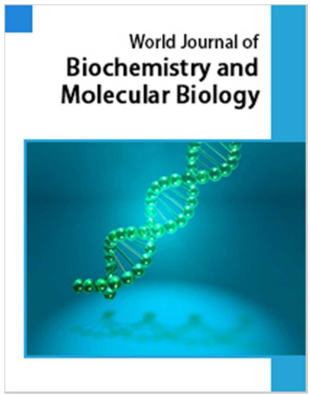
World Journal of Biochemistry and Molecular Biology
2017; 2(6): 39-45
http://www.aascit.org/journal/wjbmb

American Association for Science and Technology



Keywords
Phyllanthus niruri, Phytochemical Potentials, Antibacterial Activity and Enteric Pathogens

Received: August 19, 2017
Accepted: October 25, 2017
Published: November 25, 2017

Phytochemical Potentials and the *in-vitro* Antibacterial Activity of *Phyllantus niruri* (Chanca Piedra) Extracts on Some Enteric Pathogens

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Citation

Ibrahim Awache, Imo Chinedu, Michael Nosano Yakubu, Andefiki Ubandoma, Mohammed Gero, Moses Nnamdi Ononimu. Phytochemical Potentials and the *in-vitro* Antibacterial Activity of *Phyllantus niruri* (Chanca Piedra) Extracts on Some Enteric Pathogens. *World Journal of Biochemistry and Molecular Biology*. Vol. 2, No. 6, 2017, pp. 39-45.

Abstract

The Phytochemical Potentials and the *in-vitro* Antibacterial Activity of *Phyllantus niruri* extratcs (Chanca piedra) on Some Selected Enteric Pathogens was investigated. The entire fresh plants (*P. niruri*) obtained from the Federal University Wukari, were washed under running tap water; air-dried for ten days; pulverized in a mortar and then finely sieved. Each of 20g, 40g, 80g, 100g, 200g, 400g, 600g, 800g and 1000g of the powder was dissolved separately into 1000mL each of distilled water and ethanol for 24hr and then filtered using Whatman filter paper; to obtain 2%, 4%, 8%, 10%, 20%, 40%, 60%, 80% and 100% concentrations of the ethanolic and aqueous extracts accordingly. The test organisms were pure isolates *Escherichia coli*, *Pseudomonas aerognosa* and *Salmonella typhi*. Agar-well diffusion method was used to determine the antibacterial effect of the extract on a prepared nutrient agar. The extracts showed antibacterial activity against all the test isolates at different concentrations. The zone of inhibition increased with increased concentrations of the extracts. However, aqueous extracts showed more antibacterial activity than the ethanolic extracts. Following the same concentrations and procedure; the minimum inhibitory concentration (MIC) of the extracts were further determined by broth dilution technique. Exactly 5mls from each of the extracts were serially diluted to a five-fold (10^{-5}) and mixed with 5mls of Nutrient broth. And an inoculum of the test microorganisms were added to each of the tubes; thoroughly mixed and incubated for 24 hours at 37°C. The tubes were observed visually for growth by comparing the turbidity with the control. The lowest concentration of the extract that did not show any visible growth when compared to control test tube of Chloramphenicol (50mg/ml) was recorded as the MIC. Phytochemical extraction was done using GC-MS QP2010 Japan PLUS machine. Phenols, tannins and flavonoids were extracted in a very large amount; alkaloids, large amount; anthroquinones, low amount while saponnins, Phlabobatanins, Carbohydrates and Cardiacglycosides were absent. Most of these compounds extracted have antibacterial activity.

1. Introduction

From time immemorial, medicinal plants have being used to cure various kinds of life threatening diseases. Their use are still employed in present day. They are considerably indispensable, useful and economically essential. They are made up of various bioactive compounds. These compounds are the key players in the treatment of various humans and animals diseases [22]. Different plant extracts act against different microorganisms, some act against more than one microorganisms. They could be antibacterial, antifungal, antiviral, anti-parasitic and anti-protozoans among others. While others are hormone inducers. A common examples of such plants are that are being used are *Terminalia bellerica, Vitex negundo, Terminalia chebula, Emblica officinalis, Punica granatum* and *Lawsonia inermis* [24]. The increasing global population have made the demand for drugs from varied sources to be continuously increasing. The need to scientifically evaluate plants of medicinal value for the treatment of various ailments to augment the pharmaceutical drugs cannot be over-emphasized. The plant *Phyllanthus niruri* (Chanca piedra) is not an exception. Remarkably, various countries of the world where it is being used, use it to treat and cure closely related diseases such as blennorrhagia, colic, diabetes, malaria, dysentery, fever, flu, tumors, jaundice, vaginitis, and dyspepsia, analgesic and as an aperitif, carminative, digestive, emmenagogue, laxative, stomachic, tonic, vermifuge, biliary and urinary conditions (such as including kidney and gallbladder stones), viral hepatitis (liver diseases), HIV (and other viral infections), tuberculosis, and disorders including anemia, cystitis, prostatitis, venereal diseases and urinary tract infections, diabetes, hypertension, diuretic, analgesic, stomach ache, antispasmodic, febrifugal and also as cell protective properties in many other conditions [1, 4, 5, 14, 15, 17, 18, 25, 27, 28, 31, 32, 33]. They either taken in a form of tincture, standard infusion or weak decoction of the whole plant or its aerial parts.

The plant belongs to the family: Euphorbiaceae; genus: Phyllanthus; tribe: Phyllanthae and species: niruri, amarus. Its genus is large, as it consists of more than 700 species and widely distributed in the tropical and subtropical zones of Asia, America and Africa. But indigenous to the rainforests of the Amazon and other tropical areas throughout the world, including the Bahamas, southern India, and China. The genus is further classified into eleven (11) other sub-genuses [27, 28]. Mainly, the most widespread and common twenty four (24) species belongs to sub-genus *Kirganelia, Cicca,* and *Phyllanthus* [27, 28]. Chanca piedra is a small, erect and an annual and biennial herb that grows and spreads freely (like weed) between 30–40 cm height. Although *P. amarus* and *P. sellowianus* are closely related to *P. niruri* in history of usage, appearance and phytochemical structure, they are however typically found in drier tropical climates of India, Brazil, Florida and Texas. One peculiar feature of P. *niruri* that differentiates it from other plants of its kind, is that its seeds are below the leaves.

Synonyms: *Phyllanthus carolinianus, P. fraternus, P, sellowianus, P. kirganella, Nymphanthus niruri, P. lathyroides, P. lonphali* [12].

Common Names: Chanca piedra, quebra pedra, stone-breaker, arranca-pedras, punarnava, amli, bhonya, bhoomi amalaki, bhui-amla, bhui amla, bhuianvalah, bhuimy-amali, bhuin-amla, bhumyamalaki, cane peas senna, carry-me-seed, creole senna, daun marisan, derriere-dos, deye do, erva-pombinha, elrageig, elrigeg, evatbimi, gale-wind grass, graine en bas fievre, hurricane weed, jar-amla, jar amla, kizha nelli, malva-pedra, mapatan, para-parai mi, paraparai mi, pei, phyllanto, pombinha, quinine weed, sacha foster, cane senna, creole senna, shka-nin-du, viernes santo, ya-taibai, yaa tai bai, yah-tai-bai, yerba de san Pablo [12].

Numerous scientific studies have being carried out on *P. niruri.* In their study, [21], reported the plant extract to have inhibitory property on *Helicobacter pylori,* Lactic-acid bacteria. The entire plant extracts was tested to have an antibacterial effect on *Escherichia coli, Staphylococcus aureus* and *Salmonella typhi* [10]. [24] revealed that the minimum inhibitory concentration (MIC) of P. niruri leaf extract was 50 µg/ml against *Salmonella typhi* and *Staphylococcus aureus*. There was an inhibitory effect of P. *niruri* on *E. coli, S. aureus, S. typhi* and *Pseudomonas aeruginosa* but bactericidal effect on *Klebsiella aerogene* [16]. So also, the studies of [10] revealed that P. *niruri* inhibited the growth of *E. coli, S. aureus, S. typhi.*

The bioactive phytochemical constituents that produce definite physiological action on the human body confers medicinal value on these plants [2]. Some of the most important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoid, saponins, phenolic compounds and many more [8, 31, 32, 33]. Studies have linked antioxidant activity with phenolic compounds, antimicrobial activity with alkaloids, flavonoids with variety of fever and menstrual cycle problems and terpenoids as antipyretic [2]. [3] revealed the presence of (from genus Phyllantus) high amounts of phenolic compounds, alkaloids, saponins, flavonoids and terpenoids in aerial parts of the plant i.e. stem and leaf. Roots and fruits showed high amount of phenolics and terpenoids. Phyllanthus amarus have different classes of organic compounds of medicinal importance such as alkaloids, flavonoids, hydrolysable tannins (Ellagitannins), major lignans, polyphenols, triterpenes, sterols and volatile oil. Many lignans such as phyllanthin (a bitter constituent) and hypophyllanthin (a non-bitter constituent); were isolated from the plant [2, 31, 32, 33]. The highest amounts of phyllanthin (0.7% w/w) and hypophyllanthin (0.3% w/w) have been reported in leaves whereas, in the stem these are in minor quantities [8]. However, studies have shown that reported that these active biochemicals present in the plants are water soluble, fragile and deactivated by alcohol [7, 20].

The study investigated the phytochemical properties and the in-vitro antibacterial effects of the extracts of *P. niuriri* on three (03) enteric pathogens.

2. Materials and Methods

2.1. Plant Collection and Preparation of Extract

The entire fresh plants (*P. niruri*) were obtained from the Federal University Wukari, compound. The plant materials were carefully washed under running tap water (to remove dust and any other foreign materials) and were allowed to drain off. The plants were spread on the laboratory bench to air-dry at room temperature for two weeks. This was then pulverized in a mortar and finely sieved. The extract was stored in cupboard at room temperature until needed for use. Exactly ten (20g) of the powder was weighed and dissolved into 1000mL of distilled water and ethanol for 24h for each of the extracts. The same procedure was repeated for extracts of 40g, 80g, 100g, 200g, 400g, 600g, 800g and 1000g to obtain 2%, 4%, 8%, 10%, 20%, 40%, 60%, 80% and 100% concentrations accordingly [13]. This was then filtered using Whatman filter paper (No. 1). The aqueous filtrate was evaporated to dryness on steam at 60°C in a water bath while the methanolic extract was allowed to evaporate at room temperature as described by [23, 26]. The test organisms were *Escherichia coli, Pseudomonas aerognosa* and *Salmonella typhi*. The organisms were obtained from the Microbiology Laboratory Federal University Wukari, Taraba State, Nigeria. They were sub-cultured on nutrient agar slant and kept at 4°C.

2.2. Antibacterial Activity Analysis

To determine the antibacterial activity of the aqueous extracts of the P. *niruri*, an aliquot (0.1mL) of each of the prepared extracts at the various concentrations (2%, 4%, 8%, 10%, 20%, 40%, 60%, 80% and 100%), were obtained using sterile syringe and inoculated onto a freshly prepared nutrient agar plates (containing each test organism) by agar well diffusion method. Using a sterile cork borer of diameter size 6.00mm, three wells were bored on the solid Agar surface; as carried out by [29]. Incubation was done at 37°C for 24 hours. Zone of clearance (inhibition) was determined and measured round the well. The minimum inhibitory concentration (MIC) was interpreted as the lowest concentration of the extract that did not show any visible growth when compared to control plates of Chloramphenicol (50mg/ml) in the third well. This procedures were repeated for both the aqueous and ethanolic extracts. The diameters of the zones of inhibition were measured with metre rule to the nearest millimeter.

Again, the minimum inhibitory concentration (MIC) of the aqueous and ethanolic extracts were further determined by the broths dilution technique [19]. Exactly 20g was dissolved in 1000ml (20g/1000ml) standard was used for all the extracts. The same was done for 40g, 80g, 100g, 200g, 400g, 600g, 800g and 1000g. Nine (09) sets of nine (06) sterile test tubes were used. A control test tube of chloramphenicol served for each of the nine (09) concentrations. Exactly 5mls of the extract was mixed with 5mls of medium (Nutrient broth) and serially diluted to a five-fold (10^{-5}) dilution. And an aliquots (0.1ml) inoculum of the test microorganisms were added to each of the five test tubes. The bottles were thoroughly mixed by gentle shaking and incubated for 24 hours at 37°C. The tubes were observed visually for growth by comparing the turbidity with the control. The highest dilution or lowest concentration, which produced no growth, was taken as the minimum inhibitory concentration. Tubes showing no visible growth from the MIC test were sub-cultured on the fresh nutrient agar and incubated at 37°C for 24 hours. The lowest concentration of the extract that yielded no growth was recorded as the minimum bactericidal concentration (MBC). Diameters of zones of inhibition given by the extracts were compared with the one produced by the antibiotics Chloramphenicol (50 mg/ml) used.

2.3. Phytochemical Analysis of *A. indica* (GC-MS Analysis)

The pulverized sample of P. *niruri* was weighed. Exactly 1000g was sent to the Department of Consultancy and Production, National Research Institute for Chemical Technology Zaria, Nigeria, for phytochemical analysis using a Gas Chromatography – Mass Spectrometry Analysis (GC – MS). GC-MS analysis was carried out using the GCMS-QP2010 PLUS SHIMADZU. The column used was Perkin Elmer Elite - 5 capillary column measuring 30m × 0.25mm with a film thickness of 0.25mm composed of 95% Dimethyl polysiloxane. Helium was the carrier gas used at a flow rate of 0.5mL/min. And the sample injection volume that was utilized was 1µL, maintaining 250°C inlet temperature. Initially, the oven temperature was programmed at 110°C for 4 min, then an increased to 240°C. And further programmed to increase to 280°C at a rate of 20°C ending with at 5 minutes. The total run time was 35 minutes. At a temperature of 200°C, the MS transfer line was maintained with the source temperature maintained at 180°C. The GC-MS was analyzed using an acceleration electron impact ionization at 70eV and data were evaluated using Total Ion Count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library. See Table 5.

Table 1. *Showing measurements (mm) of the Zone of Clearance of the ethanolic extract of P. niruri.*

Concentrations of *P. niruri* extract (g/mL)	Diameter of zone of clearance (mm) on Test organisms		
	Salmonella typhi	***Escherichia coli***	***Pseudomonas aeruginosa***
2	15	13	16
4	16	13	18
8	23	14	23
10	24	16	27
20	28	19	29
40	33	23	33
60	37	25	34
80	41	26	39
100	50	31	56
Average:	29.67	20	30.55
Control	43	36	41

The results of the Minimum Inhibition Concentration (MIC) of the ethanolic extracts of *P. niruri* showed that *S. typhi, E. coli* and *P. aeruginosa* were inhibited at all the concentrations used. However the rate of inhibition differs. Some are highly sensitive at some concentrations while others are not. These results indicated that *P. aeruginosa* is the most sensitive at all concentrations while *E coli* is the least sensitive. This is evident in Table 3. While *S. typhi* and *P. aeruginosa* were totally inhibited (bactericidal concentration) at 8g/ml, *E. coli* was totally inhibited at 20g/ml.

Table 2. *Showing measurements (mm) of the Zone of Clearance of the aqueous extract of P. niruri.*

Concentrations of *P. niruri* extract (g/mL)	Diameter of zone of clearance (mm) on Test organisms		
	Salmonella typhi	***Escherichia coli***	***Pseudomonas aeruginosa***
2	18	18	20
4	21	18	21
8	23	20	23
10	29	25	24
20	33	31	30
40	39	36	36
60	46	39	41
80	51	43	47
100	58	47	56
Average:	35.33	30.78	33.11
Control	41	37	43

The results of the Minimum Inhibition Concentration (MIC) of the aqueous extracts of *P. niruri* showed that *S. typhi, E. coli* and *P. aeruginosa* were inhibited at all the concentrations used. However the rate of inhibition differs. Some are highly sensitive at some concentrations while others are not. These results indicated that *S. typhi* is the most sensitive at all concentrations while *E coli* is the least sensitive. This is evident in Table 4. While *S. typhi was* totally inhibited (bactericidal concentration) at 4g/ml, *P. aeruginosa* was 2g/ml and *E. coli* was totally inhibited at 8g/ml.

Table 3. *Showing Minimum Inhibition Concentration (MIC) of the ethanolic extract of P. niruri.*

Concentrations of *P. niruri* extract (g/mL)	Minimum Inhibition Concentration (MIC) on Test organisms		
	Salmonella typhi	***Escherichia coli***	***Pseudomonas aeruginosa***
2	+	+	+
4	+	+	+
8	-	+	-
10	-	+	-
20	-	-	-
40	-	-	-
60	-	-	-
80	-	-	-
100	-	-	-
MIC	8.00	20.00	8.00
Control	-	-	-

Keys: + = Slight Growth; - = No growth.

Table 4. *Showing Minimum Inhibition Concentration (MIC) of the aqueous extract of P. niruri.*

Concentrations of *P. niruri* extract (g/mL)	**Minimum Inhibition Concentration (MIC) on Test organisms**		
	Salmonella typhi	***Escherichia coli***	***Pseudomonas aeruginosa***
2	+	+	-
4	-	+	-
8	-	-	-
10	-	-	-
20	-	-	-
40	-	-	-
60	-	-	-
80	-	-	-
100	-	-	-
MIC	4.00	8.00	ND
Control	-	-	-

Keys: + = Slight growth; - = No growth; ND = Not Determined (as even the lowest concentration used in this study was bactericidal).

Table 5. *Showing the Phytochemical properties of P. niruri.*

Phytochemical Composition of *A. indica*	**Quantity**
Saponnins	-
Phlabobatanins	-
Carbohydrates	-
Anthraquinones	+
Phenols	+++
Tannins	+++
Flavonoids	+++
Cardiacglycosides	-
Alkaloids	++

Keys: +++ = Very large amount; ++ = Large amount; + = Low amount; - = Not present

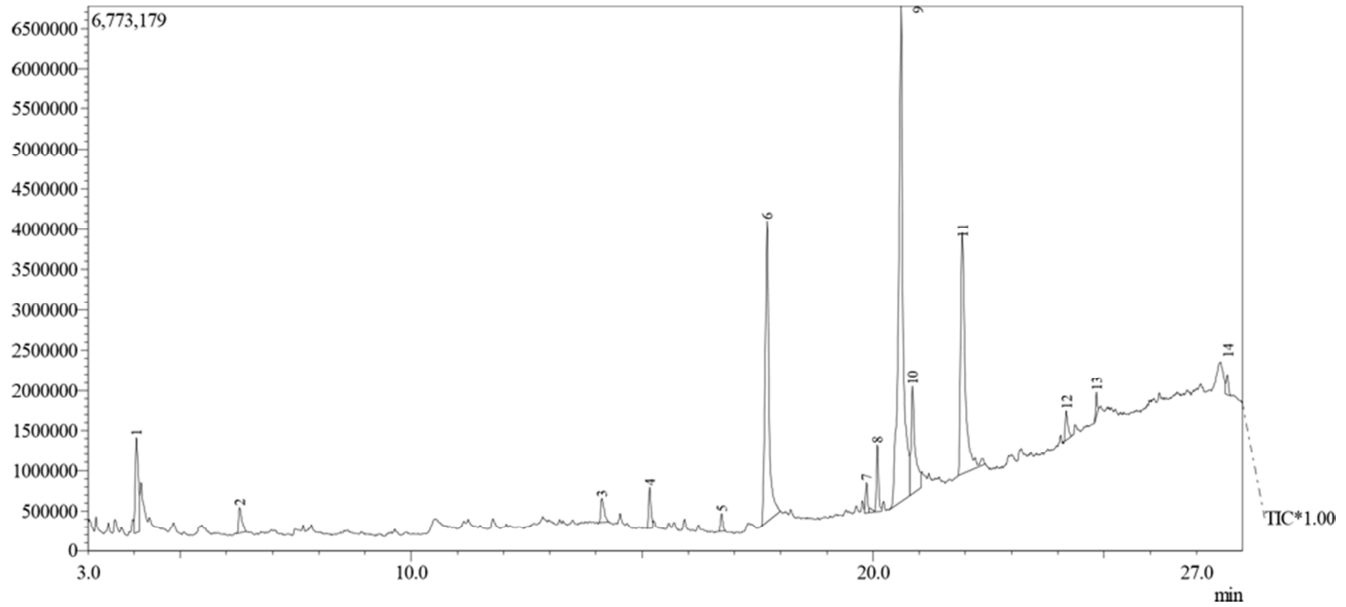

Figure 1. *GC-MS chromatogram of aqueous extract of P. niruri.*

3. Discussion

Table 1 presented the measurements (mm) of the Zone of Clearance of the ethanolic extract of *P. niruri*; Table 2 showed measurements (mm) of the Zone of Clearance of the aqueous extract of *P. niruri*. Table 3 presented the Minimum Inhibitory Concentration (MIC) of the ethanolic extracts of *P. niruri*; Table 4 showed Minimum Inhibition Concentration (MIC) of the aqueous extract of *P. niruri* and Table 4 presented the Phytochemical properties of *P. niruri*. The results from Table 1 to Table 4 indicated that both the ethanolic and aqueous extracts of *P. niruri* were potent against the test organisms. However, the rate of inhibition

differs. Table 1, showed the results of the Minimum Inhibition Concentration (MIC) of the ethanolic extracts of *P. niruri* showed that *S. typhi, E. coli* and *P. aeruginosa* were inhibited at all the concentrations used. However the rate of inhibition differs. Some are highly sensitive at some concentrations while others are not. Table 2 results indicated that *P. aeruginosa* is the most sensitive at all concentrations while *E coli* is the least sensitive. This is evident in Table 3. While *S. typhi* and *P. aeruginosa* were totally inhibited (bactericidal concentration) at 8g/ml, *E. coli* was totally inhibited at 20g/ml. Table 2 showed the results of the Minimum Inhibition Concentration (MIC) of the ethanolic extracts of *P. niruri* showed that *S. typhi, E. coli* and *P. aeruginosa* were inhibited at all the concentrations used. However the rate of inhibition differs. Some are highly sensitive at some concentrations while others are not. These results indicated that *S. typhi* is the most sensitive at all concentrations while *E coli* is the least sensitive. This is evident in Table 4. While *S. typhi was* totally inhibited (bactericidal concentration) at 4g/ml, *P. aeruginosa* was 2g/ml and *E. coli* was totally inhibited at 8g/ml. Generally several studies have reported susceptibility of varied bacteria to *P. niruri* [6, 9, 10, 16, 21]. Therefore, this study falls in alignment with several other studies at different locations [6, 9, 10, 16, 21]. Again results of this study showed that the zone of inhibition increased with increase in concentrations of the extracts. This confirms the findings of [30] who reported that higher concentrations of antibacterial substances exhibit more growth inhibition of some microbial pathogens. Remarkably, the results of this study also pointed out that the aqueous extracts exhibited more potency than the ethanolic extracts. This is most likely in line with the studies of [7, 20]. Which reported that the active biochemicals present in the plants are water soluble, fragile and deactivated by alcohol [7, 20, 31].

Like [11] reported in their ethnobotanical findings that the plant was used in the treatment of several ailments including constipation, flu and typhoid fever caused by *S. typhi* in the Bahamas. It is therefore safe to say *P. niruri* could also serve the same purpose.

Essential phytochemical extracts were extracted from different plants. This study extracted phenols, tannins and flavonoids in a very large amount; alkaloids in a large amount; anthroquinones in a low amount while saponnins, Phlabobatanins, Carbohydrates and Cardiacglycosides were found to be absent. These extracts has being applied to numerous aspects of life which are but not limited to pharmaceautical, food preservation and alternative source of natural medicine among others. The most essential of these substances include flavonoids, glycosides, tannins, alkaloids, polyphenols, saponnins and anthroquinones which are extracted from this studies and several other studies [2, 3, 8, 31, 32, 33].

4. Conclusion

The importance of medicinal plants cannot be over-emphasized. The increasing global population have made the demand for drugs from varied sources to be continuously increasing. Various countries of the world have employed the use of medicinal plants. This is because they act as antibacterial, antifungal, antiviral, anti-parasitic and anti-protozoans activity and other hormonal inducers. Also, they had and are still playing vital roles in treating, curing and managing even life-threatening diseases. Therefore there is need to scientifically evaluate *P. niruri* and other plants of medicinal value to get more conclusive evidences and results with the right doses, concentrations and route of administration for the treatment of various ailments to augment the pharmaceutical drugs is also important.

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