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Antimicrobial Activity of the Essential Oil and the Fractional Samples Obtained from the Leaves and Seeds of *Phyllanthus amarus* (Euphorbiaceae)

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Abstract: The aim of this study is to investigate the alleged antimicrobial activity of *P. amarus*. Fresh leaves and seeds of the plant were air-dried, pulverized and the essential oil extracted into hexane by hydrodistillation over a period of 4 h. In addition, hourly fractions were collected and sensitivity tests were carried out on twelve microorganisms including yeast, Gram-positive and Gram-negative bacteria. All the samples of essential oil and fractions demonstrated activity against the microorganisms except *Pseudomonas aeruginosa*. The activity of the essential oil collected over 4 h exceeded that of the control 0.05% ciprofloxacin, for *Staphylococcus aureus* (isolate) and *Bacillus subtilis*. The results indicate the use of the plant as an antimicrobial. Thus, there is scientific basis for the use of the plant in the treatment of bacterial and fungal diseases.

Key words: *Phyllanthus amarus*, essential oil, medicinal uses, antimicrobial activity, antiviral activity, male infertility factor

INTRODUCTION

Phyllanthus amarus Schum and Thonn (Euphorbiaceae) is an annual herb commonly found in tropical rain forest. The whole plant is widely used for medicinal purposes including the treatment of genito-urinary diseases, gonorrhoea, hepatitis, liver injury, asthma, diabetes, jaundice, typhoid fever, dysentery, joint pains, stomach-ache, weight loss, ringworm and hypertension. In ophthalmic conditions the juice of the stem is mixed with oil and applied to the eye (Odugbemi, 2008; Idika and Niemogha, 2008; Addo-Fordjour *et al.*, 2008).

Several phytochemicals have been found present in the plant. These include phyllanthin, hypophyllanthin (Mahidol *et al.*, 1994) quercetin and tannins (Rajeshkumar *et al.*, 2002). Two securiniga-type alkaloids have been isolated from *P. amarus* (Rajakannan *et al.*, 2003; Houngton *et al.*, 1996). Two new lignans, 3-(3, 4-dimethoxy-benzyl)-4-(7-methoxy-benzo [1, 3] dioxol-5-yl-methyl)-dihydrofuran-2-one and 4-(3, 4-dimethoxy-phenyl)-1-(7-methoxy-benzo [1, 3] dioxol-5-yl)-2, 3-bis-methoxymethyl-butan-1-ol have been isolated from the leaves of *P. amarus* and their structures established by spectral analysis. In the study, eight known lignans were isolated and characterized (Singh *et al.*, 2009). Phyllanthusiin D, a tannin, has been reported present in the plant (Foo and Wong, 1992).

Phyllanthus amarus has been used widely as an antiviral agent (Thyagajaran *et al.*, 1988; De *et al.*, 1990). The plant extract has been shown to inhibit hepatitis viruses (Venkatswaran *et al.*, 1987; Blumberg *et al.*, 1990). In a study on the inhibitory activity of

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P. amarus on Human Immunodeficiency Virus (HIV), it was found that the plant extract inhibited the replication of a variety of RT inhibitor-resistant HIV-1 strains (Notka *et al.*, 2003). It has also been shown to inhibit chemically-induced liver tumour in rats and increase the life span of animals with hepatocellular carcinoma (Joy and Kuttan, 1988; Rajeshkumar and Kuttan, 2000).

In an extensive study of the antitumour and anticarcinogenic activity of *P. amarus*, the plant extract was found to increase the life span of ascites tumour-harboring mice. It also reduced the tumour volume in some specific cell lines. Administration of the extract also increased the life span of sarcoma-bearing mice. The results of the study suggest that the extract may be more effective in liver tumours.

The aqueous extract of *P. amarus* was found to exhibit a protective effect on gentamicin- and acetaminophen-induced nephrotoxic rats (Adeneye and Benebo, 2008). The aqueous extract of the dried leaves and seeds was found to lower plasma glucose in normal mice in a dose-related pattern. It was also found to lower plasma cholesterol (Adeneye *et al.*, 2006). Fractionation of the hexane extract of *P. amarus* led to the isolation of dotriacontanyl docosanoate, triacontanol and a mixture of oleanolic acid and ursolic acid (Ali *et al.*, 2006).

The aqueous extract of *P. amarus* has been found to exhibit anti-diarrhoeal and gastrointestinal potentials (Odetola and Akojenu, 2000). Diarrhoea may be caused by bacteria, viruses, protozoa, toxins arising from food poisoning and drugs (Jouret-Mourin and Geboes, 2002). Thus, the reported anti-diarrhoeal potential may be due to antimicrobial activity of the plant extract. Some studies have also shown that *Phyllanthus* species have antimalarial activity (Tona *et al.*, 1999). Crude aqueous and ethanolic extracts of the plant were found to inhibit *Salmonella typhi*, a microorganism that causes typhoid fever (Oluwafemi and Debiri, 2008).

In South-West Nigeria, a cold decoction of the leaves and seeds of *P. amarus* is one of the medications employed as immune booster in the management of immune suppressive diseases (Idika and Niemogha, 2008). In South-Western Nigeria, the plant is used to promote both male and female fertility. It has been established that sexually-transmitted diseases may be the causative organisms for male infertility factor (Greendale *et al.*, 1993). The antimicrobial activity of the plant extract may contribute to its curative effect. Although several properties of the plant have been extensively studied, report of antimicrobial property is sparse in literature. In this study, the antimicrobial property of the essential oil from the powdered dried leaves and seeds were investigated.

MATERIALS AND METHODS

Collection of Plant Materials and Hydrodistillation of Samples

Several batches of the fresh plant were purchased from Mushin market, Lagos, in May 2007. They were identified by Mr. T.K. Odewo of the Federal Research Institute (FRIN) Ibadan where a voucher labelled FHI 107683 was deposited in the Herbarium in Botany Department. The leaves including the seeds were picked off the stalk and air-dried in a dust-free environment for about two weeks after which they were pulverized in a blender equipped with stainless steel cutters. The percentage moisture was about 60%. In sample extractions of the essential oil about 100 g of the powdered material was mixed with 2 L of distilled water and hydrodistillation was carried out at 100°C for 4 h and the essential oil was collected in hexane. In another series of extractions, hourly fractions of the essential oil were collected over a period of 4 h. Several corresponding batches of the essential oil were pooled. The various samples of essential oil were screened for antimicrobial activity.

Antimicrobial Screening

The microorganisms used for the antimicrobial screening were: *Bacillus subtilis*, *Citrobacter* sp., *Escherichia coli* (isolate), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus albus*, *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (isolate) and *Candida albicans*. These were obtained from the collection in the Department of Medical Microbiology and Parasitology in the College of Medicine, University of Lagos. The antimicrobial assay was carried out according to the method of Sharhidi-Bonjar (2004).

The test organisms were subcultured into fresh plates of nutrient agar (Oxoid, UK) for 23 h at 37°C for bacteria and fungus. Colonies from these plates were suspended in Mueller-Hinton broth (Oxoid, UK) to a turbidity matching 0.5 McFarland standard to give suspensions containing approximately 1×10^8 cfu cm^{-3} for bacteria and fungus. The media used was Mueller-Hinton agar and each labeled medium plate was uniformly seeded with a test organism by means of sterile swab rolled in the suspension and streaked on the plate surface. Wells of 5 mm in diameter and depth of about 2 mm placed about 2 cm apart were punched in the culture media with sterile cork borer. The samples of the essential oil were diluted with hexane to give concentrations of 3 mg cm^{-3} . Fifty microliter of each sample of essential oil was dropped into each well to fullness. The controls, 0.05% ciprofloxacin antibiotic suspension and the neat solvent, hexane, were placed in wells on each plate along with the test extracts. Each plate was thereafter kept in the refrigerator at 4°C for 1 h before incubating at 37°C for 24 h. Zones of inhibition around the wells were measured in millimeter and used as positive bioactivity.

RESULTS

All the samples of the essential oil except the first hourly fraction had pungent smell. The essential oil collected over 4 h had a light brown colour. The 1st h fraction was almost colourless and odourless. The 2nd and 3rd h fractions were golden in colour and the 4th h fraction was yellowish. Hexane did not inhibit the growth of any of the microorganisms. All the samples of essential oil showed inhibitory activity against the microorganisms tested except *P. aeruginosa*. The results of the antimicrobial screening are presented in Table 1.

The fraction collected in the 1st h was uniformly and appreciably active against all the microorganisms tested except *P. aeruginosa* and *K. pneumoniae* against which there was no activity. It exhibited strong activity against *C. albicans* for which the ciprofloxacin control

Table 1: Antimicrobial activity of the essential oil and fractional hourly samples obtained from the dried leaves and seeds of *Phyllanthus amarus*

Extracts	<i>B.s</i>		<i>E.c</i>		<i>E.f</i>	<i>K.p</i>	<i>P.a</i>	<i>P.m</i>	<i>S.al</i>	<i>S.au</i>		<i>C.a</i>
	<i>B.s</i>	<i>C.s</i>	(Isolate)	(ATCC)						(ATCC)	(Isolate)	
1st h fraction	2+	2+	2+	2+	2+	-	-	2+	2+	2+	2+	3+
2nd h fraction	2+	2+	1+	1+	2+	2+	-	1+	2+	2+	2+	2+
3rd h fraction	2+	1+	2+	2+	1+	2+	-	2+	2+	2+	2+	2+
4th h fraction	2+	1+	2+	2+	2+	1+	-	1+	2+	2+	2+	2+
4 h collection	3+	2+	2+	2+	2+	2+	-	2+	2+	2+	3+	3+
0.05% ciprofloxacin (Control)	2+	2+	3+	3+	2+	3+	3+	3+	2+	3+	2+	-
Hexane control	-	-	-	-	-	-	-	-	-	-	-	-

-: No inhibition; 1+: 5-10 mm diameter zone of inhibition; 2+: 11-20 mm diameter zone of inhibition; 3+: ≥ 21 mm. Microorganisms: *B.s*: *Bacillus subtilis*, *C.s*: *Citrobacter* (sp.), *E.c*: *Escherichia coli* (isolate); *E. c* (ATCC): *Escherichia coli* (ATCC 25922), *E.f*: *Enterococcus faecalis*; *K.p*: *Klebsiella pneumoniae*; *P.a*: *Pseudomonas aeruginosa*; *P.m*: *Proteus mirabilis*; *S.al*: *Staphylococcus albus*; *S.au* (ATCC): *Staphylococcus aureus* (ATCC 25923); *S.au* (Isolate): *Staphylococcus aureus* (isolate); *C.a*: *Candida albicans*

was not active. The 2nd h fraction was active against all the microorganisms tested except *P. aeruginosa*. It was appreciably active against all the other microorganisms tested except *E. coli* (isolate), *E. coli* (ATCC 25922) and *P. mirabilis* for which it was weakly active. The 3rd h fraction exhibited appreciable activity against almost all the microorganisms tested, weak activity against *Citrobacter* sp. and *E. faecalis* but no activity against *P. aeruginosa*. The 4th h fraction exhibited appreciable activity against several of the microorganisms tested, weak activity against *Citrobacter* sp., *K. pneumoniae* and *P. mirabilis* but no activity against *P. aeruginosa*.

The essential oil collected over the entire 4 h period exhibited strong activity against *B. subtilis*, *S. aureus* (isolate) and *C. albicans*, appreciable activity against all the other microorganisms except *P. aeruginosa* against which there was no activity. The control, 0.05% ciprofloxacin exhibited strong activity against six microorganisms, namely *E. coli* (isolate), *E. coli* (ATCC 25922), *K. pneumoniae*, *P. aeruginosa*, *P. mirabilis* and *S. aureus* (ATCC 25923), appreciable activity against five microorganisms, namely, *B. subtilis*, *Citrobacter* sp., *E. faecalis*, *S. albus* and *S. aureus* (isolate), but no activity against *C. albicans*.

DISCUSSION

Inspection of the results shows that among the samples of the essential oil, the single collection over the entire 4 h period exhibited maximum activity, with significantly higher activity against *B. subtilis*, *S. aureus* (isolate) and *C. albicans*. The 1st h collection also showed equal activity against *C. albicans* while the other hourly fractions showed less activity. This observation is an indication of synergy thus suggesting that the single collection of 4 h which is a mixture of the hour collections is the most effective. The 1st h fraction was active against ten microorganisms, while all the other samples of the essential oil were active against eleven. The essential oil samples were less active than the ciprofloxacin control for both strains of *E. coli*, *K. pneumoniae*, *P. mirabilis* and *S. aureus* ATCC 25923 and exhibited essentially comparable activity against *E. faecalis* (except the 2nd h fraction), *S. albus* and *S. aureus* (isolate) except the 4 h collection which exhibited higher activity. One significant observation is that the single 4 h collection exhibited higher activity against *B. subtilis* and *S. aureus* (isolate) than the ciprofloxacin control, comparative activity against *Citrobacter* sp., *E. faecalis* and *S. albus* and lower activity against both strains of *E. coli*, *K. pneumoniae*, *P. mirabilis* and *S. aureus* ATCC 25923. The significant activity of the essential oil and fractions against *S. aureus* and *S. aureus* (isolate) is a useful finding. This is because reports of resistant strains of *S. aureus* have been on the increase (Onanuga *et al.*, 2005; Donaldson and Gosbell, 2006) and thus *P. amarus* may be a source of antimicrobial against these resistant strains. Another significant observation is that all the samples of essential oil exhibited strong activity against *C. albicans* for which the ciprofloxacin control did not exhibit any activity. Thus the essential oil from the plant is a useful source of antimicrobials of significant antibacterial and antifungal activity.

Antifungal effect exhibited on *C. albicans* and some other fungi by essential oils from several plants of Lamiaceae, Asteraceae, Verbenaceae, Rutaceae, Lauraceae and Cupressaceae families have been reported in a review by Abad *et al.* (2007).

The 1st h fraction of the essential oil exhibited strong activity against *C. albicans*, thus if the essential oil is required only for that purpose, the hydrodistillation can be terminated after the 1st h. The activity against *C. albicans* may support the use of the plant

extract in treating ringworm. It is significant to note that all the samples of the essential oil are active against *E. coli*. Some strains of *E. coli* have been implicated in diarrhoea (Jouret-Mourin and Geboes, 2002). Thus these results may support the traditional use of the plant extract for treating diarrhoea (Odetola and Akojenu, 2000). The broad-spectrum antimicrobial activity of the essential oil samples can thus explain the medicinal uses of *P. amarus* in the management of genitor-urinary diseases, venereal diseases, dysentery, ringworm and ophthalmic conditions (Odugbemi, 2008; Idika and Niemogha, 2008; Addo-Fordjour *et al.*, 2008).

The chloroform extract of the aerial part of *P. amarus* has been reported to show a significant inhibitory effect against dermatophytic fungus, *Microsporum gypseum* (Agrawal *et al.*, 2004). This result is in agreement with our finding that the essential oil from *P. amarus* exhibits antifungal activity.

The methanolic extract of *P. amarus* was found to exhibit significant antibacterial activity against some drug-resistant pathogenic bacterial strains particularly gram-negative microbes. The antimicrobial activity was attributed largely to phyllantin (Mazumder *et al.*, 2006). The ethanolic extract of *P. amarus* has also been reported to demonstrate promising antibacterial properties, inhibiting all nine bacterial strains tested with minimum inhibitory concentrations ranging from 0.25 to 16 mg cm⁻³ (Kloucek *et al.*, 2005). In our report, the concentration of the various samples of essential oil and fractions was 3 mg cm⁻³ and significant inhibitory zones were observed. In a study of the antibacterial activity of the aqueous and methanolic extracts of the leaves of *P. amarus* against *E. coli*, *Streptococcus* sp., *Klebsiella* sp., *Pseudomonas* sp. and *Staphylococcus* sp., the aqueous extract was found to be inactive against *Klebsiella* sp., fairly active against *Streptococcus* sp., *E. coli* and *Pseudomonas* sp. and significantly active against *S. aureus*. The ethanolic extract was inactive against *Klebsiella* sp., fairly active against *Streptococcus* sp. and *S. aureus* and significantly active against *E. coli* and *Pseudomonas* sp. (Okoli *et al.*, 2009). In our study, the samples of essential oil and hourly fractions were active against both strains of *E. coli* and *S. aureus* which is in agreement with the results of Okoli *et al.* (2009). However, while the essential oil samples were active against *K. pneumoniae* (except the 1st h fraction) the ethanolic fraction was reportedly inactive against the *Klebsiella* sp., used by Okoli *et al.* (2009). While the ethanolic extract of Okoli *et al.* (2009) was active against *Pseudomonas* sp., all the essential oil samples used in our study were inactive against *P. aeruginosa*. It is to be appreciated that the strains of *Klebsiella* sp. and *Pseudomonas* sp., used by Okoli *et al.* (2009) are not stated hence the results may not be comparable with those of *K. pneumoniae* and *P. aeruginosa*. For example, in our study on an assessment of the antimicrobial properties of extracts of various polarities from *Chasmanthera dependens*, *Emilia coccinea* and *Cuscuta australis*, the ethyl acetate fraction of *C. dependens* was active against *Staphylococcus albus*, *Staphylococcus aureus* but inactive against *Staphylococcus aureus* ATCC 25923 (Okie *et al.*, 2009). It is also pertinent to note that the 1st h fraction of the essential oil was inactive against *K. pneumoniae* while the others were active. Another explanation of the inactivity of the essential oil samples against *P. aeruginosa* is that the chemical substance inhibiting *P. aeruginosa* may not be present in the essential oil while it might have been extracted with ethanol as reported by Okoli *et al.* (2009). The results of the inactivity of the ciprofloxacin control against *Klebsiella* sp. and *Pseudomonas* sp., reported by Okoli *et al.* (2009) is at variance with our findings. The difference may be found in the concentrations used. Okoli *et al.* (2009) stated 5 µg disc potency which is less than the 0.05% used in our study.

CONCLUSION

This is one of the few extensive scientific reports of the potentialities of *P. amarus* as an antimicrobial in which several microorganisms were tested and it is the first report of the antimicrobial property of the essential oil. The essential oil is as potent as 0.05% ciprofloxacin against some microorganisms. The activity of the essential oil against bacterial species and fungal yeast, *C. albicans*, shows that the essential oil samples have antibacterial and antifungal activity. The inactivity of all the essential oil samples against *P. aeruginosa* is also reported.

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