



***In vitro* antibacterial activity of the aqueous extract of *Phyllanthus muellerianus* leaves against some selected organisms**

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ABSTRACT

The *in vitro* activity of the aqueous extract of *Phyllanthus muellerianus* leaves against some selected organisms was investigated. The aqueous extract of *P. muellerianus* leaves was subjected to phytochemical screening. In addition, its antibacterial activity against clinical isolates, including *Escherichia coli*, *Bacillus cereus*, *Salmonella typhi* and *Staphylococcus aureus* was determined at 1.25, 2.50, 5.00, 10.00, 12.50, 25.00 and 50.00 mg/ml body weight in albino rats. Phytochemical screening revealed amongst others the presence of cardenolides (0.33%), cardiac glycosides (0.51%), flavonoids (0.16%), saponins (1.21%), tannins (0.68%), phenolics (0.55%) and terpenoids (0.08%). Antibacterial study revealed that the aqueous extract of *P. muellerianus* leaves was bacteriostatic for about 15 hours on all the tested organisms except *Proteus mirabilis* which showed growth at all the concentrations used. Consequently, the data in the present study indicates that the aqueous extract of *P. muellerianus* leaves possessed antibacterial properties, being most effective at 50.00 mg/kg body weight.

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1. INTRODUCTION

Medicinal plants form important component of the natural wealth of Nigeria [1] and constitute an effective source of both traditional and modern medicines. About 80% of the rural population depends on herbal medicine as primary health care [2]. Going by this, herbal medicine has been widely used and forms an integral part of primary health care in Nigeria, China, Argentina, Ethiopia and Papua New Guinea [3-7]. Herbal medicine is gaining popularity in developing countries [8]. Herbal treatments involve mainly the use of plant extracts and other plant products [9-10] which contain bioactive substances. *Phyllanthus muellerianus* is one of these herbs that are locally used frequently for the treatment of different ailments in Nigeria.

Phyllanthus muellerianus otherwise known as "Eegun eja" (Yoruba-Western Nigeria) was formally classified as one of the flowering trees

of spurge family (Euphorbiaceae). However, a recent revision of classification has categorised *Phyllanthus* under the family Phyllanthaceae [11]. *P. muellerianus* is widely spread in tropical Africa [12]. Apart from Nigeria, it is also found in other countries like China, Cuba, Philippines, central and southern India [13]. Although common in the tropics, the *Phyllanthus* specie of interest is generally often scattered in distribution [14].

The ethnobotanical uses of the plant include the treatment of diarrhoea, toothache, sexual dysfunction, chest pain, conjunctivitis, fever, paralysis, sore throat, urethritis, gonorrhoea and wound [15], many out of which are caused by bacteria. In view of the fact that many bacteria are now showing resistance against many bacteria, alternatives to the existing antibacterial drugs have to be provided as a matter of urgency.

This research is therefore designed to:

- a) determine the phytochemical constituents of *P. muellerianus* leaves;
- b) evaluate the *in vitro* antibacterial activity of the aqueous extract of *P. muellerianus* leaves against some selected diarrhoea causing organisms.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 The Plant: Collection and Identification

Phyllanthus muellerianus was collected within the premises of Government Day Secondary School, Oke Adinni, Ilorin, Kwara State, Nigeria. It was authenticated at Forestry Research Institute of Nigeria (FRIN), Ibadan, where a voucher specimen (FHI 108364) was deposited.

2.1.2 Bacteria isolates

Bacteria Isolates (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella flexneri*, *Bacillus cereus*, *Proteus mirabilis*, *Streptococcus pyogenes* and *Klebsiella pneumonia*) were obtained from the Department of Pathology, University of Ilorin Teaching Hospital, Ilorin, Kwara State, Nigeria.

2.1.3. Reagents and Chemicals

Nutrient agar, nutrient broth, Muller-Hinton agar and agar-agar were all products of Biotec Laboratories Ltd., United Kingdom. All other reagents used were of analytical grade and were prepared using distilled water.

2.2 Methods

2.2.1 Preparation of aqueous extract of *Phyllanthus muellerianus* leaves

The preparation was carried out following the method described by Adedapo *et al* [13]. Leaves of *P. muellerianus* were harvested (as always freshly harvested by the traditional medicine practitioners) after which 500 g was blended and 2.75 litres of distilled water added, allowed to stand for 48 hours but shaken intermittently with electric stirrer. The suspension was then filtered using Whatman No. 1 filter paper and the filtrate was lyophilized (with 1.5 litre ice capacity model of FS400-05 Freeze Dryer, Micromodulyo; USA). The lyophilized sample was reconstituted in distilled water to prepare different doses (12.50, 25.00 and 50.00 mg/kg body weight) that were administered to different groups of animals. The doses were informed from the bacteriostatic activity displayed by the extract during the antibacterial studies.

2.2.2 Quantitative estimates of Phytochemicals in *Phyllanthus muellerianus* leaves

Cardiac glycosides and Terpenoids: The procedure described by Sofowora [16] was used.

Flavonoids: The procedure described by Allen [17] was used.

Cardenolides: The procedure described by Edeoga *et al* [18] was used.

Phenolics: The procedure described by Harborne [19] was used.

Saponins: The spectrophotometric method described by Brunner [20] was used for the quantitative determination of saponins.

Tannins: The procedure described by Swain [21] was used.

2.2.3 Preparation of Mueller-Hinton agar, nutrient broth and nutrient agar

The method of Fawole and Oso [22] was used to prepare agars and broth.

2.2.4 Standardisation of bacterial inoculation

Standardisation of bacterial inoculum was carried out using the procedure described by Bauer *et al* [23] and Barry and Thornsberry [24].

2.2.4 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The aqueous extract of *P. muellerianus* leaves at the concentrations of 1.25, 2.50, 5.00, 10.00, 12.50, 25.00 and 50.00 mg/ml were prepared into different test tubes containing broth agar and 0.1 ml of standardised inoculum (*Escherichia coli*) was added to the tubes. The procedure was repeated for the remaining bacteria (*Staphylococcus aureus*, *Salmonella typhi*, *Bacillus cereus*, *Shigella flexneri*, *Proteus mirabilis*, *Klebsiella pneumonia* and *Streptococcus pyogenes*). All the tubes were incubated at 37°C for 24 hours. Two control tubes were maintained for the test. These included the tube containing extract and broth without inoculum (negative control or aqueous extract control) and the tube containing the broth, distilled water and the inoculum (positive control or organism control). The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tube was regarded as the minimum inhibitory concentration (MIC).

However, the minimum bactericidal concentration (MBC) was determined by sub-culturing the dilution that gives the MIC (the test dilution) on to the fresh nutrient agar and incubated further for 24 hours. The highest dilution that yielded no single bacteria colony on a solid medium was taken as MBC [25].

2.3 Statistical analysis

Data were expressed as means \pm S.D of five determinations except otherwise stated. The statistical tools used were one-way analysis of variance (ANOVA) and Duncan Multiple Range Test. Differences were considered statistically significant at $P < 0.05$ [26].

3. RESULTS

Of all the phytochemicals identified in the aqueous extract, saponins constituted the highest percentage with 34.38 while terpenoids was found to be the least (2.27%) (Table 1).

The aqueous extract of *P. muellerianus* leaves produced concentration dependent effect on the diameter of zones of inhibition of the tested organisms. For instance, whereas the least dose of the extract (1.25 mg/ml) produced minimal zone of inhibition ranging from 14 – 17 mm on all the tested organisms, the highest dose (50 mg/ml) produced diameter of zone of inhibition within the range of 24 - 36 mm. At the highest dose of the extract, *B. cereus* had the least diameter of zone of inhibition whereas the extract produced the highest diameter of zone of inhibition on *E. coli* (Table 2).

The growth of *Bacillus cereus*, *Klebsiella pneumonia* and *Staphylococcus aureus* was inhibited at all the doses (1.25 – 50 mg/ml) of the extract used in the study. Furthermore, the growth of *E. coli* and *Shigella flexneri* was similar as they were both inhibited within the concentration range of 10 – 50.00 mg/ml whereas *Streptococcus pyrogenes* and *Salmonella typhi* were inhibited at 2.50 and 5.00

mg/ml, respectively. However, the extract did not inhibit the growth of *Proteus mirabilis* at all the doses investigated in the present study (Table 3). At the concentrations tested, the aqueous extract of *P. muellerianus* leaves prevented growth of all the bacteria tested for 15 hours. By 20th hour, *B. cereus* and *Shigella flexneri* had started to grow. This pattern was extended to all other organisms by 24th hour experimental period (Table 4).

Table 1. Phytochemical constituents of aqueous extract of *Phyllanthus muellerianus* leaves.

Phytochemicals	Concentration (%)
Alkaloids	Not detected
Anthraquinones	Not detected
Cardenolides	0.33 ± 0.02
Cardiac	0.51 ± 0.00
Glycosides	
Flavonoids	0.16 ± 0.01
Phenolics	0.55 ± 0.02
Phlobatannins	Not detected
Saponins	1.21 ± 0.03
Steroids	Not detected
Tannins	0.68 ± 0.03
Terpenoids	0.08 ± 0.00

Values are mean of three determinations ± S.D.

Table 2: Diameter of zones of inhibition by aqueous extract of *Phyllanthus muellerianus* leaves on selected bacteria.

Extract (mg/ml)	Diameter* of Zones of inhibition on the test organisms (mm)							
	<i>Bacillus cereus</i> (G +ve)	<i>Salmonella typhi</i> (G -ve)	<i>Escherichia coli</i> (G -ve)	<i>Klebsiella pneumonia</i> (G -ve)	<i>Proteus mirabilis</i> (G -ve)	<i>Shigella flexneri</i> (G -ve)	<i>Staphylococcus aureus</i> (G +ve)	<i>Streptococcus pyogene</i> (G +ve)
1.25	14.00±0.00 ^a	16.67±0.17 ^d	16.00± 0.00 ^c	13.83±0.44 ^a	15.00±0.00 ^b	15.00± 0.00 ^b	16.00± 0.00 ^c	17.00± 0.00 ^d
2.50	14.83±0.17 ^a	18.33±0.33 ^d	18.00± 0.00 ^d	18.00± 0.00 ^d	16.00±0.00 ^b	16.67± 0.17 ^c	17.00± 0.00 ^d	18.00± 0.00 ^d
5.00	16.00±0.58 ^a	20.67±0.33 ^d	20.00±0.58 ^{cd}	19.17±0.17 ^c	18.00±0.00 ^b	18.00± 0.00 ^b	20.00±0.00 ^{cd}	20.00±0.00 ^{cd}
10.00	20.00±0.58 ^a	21.50±0.29 ^b	24.00±0.00 ^d	21.00±0.58 ^{ab}	23.00±0.58 ^c	21.00±0.00 ^{ab}	23.00±0.00 ^c	25.00±0.00 ^{cd}
12.50	22.00±0.58 ^a	23.00±0.33 ^b	26.00±0.00 ^e	22.00±0.00 ^a	25.00±0.00 ^d	24.00±0.00 ^{bc}	24.33±0.33 ^{cd}	26.67±0.33 ^e
25.00	23.33±0.89 ^a	24.00±0.00 ^a	33.67±0.17 ^d	23.17±0.17 ^a	26.83±0.44 ^b	26.00±0.00 ^b	26.00±0.00 ^b	30.00±0.00 ^c
50.00	24.33±0.89 ^a	25.33±0.33 ^a	36.00±0.00 ^e	30.00±0.58 ^b	34.00±0.00 ^d	33.33±0.17 ^{cd}	33.00±0.00 ^{cd}	32.33±0.33 ^c

Values are mean of three determinations ± S.D.

* indicates the extent of inhibition of growth

G +ve indicates Gram positive; G -ve indicates Gram negative

Table 3: Minimum inhibitory concentration of aqueous extract of *Phyllanthus muellerianus* leaves on selected bacteria

Microorganisms	Extract concentration (mg/ml)						
	1.25	2.50	5.00	10.00	12.50	25.00	50.00
<i>Bacillus cereus</i>	- ^a	-	-	-	-	-	-
<i>Salmonella typhi</i>	+ ^b	+	-	-	-	-	-
<i>Escherichia coli</i>	+	+	+	-	-	-	-
<i>Klebsiella pneumonia</i>	-	-	-	-	-	-	-
<i>Proteus mirabilis</i>	+	+	+	+	+	+	+
<i>Shigella flexneri</i>	+	+	+	-	-	-	-
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	-
<i>Streptococcus pyogenes</i>	+	-	-	-	-	-	-

-^a Indicates no growth; +^b Indicates growth

Table 4. Bacteriostatic activity of aqueous extract of *Phyllanthus muellerianus* leaves on selected bacteria.

Bacteria	MIC of the extract	Period of observation (hour)				
		5	10	15	20	24
<i>Bacillus cereus</i>	1.25	- ^a	-	-	+ ^b	+
<i>Salmonella typhi</i>	5.00	-	-	-	-	+
<i>Escherichia coli</i>	10.00	-	-	-	-	+
<i>Klebsiella pneumonia</i>	1.25	-	-	-	-	+
<i>Shigella flexneri</i>	10.00	-	-	-	+	+
<i>Staphylococcus aureus</i>	1.25	-	-	-	-	+
<i>Streptococcus pyrogenes</i>	2.50	-	-	-	-	+

-^a Indicates no growth; +^b Indicates growth

4. DISCUSSION

The bioactives found in the medicinal plants have potentials to prevent or cause adverse effects [27]. For example, tannins have been reported to possess antibacterial properties which act by different mechanisms, including enzyme inhibition, reduction in oxidative phosphorylation and iron deprivation amongst others [28]. Similarly, saponins have also been implicated to exhibit antibacterial activities [29]. The present study showed that aqueous extract of *P. muellerianus* leaves contained phytochemicals, which on application inhibited the growth of both gramme positive and gramme negative bacteria that resulted in the observed diameter of zones of inhibition of the test bacteria. The concentration dependent diameter of zones of inhibition observed on some of these bacteria suggested antibacterial activity of the extract. However, the doses of extract used in this study exhibited bacteriostatic activity for only 15 hours. Therefore, one or more of these phytoconstituents might be responsible singly or in combination for the observed effect of the extract.

Several workers had employed the principles of minimum inhibitory concentration (MIC) values as a measure of effectiveness of antibacterial agents [29-31]. Therefore, the pattern of MIC and the bacteriostatic results on the test organisms indicated antibacterial activity of the extract. The least values obtained on *B. cereus*, *K. pneumonia*, and *S. aureus* as well as on the other organisms suggested that the plant extract is less potent as an antibacterial agent on these organisms. Furthermore, it is worthy of note that the extract is not effective on *P. mirabilis* since it did not inhibit the growth of this organism.

Diarrhoea-causing organisms constitute the major causes of mortality in several countries of the world [32], some of which are gram-negative bacteria. Gram-negative bacteria are frequently reported to have developed multi-drug

resistance to many of the well-known antibiotics currently available in the market of which *Escherichia coli* is the most prominent [33-35]. The *in vitro* antibacterial activity of the extract on the diarrhoea-causing bacteria such as *Escherichia coli*, *Shigella flexneri*, *Salmonella typhi* and *Staphylococcus aureus* might justify the folkloric use of the plant as an antidiarrhoea agent. Therefore, the effectiveness of the extract against some diarrhoea-causing organism may account for the acclaimed use of the plant as an anti-diarrhoea agent.

4. CONCLUSION

The effects of aqueous extract of *P. muellerianus* leaves on bacterial isolates revealed that it was bacteriostatic on *Escherichia coli*, *Shigella flexneri*, *Salmonella typhi*, *Staphylococcus aureus* which are diarrhoea-causing bacteria and other tested bacteria such as *Bacillus cereus*, *Klebsiella pneumonia*, *Streptococcus pyrogene* except *P. mirabilis*;

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