Antibacterial Effect of *Phyllanthus niruri* (Chanca Piedra) on Three Enteropathogens in Man

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Abstract: Three microorganisms causing gastroenteritis were used for the study of the antibacterial effects of *Phyllantus niruri,* extracts. The bacterial pathogens used were *Escherichia coli, Staphylococcus aureus* and *Salmonella typhi.* The solvents used for the extraction of active components of the plant (whole plant) were hot water, cold water (both as aqueous solvent) and ethanol. The antibacterial effect of the plant extracts showed that all the extracts were inhibitory to *E. coli, S. aureus* and *S. typhi.* The results of the Minimum Inhibitory Concentration (MIC) value of the extracts on the organisms are 31.25 mg mL^{-1} for *S. aureus,* 15.625 mg mL^{-1} for *E. coli* and 31.25-62.50 mg mL^{-1} for *S. typhi.* The pathogens used were not multi-drug resistant strains to most of the orthodox drugs. The antibacterial effect of plant extracts of *P. niruri* tends to give a clue to the problem of drug resistant strains of microorganisms causing gastroenteristis and thus provides the possibility of drug development for such human diseases.

Key words: Antibacterial, *Phyllanthus niruir*, Chanca piedra,

INTRODUCTION

The use of plants to heal or combat illness is as old as mankind. Since age long, plants have remained the basis for the development of modern drugs for human health[1]. Medicinal plants are considerably useful and economically essential. They contain active constituents which are used in the treatment of many human diseases. Besides being used as direct remedies, they are also used in the pharmaceuticals, agriculture, cosmetics, perfumery and food industries[2].

Pharmaceuticals mainly use plants containing known medically effective chemical substances which are first isolated from the plants and then used in the manufacture of drugs. Among these active substances include ephedrine which is extracted from the Chinese herb, Ma Huang, is used to ease the difficult breathing of Asthma sufferers[1]. Ginseng from the root of panax ginseng, a perennial herb has been widely used as a toxic and precious medicine since ancient times. This substance is effective for gastroenteric disorders, diabetes and weak circulation and has equally been used as adjuvant to prevent various disorder. Ginseng has been shown to contain various saponins and sapogenins.

Until the advent of synthetic anti malarials quinine, isolated from the bark of various cinchona trees species, constituted the most effective agent for the treatment of malaria. Aspirin on the other hand is a chemical copy of the active analgesic chemical found in the bark of willow

trees[1]. Digoxin, the main ingredient of many preparations used in the treatment of heart diseases is obtained from the crude version of the drug extracted from the fermented leaves of the common foxglove. The substances used in the pharmaceutical preparation for treating liver diseases are the flavonoligans from the milk thistle[2].

In agriculture, a large number of chemically synthesized compounds and natural molecules have been examined for their inhibitory effects on plant viruses and some of them have potent activity as protectors against virus infections[3].

Man has always been highly dependent on nature. The search for cure was not only the instinct of self-preservation but also self-protection. These first effects to ensure a continued existence on earth served as the foundation of medicine and healing. The only place man could find these medicines was his environment. It seemed that the traditions of years would not save medicinal plants from falling into disuse. However, the opposite proved to be the case when it was discovered that they have their advantages, synthetic medicines also frequently have their weakness in the form of serious adverse effects on human[2].

To date, man has discovered only about 10% of many and diverse existing plant species and thus the prospects of research in the field of natural medicine are bright indeed. Antibiotics, hormones, vitamins prostagladins-all have their origin and basis in nature[4]. New methods of plant tissue culture and biotechnology are beginning to

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point the way towards producing valuable natural substance in the test tube.

Thus, modern research has paved a way for the discovery of another plant of potential value to help in a wider range of ailments. Among the plants is *Phyllanthus niruri, Phyllanthus* has been used in Ayurvedic medicine and has a wide number of traditional uses including internal use for jaundice, gonorrhea, frequent menstruation, diabetes, tropical use as a poultice for skin ulcers, sores, swelling and itching. The young shoots of the plants are administered in the form of an infusion for the treatment of chronic dysentery[5].

Phyllanthus niruri is an annual herb, 50cm tall which belongs to the Euphorbiaceae family. It has over 500 species and 300 genera. It usually grows in the shade in the most tropical regions of the world. It is a wild herb in the Amazonian forest, but it can be cultivated easily. The generic name, *Phyllanthus* means 'leaf and flower' because the flower as well as the fruit, seem to become one with leaf. The specific name *niruri* may have come from a Hindu term and is adopted by Linneo.

The most common species of the genus *Phyllanthus* found in most West African countries including Nigeria are *Phyllanthus niruri* and *Phyllanthus amarus. P. niruri* and *P. amarus* are very closely related in appearance and in phythochemical structure. The major difference between the two is that *P. niruri* has larger leaves and the plants as a whole is bigger compared to *P. amarus.* Reorganization of the P*hyllanthus* genus has however classified *P. amarus* as type of *P. niruri*, thus *P. niruri* has been chosen as a prototype of the P0*hyllanthus* genus.

This investigations was aimed at finding out whether the plant, *Phyllanthus niruri* could inhibit *Salmonella typhi,* one of the agents of intestinal inflammation and pains through typhoid fever, *Escherichia coli,* the causative agent of travellers' and infantile diarrhoea and S*taphylococcus aureus,* a major agent of food intoxication in man.

MATERIALS AND METHODS

Collection of plant materials: *Phyllanthus niruri* plants were collected from around the environments of Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria. The plant was properly identified and authenticated by Dr. A.O.A. Meregini at the Department of Forestry, Michael Okpara University of Agriculture, Umudike. The plants were thoroughly washed with clean water to remove earthly matters and spread on clean surface in order to air dry. These were properly air dried for four days at room temperature (27 2°C).

Test for potency of organisms: The microorganisms (*Staphylococcus aureus, Escherichia coli* and *Salmonella typhi*) used in this work were obtained from stock culture at the Federal Medical Centre (FMC) Umuahia, Abia State. Viability test of each isolate were carried out by resuscitating the organisms in nutrient agar slants. The organisms were sub-cultured onto appropriate solid media followed by over night incubation at 37°C. This was followed by refrigerator storage at 4°C until required for use.

Confirmation of test organisms: Confirmation of the bacterial isolates which were collected from the stock cultures was performed using the Gram stain techniques and by specific biochemical tests.

Aqueous extract preparation: Aqueous extract preparation was carried out using the cold water and hot water extraction methods[7]. One hundred and fifty grams (150 g) of dried *Phyllanthus niruri* were weighed using a laboratory weighing balance. The plants were ground to fine particles using sterile grinding machine.

The ground sample of 50 g were weighed and soaked in 100 mL of clean sterile cold water in a conical flask with rubber cork and left for 24 hrs unagitated. so that it can be dissolved properly and the active ingredient extracted. The extract was filtered using sterile filter paper. The filtrate was transferred into the sample holder of vacuum evaporator, where the cold water solvent was evaporated to dryness[8]. The standard extracts obtained were stored in a refrigerator at 4°C until required for use.

Another ground sample of 50g were weighed and soaked in 100 mL of hot water in conical flask and corked. This was left unagitated for 24 hrs. The extract was filtered and the filtrate evaporated to dryness by heating under Bunsen burner. Standard extracts were stored in a refrigerator at 4°C until required for use[9].

Ethanol extract preparation: Dried ground sample of 50 g were soaked in 100 mL of ethanol (BDH chemical Ltd, England) in a clean conical flask and corked. This was left for 24 hrs unagitated. The extract was filtered using sterile filter paper (Whatman No. 1 filter paper). The filtrate was evaporated to dryness by heating. The extract was then collected and stored in the refrigerator at 4°C[7].

Antimicrobial susceptibility test: Antimicrobial activity of the extracts was evaluated using the test organisms: *Staphylococcus aureus, Escherichia coli* and *Salmonella typhi.* The organisms were first streaked separately on nutrient agar plates and inoculated into nutrient broth and incubated at 37°C for 24 hrs.

The ability of the various extracts to inhibit growth of the bacterial isolates was determined using the 5 mm diameter paper disc (Whatman No. 1 filter paper) in paper disc diffusion techniques[10]. Each of the paper discs was found to absorb a maximum volume of 0.01 mL. 1000 mg mL⁻¹ of each of ethanol, hot water and cold water extracts was prepared by dissolving 1.0 g of extract in 1 mL of appropriate solvent. Each disc contains 10 mg/0.01 mL. Therefore the concentration of the extract in each disc is 10 mg. Ethanol extract was dissolved using Di-methyl sulfoxide, a basic solution. This was to avoid interference of ethanolic solvent (an acidic solution) during inhibition if used for dissolving the extract.

Three agar plates were inoculated with the different test organisms. Unto each of the plates was placed 3 discs impregnated with the extracts of 1000 mg mL⁻¹ concentration using sterile forceps. Plates were labeled properly. Paper discs were sterilized, dried in oven at 170°C for 90 minutes and cooled before use. These were incubated at 37°C for 12 hrs in the incubator. Following inhibition, the diameter of the zones of inhibition were measured in (mm) and recorded accordingly.

Determination of Minimum Inhibitory Concentration (MIC): Minimum Inhibitory Concentration (MIC) is the concentration with the least inhibitory activity below which there is no further inhibition[11]. The concentration of extracts giving the least possible zone of inhibition is taken as the MIC. The MIC of the extracts was determined according to Brown[10] by incorporating constant volumes (10 mL) of each dilution of extract impregnated with the disc on the agar medium with streaked organisms as described in the antimicrobial susceptibility testing. One gram of each extract was dissolved in 1 mL of sterile water to give 1000 mg mL⁻¹. This concentration of

$$\frac{1}{2}, \frac{1}{4}, \frac{1}{8}, \frac{1}{16}, \frac{1}{32}, \frac{1}{64}, \frac{1}{128}$$

$(2^{-1}, 2^{-2}, 2^{-3}, 2^{-4}, 2^{-5}, 2^{-6}, 2^{-7},)$ mg mL⁻¹[11]. A total of nine (9) plates were prepared each with specific test organism in which discs impregnated with extracts of known concentrations were incorporated. The plates were incubated at 37°C for 24 hrs. After which zones of inhibition around each disc were examined and the diameter of the clearing measured and recorded.

Control experiment: Control experiment was carried out using known antibiotics. Antibiotics used were erythromycin (10µg mL⁻¹), tetracycline(30µg mL⁻¹), penicillin(30µg mL⁻¹) and chloramphenicol (25µg mL⁻¹).

This was used to compare with the result of inhibition using plant extracts on the same microorganisms.

Anova: Analysis of variance (ANOVA) was used to analyse if there was any significant difference in the effects of different plant extracts on the diameter of zones of inhibition (mm) at 10 mg mL⁻¹ concentration on the tested pathogens.

RESULTS

The yields in weight of plant extracts with hot water, cold water and ethanol showed that the highest percentage of yield was obtained with hot water (5%) followed by ethanol (3.2%) and cold water 4.4%) (Table 1). All the bacterial pathogen used in this work demonstrated susceptibility to both the aqueous (hot water, cold water) and ethanol extracts of *Phyllanthus nitruri*. Diameter of zones of inhibition around each impregnated disc were recorded with respect to extracts on each organism as shown in Table 2. Ethanol extract gave the highest zones of inhibition (12.0 mm) on *Escherichia coli*. With *Staphylococcus aureus*, ethanol showed the highest zone of inhibition with 14.0 mm diameter. With *Salmonella typhi*, hot water extract showed the highest zone of inhibition of 18.0 mm (Table 2).

Minimum Inhibitory Concentration (MIC) of plant extract gave different zones of inhibition based on methods of extractions at different concentrations of dilution using original concentration of 1000 mg mL⁻¹. All the extracts gave inhibitory actions at various dilutions. Diameters of zones of inhibition recorded at various concentrations are shown in Tables 3-5.

In general, plant extracts with hot water produced the highest zone of inhibition as shown in Table 4. Zones of inhibition increased with concentration of extracts. That is, as concentration of extracts were diluted, zones of inhibition decreased. All the bacterial pathogen showed susceptibility with the *Phyllanthus niruri* extracts, with *E. coli* producing the highest zone of inhibition on hot

Table 1: Yields in weight of plant extracts with respective solvents

Plant	Solvent	Yield (G)	Percentage yield (%)
Phyllanthus niruri	Hot water	2.50	5.00
	Cold water	1.60	3.20
	Ethanol	2.20	4.40

Table 2: Effect of Different extracts of *Phyllanthus niruri* on the diameter of zones of inhibition (mm) at 1000 mg mL⁻¹ concentration on test bacteria

Bacteria	Ethanol	Hoth water	Cold water
E. coli	12.00	10.00	9.50
S. aureus	14.00	11.00	7.00
S. typhi	12.00	18.00	9.00

Table 3: Effects of ethanol extracts on the diameter of zones of inhibition (mm) at varying concentrations (mg mL^{-1}) of *Phyllanthus niruri on test bacterial isolates*

	Bacterial pathogens								
	1000	500	250	125	62.5	31.25	15.625	7.812	MIC
S. aureus	13.00	12.00	7.50	7.00	6.50	5.00	-	-	31.25
E. coli	12.00	11.50	10.00	9.00	8.00	8.50	7.50	-	15.625
S. typhi	13.00	11.50	11.00	10.00	9.50	-	-	-	62.5

Key: (-) = No zone of inhibition (M/C) = Minimum inhibitory concentration.

Table 4: Effects of hot water extracts on the diameter of zones of inhibition (mm) at varying concentration (mg mL^{-1}) of *P. niruri* on test bacterial isolates

	Bacterial pathogens								
	1000	500	250	125	62.5	31.25	15.625	7.812	MIC
S. aureus	12.5	9.0	8.0	8.0	6.0	5.0	-	-	31.25
E. coli	13.5	10.0	10.0	9.7	7.0	6.5	6.0	-	15.625
S. typhi	10.0	8.5	7.0	6.0	5.5	-	-	-	62.50

Key: (-) = no zone of inhibition (MIC) = Minimum inhibitory concentration

Table 5: Effects of cold water extracts on diameter of zone of inhibition (mm) at varying concentration (mg mL^{-1}) of *P. niruri* on test bacterial pathogens

	Bacterial pathogens								
	100	500	250	125	62.5	31.25	15.625	7.812	M/C
S. aureus	9.5	8.5	7.0	7.5	5.0	3.0	-	-	31.25
E. coli	11.0	10.0	9.5	9.5	8.0	6.0	4.0	-	15.625
S. typhi	12.0	10.5	10.0	9.0	6.0	3.0	-	-	31.25

Key: (-) = no zone of inhibition (MIC) = Minimum inhibitory concentration

Table 6: Severity of plant extracts on inhibition of pathogen

Pathogens	Ethanol extract	Hot water extract	Cold water extract
S. aureus	+++	+++	++
E. coli	+++	+++	+++
S. typhi	+++	++	+++

Key: +++ = Complete inhibition, ++ = Moderate inhibition + = Slight inhibition, (-) = no inhibition

Table 7: Minimum inhibitory concentration (MIC) of all the plant extract on the test pathogens

Pathogens	Ethanol extract	Hot water extract	Cold water extract
S. aureus	31.25	31.25	31.25
E. coli	15.625	15.625	15.625
S. typhi	62.5	62.50	31.25

Table 8: Mean zone diameter of inhabition of different plant extracts on test bacteria

Bacteria	Ethanol extract	Hot water extract	Cold water extract
S. aureus	8.50±3.22	8.08±2.62	6.75±2.17
E. coli	9.50±1.73	8.95±2.64	8.28±2.29
S. typhi	11.00±1.40	7.40±1.65	8.42±3.03

Each value shows an average of three replications±standard deviation

water extracts (Table 4, 6). *S. typhi* showed the highest minimum inhibitory concentration, followed by *S. aureus* and then *E. coli* with the different extracts. Thus, there is slightly varied minimum inhibitory concentration and zones of inhibition of the different extracts on the pathogens tested (Table 7). Based on these results, the mean zone diameter of inhibition of the different extracts in the organisms was calculated and shown on Table 8.

The result of the control experiment as shown in Table 9 shows that *S. aureus* has 27.1mm inhibition with *erythromycin*, 16.2 mm with *tetracycline*, 14.5 mm with *penicillin* and 10.3 mm with *chloramphenicol*. Thus, they can be applied in any staphylococcal infection.

Escherichia coli. showed no inhibition with *erythromycin*, *tetracycline* and *penicillin* but showed 5.00mm with *chloramphencol*. Thus, *chloramphencol* is applicable in *E. coli* related infections. With *S. typhi*., *erythromycin* showed 13.2 mm, 16.0 mm with chloramphencol, thus both antibiotics may be used against *S. typhi*.

The observation that *P. niruri* has good inhibition against *S. aureus, E. coli* and *S. typhi* tends to prove a worthy remedy to the problem of drug resistance against these pathogens which are already known to be resistant to the standard antibiotics employed in the control experiment when used by infected persons.

Table 9: Result of control experiment using known antibiotics

	Erythromycin (10 g mL^{-1})	Tetracycline (30 g mL^{-1})	Penicillin (30 g mL^{-1})	Chloramphenicol (25 g mL^{-1})
S. aureus	27.1	16.2	14.5	10.3.
E. coli	-	-	-	5.0
S. typhi	13.2	-	-	16.0

Key: (-) = No inhibition Inhibition is measured in millimeter (mm)

DISCUSSION

The results of this investigation showed that plant extracts of *Phyllanthus niruri* possess appreciable and potential antibacterial activity against commonly encountered gastroenteric microorganisms in humans. Gastroenteritis caused by *E. coli* affects the intestinal epithelial cells producing enterotoxins that lead to secretion of electrolytes and water into the lumen of the small intestine, manifested as watery diarrhoea which is experienced by travelers (traveler's diarrhoea). Thus this study has revealed that this plant is a therapy for prevention of the intestinal mucosa against invasion of *E. coli* related diseases.

It is interesting to note that the action of the extracts of *P. niruri* is non toxic. Studies have shown that the alcohol and water based extracts of the plant have very low toxicity in mammals. The effects of *P. niruri* extracts agrees with the work of Contreras and Gamarra[12] that *P. niruri* showed antibacterial effect over *E. coli* at concentrations of 30, 50, 100, 200 and 300 mg mL^{-1}.

As with *E. coli, Staphylococcus aureus* and *Salmonella typhi* produced appreciable susceptibility with the plant extracts. The plant has been found to be effective in reducing intestinal haemorrhoids, intestinal inflammation, fever and pains. Thus *P. niruri* is used as medical plant in Ayurveda medicine[13].

The most remarkable finding of this work is the high inhibitory effects of the different plant extracts on *Staphylococcus aureus* thus challenging the claims by Contreras and Garmarra[12] of Marcos University, that the plant does not inhibit *S. aureus*. *P. niruri* showed good inhibition on *S. aureus*, the bacterial agent of food intoxication. *S. aureus* exhibited the highest zones of inhibition with the ethanol extracts and also appreciable zones of inhibition with both hot water extracts and the cold water extracts. Thus *P. niruri* may provide a possible cure for staphylococcal related diseases. *S. typhi* on its own was effectively inhibited with the different extracts, showing the highest zones of inhibition with both ethanol (as with *S. aureus*) and cold water extracts of the plant. This justifies the need why it is used in folk medicine as curative plant for typhoid fever and as intestinal anesthetic[14].

The result of this research has revealed that the many active bioconstitutents of *P. niruri* constitute potential qualities in its curative action[15]. Thus, it must be exploited upon by scientists in the development of human medicines and drugs. *P. niruri* research for human gastroenteritis has thus opened a gate way for the possibility of finding cures for many strains of enteric microorganisms which are now resistant to many usable and common antibiotics in our countries.

There are many factors that have been found to influence the active principles present in plants. These include age of the plant, extracting solvents, method of extraction and time of harvesting of plant materials[7]. These can affect the result of the experiment and influence the decision of the observer.

This work revealed that many gastroenteric infections will be inhibited with *P. niruri* when thorough and further work is carried out on it. This research has definitely highlighted *P. niruri* as having antibacterial effects on some common gastroenteric pathogens of man.

In conclusion, *P. niruri* showed antibacterial effects on common pathogens of man including *E. coli* associated with most human diarrhoea, *S. typhi* causing human typhoid fever with many complications and *S. aureus,* a major agent of food intoxication. This work has highlighted the antibacterial effects of *P. niruri* using ethanol and aqueous (cold and hot water) extracts on *S. aureus.*

P. niruri is a promising cure for human gastroenteritis as have been demonstrated in this work. This work serves as an eye opener to many scientists who may utilize the result of the work in developing drugs from *P. niruri* against human gastroenteritis employed in this investigation.

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APPENDIX

Analysis of variance on effect of different plant extracts on diameter of zones of inhibition (mm) at 10 mg mL^{-1} concentration on bacterial pathogens using the table below.

Pathogens		Treatments		Total
E. coli	12	10	12	34
S. aureus	14	11	7	32
S. typhi	12	18	9	39
Total (J) =	38	39	28	105
n_1=3	n_2=3	n_3=3	T=105	

Correction factor (C.F) = $\dfrac{T^2}{\sum N} = \dfrac{105^2}{9} = 1225$

TSS (Total sum of squares) = $12^2 + 10^2 + 12^2 + 14^2 + 11^2 + 7^2 + 12^2 + 18^2 + 9^2$ -C.F

= 1303 - 1225 = 78

BSS (Between sum of squares) = $\dfrac{38^2}{3} + \dfrac{39^2}{3} + \dfrac{28^2}{3}$ -CF

= 1249,6 -1225 = 24.6.

WSS (Within sum of squares) = TSS –BSS

= 78-24, 6 = 53.4

ANOVA TABLE

Source of variance	Df (Degree of freedom)	Sum of squares (ss)	Mean sum of squares (mss)	Observation F(cal) F tab 5% 10%
BSS	(n -1)= 3 – 1 = 2	24,6	$\dfrac{BSS}{n-1}$ $\dfrac{24.6}{2}$ $\dfrac{12.3}{6.7}$	
			=12..3 1.84	12.6 16.3
WSS	($\sum$ n-1) = 9-1=8	53.4	$\dfrac{NSS}{\sum n-1} = \dfrac{53.4}{8} = 6.7$	

$H_O = \mu 1 = \mu 2 = \mu 3$

$H_A = \mu 1 = \mu 2 = \mu 3$

From the table above, F cal < F tab at 2 and 8 degrees of freedom with 5% and 10% levels of significance. Thus we accept the null hypothesis that there is no significance difference on the effects of difference plant extracts on diameter of zones of inhibition (mm) at 10 mg mL^{-1} concentration on the tested bacterial pathogens

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