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Phytochemical Study and Antifungal Activity of Stem Extracts of *Phyllanthus niruri (L)*

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Over the years, botanical remedies have served as an important source of treatment of diseases to many people worldwide, especially in developing countries. Nigeria has a vast range of medicinal plants with possible therapeutic activities that remain unexplored. Therefore, this study determined the phytochemical constituents and evaluated the antifungal activity of the extract of the stem of *Phyllanthus niruri*, which is a common plant used in Sokoto State, Nigeria to treat different ailments. The phytochemical test revealed the presence of saponins, alkaloids, cardiac glycocides, tannins and flavonoids. The antifungal activity test showed that the n-hexane and ethyl acetate extracts of the plant stem exhibited activity against tested organisms only at high concentrations (≥20 mg/mL) whereas the methanol extract inhibited the growth of all the tested fungal isolates at all concentrations. The GC-MS analysis of the methanol extract revealed the presence of thirteen compounds. The presence of these compounds in the extract may be responsible for the activity demonstrated by the extract.

Keywords: Antifungal, Antimicrobial, GC-MS, *Phyllanthus niruri*.

1. Introduction

Traditional medicine is the oldest method of curing diseases and infections. Various plants are used in different parts of the world to treat human diseases and infections (Venogopal and Venogopal, 1994). Medical remedies from plants have been reported safer than synthetic medicines because the phytochemicals in plant extracts target the biochemical pathway (Mahaja and Das *et al.,* 2003). It is estimated that about 25% of all modern medicines directly or indirectly are derived from higher plants (Ackerknecht, 1973; Majno, 1975; Duke and Martinez, 1994; WHO, 2005). In 2002, the World Health Organization (WHO) reported that an estimated 80% of people worldwide rely on herbal medicines for some parts of their primary health care (WHO, 2002). WHO has also noted that herbal or traditional medicine is fundamental to meeting the primary health care needs of the population in many developing countries (WHO, 2014). Several factors are responsible for this increasing usage of medicinal plants including lower cost, fewer side effects and better patient tolerance (Abdullahi and Lawal 2010). The therapeutic approval of traditional medicine as an alternative form of health care and the increasing microbial resistance to the existing drugs has led more and more researchers to investigate the antimicrobial and antifungal activities of an increasing number of plants (Prashanth *et al*., 2016).

Medicinal plants are widely distributed throughout the world but most abundantly in tropical countries (Duke and Martinez 1994). *Phyllanthus niruri (L*) is an annual herb which belong to the family Euphorbiaceae; the height varies between 30-60 cm, stem is angular with numerous distichous, flowers are yellow and numerous, leaves are elliptic oblong; monoecious with 1-3 staminate flowers and solitary pistillate (Caius, 1986); Fruit capsule, very small, globose, smooth seeds (Caius, 1986); Agharkar, (1991) and Gupta, (1984). *Phyllanthus niruri* is known as Geron Tsuntsaye in Hausa. It grows as wild shrubs in the bush or waste land. It is traditionally believed to boost immunity of children. Women are also known to use it as stimulants. In many parts of India, it is commonly used for the treatment of snake bite (Gupta, 1984).

Phyllanthus niruri (L) is widely known in Sokoto State, Nigeria for their use in traditional medicine. All parts of the plant are applied for treating one form of ailment or the other. For example, the stem of *P. niruri* has been used to cure malaria, snake bite, and sore throat. Though some studies exist on this plant (Kamal, *et al.,* 2012; Ibrahim *et*

al., 2013 and Shanmugam *et al.,* 2014*)*, the studies are limited in either the part of the plant analyzed, or solvent used for extraction. The current study is aimed at evaluating the antifungal activities of the extract of this plant and to isolate and characterize specific compounds that could be responsible for the antifungal activities.

2. Materials and Methods

2.1 Plant collection and Processing

The plant used (*Phyllantus nururi*) was collected randomly around Bado area of Wamakko Local Government of Sokoto State. The plant was identified and authenticated at the Herbarium of the Botany Unit, Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto and the vouchered specimen (UDUH/ANS/0202) was deposited in the herbarium. The plant sample was processed by separating stem bark and washing with clean tap water. It was cut into small pieces, air-dried in the laboratory and then crushed using mortar and pestle. The crushed sample was sieved using a 0.28 µ mesh sieve and the powder obtained was stored in a polythene bag for further use.

2.2 Extraction of *Phyllanthus niruri*

For the extraction, 500 g of the crude powdered plant material (stem bark of *Phyllanthus niruri*) was extracted using maceration process in an air-tight clean flat bottom container in 1500 mL of the solvent for 24 h and the content was filtered using Whatman filter paper. The filtrate was concentrated to dryness at 39 ^{o}C using a rotatory evaporator. After extraction with n-hexane, the residue was weighed before successively extracting with ethyl acetate and methanol. The percentage yield was then evaluated using the formula:

$$\%\text{Yield} = \frac{\text{Weight of of crude extract}}{\text{Weight of sample}} \times 100 \quad (1)$$

2.3 Phytochemical Screening

The plant extracts were screened for the presence of some classes of natural products using standard procedures as described in Harbone (1973), Sofowora (1982), Soforowa (1993) and El-Olemyl *et al*. (1994).

2.4 Determination of Antifungal Activity

2.4.1 Media Preparation

Sabroud dextroxe agar medium was prepared according to the manufacturer's instructions by dissolving 65 g SDA in 1000 mL of distilled water and 0.5 g streptomycin was added to inhibit the growth of bacteria. The mixture was heated for 5 mins using water bath to ensure complete dissolution. The media was sterilized in an autoclave machine at 121 ^{o}C for 15-20 mins and then allowed to cool to about 45 ^{o}C. The mixture was poured into sterile plates (petri dishes). The plates were allowed to stand for 20 mins to solidity at 37 ^{o}C.

2.4.2 Nutrient Broth

The nutrient broth was prepared according to the manufacturer's instruction. 13 g was dissolved in 1000 mL of distilled water and the mixture was heated for 5 mins. The media was then sterilized at 121 ^{o}C for 15 mins.

2.4.3 Antifungal Activity

Agar incorporation method was used for determination of antifungal activity. The fungi isolates which include *Aspergillus niger, Candida albican, T. rubrum and T. mentagrophytes* were collected from patients attending Usmanu Danfodiyo University Teaching Hospital Sokoto. These fungi species are known to be associated with the infections of the skin and urinary tract diseases. 5 mL of each crude extracts were aseptically mixed with 15 mL of sabroud dextrose agar. The media was poured aseptically in sterile petri dishes and allowed to set. Tabinafin was used as the positive control. 500 g of tabinafin was dissolved in 5 mL of sterile distilled water. The media was inoculated with the test organism impregnated with the control and the crude extract in three replicates. The plates were incubated at 28 ^{o}C for 72 h. Observations and readings for the zones of inhibition were carried out and the results were recorded (Janky *et a*l., 2011).

2.4.4 Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined using the micro tube method as described in Cheesbrough (1991).

2.4.5 Determination of Minimum Fungicidal Concentration (MFC)

The Minimum Fungicidal Concentration (MFC) was determined by culturing the content of the tube that showed no visible growth in the MIC. A loopul of the mixture contained in the tubes was sub cultured on fresh prepared sabouraud dextrose agar plate and incubated at ambient temperature for 7 h (Duke and Martinez 1994). The MFC was recorded as the concentration of extracts that did not permit any visible colony growth on the agar medium after the period of re-incubation (Wokoma *et al.*, 2007).

2.5 Chromatographic studies

2.5.1 Thin Layer Chromatography (TLC)

After the extraction using three different solvents the components of the extracts were identified using TLC as described in Shina (2014). To do this, a small amount of dried ethylacetate extracts was dissolve in 2 mL of ethyl acetate to obtain a mixture. This mixture was then used for the TLC using a standard method as reported by Shina (2014).

2.5.2 Column Chromatography

Column Chromatography was used to further separate the component obtained on TLC as described by Simon *et al.* (2015).

2.5.3 Gas Chromatography Mass Spectroscopy GC-MS analysis

To identify the components presents, the extract was subjected to GC-MS analysis as described in Chidambaramc and Janeena (2006). The analysis was carried out at the National Research Institute for Chemical Technology (NARICT) Zaria, Kaduna State, Nigeria.

2.6 Statistical Analysis

The numerical data obtained from various determinations are averages of triplicate observations. The data were subjected to statistical analysis using SPSS 17.0 statistical software. One-way Analysis of Variance (ANOVA) using LSD and Turkey's test at $\alpha = 0.05$ was used to compare variables with one another and with controls for any significant difference.

3. Results and Discussion

3.1 Phytochemical Screening

The phytochemical analysis of the extracts of *P. niruri* showed the presence of some important phytochemicals as shown in Table 1.

3.2 Antifungal activity

The antifungal activity of the n-hexane, ethyl acetate and methanol extracts of the plant was investigated using agar incorporation method against selected fungi, *A. niger, C. albican, T. mentagrophytes* and *T. rubrum.* The results are presented in Tables 2 – 4. As shown in Table 2, the n-hexane extract showed activity against only *A. niger* and *C. albican* at high concentrations. Table 3 also showed that the ethyl acetate extract exhibited activity against all the tested organisms at high concentrations. In contrast, Table 4 showed that the methanolic extract exhibited activity on all organisms at all concentrations. Generally, the activity of the extracts increased with increasing concentration and were observed to be significantly ($P < 0.05$) lower than the activity of the control (Tabinafin). Tables 5 and 6 show that the extracts have Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) in the range of 1.875 – 15 mg/mL.

3.3 GC-MS Analysis

The methanolic extract, which was the most active at all concentrations was further subjected to column chromatography. The second fraction obtained (MET 2) which showed the highest activity (Table 7) was analysed by GC-MS in a bid to identify specific compounds responsible for the activity. Thirteen compounds were identified as shown in Table 8.

3.4 Discussion

The methanolic extract of *P. niruri* stem showed more phytochemicals and contained saponins, alkaloids, cardiac glycocides, tannins and flavonoids. These compounds have been reported in the plant previously (Ibrahim, 2013 and Shanmugamm, 2014). The presence of these compounds in the extracts may be responsible for their antifungal activity. Phytochemicals like saponins and alkaloids have been linked to

antifungal activity and plant extracts containing these phytochemicals have been proposed for possible use as antifungal agents (Nino *et al.*, 2006 and Rani and Murty, 2006).

The methanolic extract of *P. niruri* stem showed the highest antifungal activity and inhibited the growth of all the tested organisms (*A. niger, C. albican*, *T. mentagrophytes* and *T. rubrum*) at all concentrations studied (15 to 30 mg/mL). This is in conformity with Mahesh and Satish (2008) who reported that the methanolic extract of various medicinal plants showed significant antibacterial and antifungal activity against *Aspergilllus* species. It is also in agreement with the findings of Bashir *et al*. (2009) who noted that methanol is more efficient than other solvent in extracting phytochemical plant materials. The ethyl acetate extract of *P. niruri* stem showed intermediate activity while the n-hexane extract showed low activity against the organism. The low activity of the n-hexane extract of *P. niruri* stem could be due to lesser phytochemical content in the solvent. Generally, the results are consistent with those of Ibrahim (2013) and Shanmugamm (2014).

The Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values of *P. niruri* varied with type of organism tested. The most potent activities were against *C. albican, T. mentaphytes* and *T. rubrum.* Generally, the extract of the plant exhibited good antifungal activity, which is most likely due to a wide range of phytochemicals present in the plant. Rani and Murty, (2006) had previously linked antifungal activity to the presence of phytochemicals in plant extract. Furthermore, the results from these studies confirms the plant' part of *P. niruri* stem as being fungicidal and fungiostatic. Thus, further providing evidence for the medicinal values of the tested plant' part and the local use of the extract of the plant for treating fungi infections.

The methanolic extract of *P. niruri* stem was subjected to GC-MS analysis and the results obtained revealed the presence of a range of compounds which could be classified as terpenoids or fatty acids. The bioactive compounds present in the extract may be responsible for the use of the plant in the treatment of various ailment including bacterial and fungi infections.

Table 1. Qualitative phytochemical analysis of n-hexane, ethyl acetate, and methanol stem extracts of P*hyllanthus niruri*.

	Phytochemicals	Stem extracts		
		n-hexane	Ethyl acetate	Methanol
i..	Carbohydrates	+	+	+
ii.	Saponins	-	-	+
iii.	Tannins	-	+	+
iv.	Flavonoids	-	-	+
v.	Anthraquinones	-	-	-
vi.	Alkaloids	-	-	+
vii.	Steroids	+	+	+
viii.	Cardiac Glycosides	+	+	+

- = absence; + = present

Table 2. Antifungal activity of n-hexane extract of *Phyllanthus niruri* stem bark.[a]

Plant extract	Extract conc. (mg/mL)	Zone of inhibition (mm)			
		A. niger	*C. albican*	*T. mentagrophytes*	*T. rubrum*
Phyllanthus niruri	15	-	-	-	-
	20	-	-	-	-
	25	4.52±0.01*	4.37±0.01*	-	-
	30	6.43±0.02	5.49±0.02	-	-
Tabinafin		38.18±0.41	32.37±0.43	37.71±0.24	34.28±0.39

[a] = values are significantly different from one another across row and column ($P < 0.05$); values asterisked (*) on the same row are significantly not different at ($P < 0.05$); - = no activity; values are reported as mean ± standard deviation (n = 3).

Table 3. Antifungal activity of ethyl acetate extract of *Phyllanthus niruri* stem bark.[a]

Plant extract	Extract conc. (mg/mL)	Zone of inhibition (mm)			
		A. niger	***C. albican***	***T. mentagrophytes***	***T. rubrum***
Phyllanthus niruri	15	-	-	-	-
	20	4.51±0.12	3.82±0.03	-	-
	25	8.17±0.23	5.19±0.01*	5.24±0.02*	-
	30	12.48±0.51	8.32±0.21	5.54±0.03	4.31±0.02
Tabinafin		38.18±0.41	32.37±0.43	37.41±0.24	34.28±0.39

[a] = values are significantly different from one another across row and column (P < 0.05); values asterisked (*) on the same row are significantly not different at (P < 0.05); - = no activity; values are reported as mean ± standard deviation (n = 3).

Table 4. Antifungal activity of methanol extract of *Phyllanthus niruri* stem bark.[a]

Plant extract	Extract conc. (mg/mL)	Zone of inhibition (mm)			
		A. niger	***C. albican***	***T. mentagrophytes***	***T. rubrum***
Phyllanthus niruri	15	6.12±0.03*	6.39±0.14*	6.02±0.01*	5.98±0.13
	20	12.43±0.51	8.73±0.42	7.16±0.05	6.37±0.02
	25	18.62±0.34	10.53±0.17*	10.04±0.01*	9.45±0.13
	30	25.71±0.41	18.42±0.37	16.19±0.16	14.68±0.36
Tabinafin		38.33±0.17	32.16±0.31	37.51±0.71	34.91±0.58

[a] = values are significantly different from one another across row and column (P < 0.05); values asterisked (*) on the same row are significantly not different at (P < 0.05); - = no activity; values are reported as mean ± standard deviation (n = 3).

Table 5. Minimum Inhibitory Concentration (MIC) of n-hexane, ethyl acetate and methanol extracts of *Phyllanthus niruri* stem bark against fungi species.

Plant extract	Organism	Concentration (mg/mL)								
		30	**15**	**7.5**	**3.75**	**1.875**	**0.9375**	**0.4688**	**0.2344**	**MIC**
HPN	*A. niger*	-	-	-	-	+	+	+	+	3.75
	C. albican	-	-	-	-	+	+	+	+	3.75
	T. menta	+	+	+	+	+	+	+	+	
	T. rubrum	+	+	+	+	+	+	+	+	
EPN	*A. niger*	-	-	-	-	-	+	+	+	1.875
	C. albican	-	-	-	-	+	+	+	+	3.75
	T. menta	-	-	-	+	+	+	+	+	7.5
	T. rubrum	-	-	+	+	+	+	+	+	15
MPN	*A. niger*	-	-	-	-	-	+	+	+	1.875
	C. albican	-	-	-	-	-	+	+	+	1.875
	T. menta	-	-	-	+	+	+	+	+	7.5
	T. rubrum	-	-	-	+	+	+	+	+	7.5

HPN = n-hexane extract of *Phyllanthus niruri* stem bark; EPN = ethyl acetate extract of *Phyllanthus niruri* stem bark; MPN = Methanol extract of *Phyllanthus niruri* stem bark; - = no growth of test organism; + = growth of test organism.

Table 6. Minimum Fungicidal Concentration (MFC) of n-hexane, ethyl acetate and methanol extracts of *Phyllanthus niruri* stem bark against fungi species.

Plant extract	Organism	Concentration (mg/mL)								
		30	**15**	**7.5**	**3.75**	**1.875**	**0.9375**	**0.4688**	**0.2344**	**MFC**
HPN	*A. niger*	-	-	-	-	+	+	+	+	3.75
	C. albican	-	-	+	+	+	+	+	+	15
	T. menta	+	+	+	+	+	+	+	+	
	T. rubrum	+	+	+	+	+	+	+	+	
EPN	*A. niger*	-	-	-	-	-	+	+	+	1.875
	C. albican	-	-	-	-	+	+	+	+	3.75
	T. menta	-	-	-	+	+	+	+	+	7.5
	T. rubrum	-	-	+	+	+	+	+	+	15
MPN	*A. niger*	-	-	-	-	-	+	+	+	1.875
	C. albican	-	-	-	-	-	+	+	+	1.875
	T. menta	-	-	+	+	+	+	+	+	15
	T. rubrum	-	-	+	+	+	+	+	+	15

HPN = n-hexane extract of *Phyllanthus niruri* stem bark; EPN = ethyl acetate extract of *Phyllanthus niruri* stem bark; MPN = Methanol extract of *Phyllanthus niruri* stem bark; - = no growth of test organism; + = growth of test organism.

Table 7. Antifungal activity of column chromatographic fractions of Methanol extract of *P. niruri* stem bark[a]

Fractions	Conc. (mg/ml)	Zone of inhibition (mm)			
		A. niger	***C. albican***	***T. rubrum***	***T. mentagraphytes***
MET1	15	6.50±0.02	6.80±0.01	6.50±0.01	6.50±0.02
	20	13.50±0.04	9.00±0.03	7.00±0.02	7.80±0.11
	25	19.00±0.42	11.45±0.31	10.50±0.23	11.00±0.35
	30	27.50±0.57	19.56±0.29	15.56±0.23	15.45±0.26
MET 2	15	8.50±0.04	7.00±0.03	8.50±0.03	7.20±0.02
	20	14.00±0.17	10.50±0.05	10.00±0.10	8.55±0.04
	25	20.45±0.51	14.85±0.31	13.55±0.27	12.00±0.11
	30	28.00±0.38	22.50±0.32	18.50±0.41	17.50±0.33
MET 3	15	5.50±0.01	6.50±0.03	5.99±0.01	6.50±0.01
	20	11.8±0.03	7.60±0.01	7.00±0.02	7.50±0.02
	25	17.45±0.27	10.50±0.05	9.50±0.13	10.50±0.03
	30	23.89±0.48	15.85±0.37	15.50±0.29	11.66±0.23
Tabinafin		38.54±0.23	32.81±0.27	37.31±0.43	34.13±0.51

[a] = values are significantly different from one another across row and column ($P < 0.05$); - = no activity; values are reported as mean ± standard deviation (n = 3); MET 1, MET 2 and MET 3 are Ethyl acetate fractions 1, 2, and 3 obtained from column chromatography.

Table 8. Compounds present in the methanol extract of *Phyllanthus niruri* stem bark.

Compound	Molecular formula	RT	M/Z
Cyclobutane, 1,2-diethyl	C_8H_{16}	19.1	56
Tridecane	$C_{13}H_{28}$	14.6	57
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	19.7	149
Bis-(3,5,5-trimethylhexyl) phthalate	$C_{26}H_{42}O_4$	15.7	57
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	16.8	74
Ocatadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	20.3	74
E-11-Hexadecenoic acid, ethyl ester	$C_{18}H_{34}O_2$	18.2	56
Sulfurous acid, 2-ethylhexyl isohexyl ester	$C_{14}H_{30}O_3S$	24.9	57
E-2-Tetradecen-1-ol	$C_{14}H_{28}O$	22.4	57
Heptadecane, 2,6,10,14-tetramethyl	$C_{21}H_{44}$	23.2	57
Cyclohexanone, 2-(1-methyl-2-nitroethyl)	$C_9H_{15}NO_3$	20.8	55
Di-n-octyl phthalate	$C_{24}H_{38}O_4$	21.5	149
Di-2-ethylhexyl chloroformate	$C_9H_{17}ClO_2$	21.3	57

Key: RT= Retention Time; M/Z = Molecular Ion.

4. Conclusion

The present study has shown that the stem of *P. niruri* contains important phytochemicals such as saponins, alkaloids, cardiac glycocides, tannins and flavonoids. The methanol and ethyl acetate extracts of *P. niruri* stem showed good antifungal activity against common organisms and the activity of the extracts have been linked to the presence of important bioactive phytochemicals such as terpenoids and fatty acids present in the plant.

Conflict of interest

The authors declare no conflict of interest.

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