterial properties; they are active against *Pseudomonas aeruginosa* and *Escherichia coli* but not active against *Klebsiella pneumonia. Phyllanthus amarus* had a better antibacterial activity on the test gram-negative organisms because it was effective at a lower concentration of 50mg/ml when compared with *Costus afer* that was effective at higher concentration of 150mg/ml. The two plants should not be used in combination but individually to avoid antagonistic effect.

Thus, *Phyllanthus amarus* and *Costus afer* that are traditionally used in the treatment of certain infections has been justified as they established antimicrobial activity against some clinical gram-negative isolates and they portray potentials as a new drug entity

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