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Invitro Activity of *Phyllanthus amarus* Extract on Nephrolithiasis and Urea-Splitting Bacteria

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Abstract

Extraction of *Phylllantus amarus* plant powder using hot percolation and cold percolation techniques revealed the presence of alkaloids, flavonoids, terpenoids, volatile oil, saponin, tannin, anthraquinone, cardiac glycosides, phenolic compounds, reducing and non- reducing sugars. The hot percolation crude extract also revealed higher scavenging activity compared with ascorbic acid (standard reference) and cold percolation crude extract. Pre-incubation of human urine with hot percolation and cold percolation crude extract of *Phyllanthus amarus* to determine its effect on crystallization process of crystal salts (calcium oxalate, calcium phosphate and cysteine) which causes nephrolithiasis (kidney stone) yielded excellent results on calcium phosphate (100% effective), calcium oxalate (97% and 85.67%) while cysteine (91% and 84.67%). A total of fifteen (15) flavonoid compounds identified in the hot and cold percolation extract of *Phyllanthus amarus* using gas chromatography analysis include flavan-3-ols, flavones, flavonols, flavanones and isoflavanones. High flavonoid content in the hot percolation crude extract was due to the presence of significant concentrations (mg/100ml) of quercetin (282.50), catechin (16.32), kaemferol (214.33), luteolin (51.79), apigenin (1.33), epicatechin (4.13), isorhamnetin (5.49), and rutin (11.72). The sensitivity pattern of *Phyllanthus amarus* leaf extract towards test organisms (*Pseudomonas aeruginosa, Escherichia coli Proteus mirabilis and Klebsiella pneumonia*) were determined using agar well diffusion technique. All test organisms were extremely sensitive to hot percolation extract of *Phyllanthus amarus* compared to cold percolation extract and antibiotics used as standard reference. Prevention of renal stone and urinary tract infection recurrence is a serious problem in human health but results obtained in this research shows that *Phyllanthus amarus* leaf is a good source of effective crude inhibitors for crystal formation which can be used in the treatment of kidney stone, urinary tract infection and other reactive oxygen species (ROS)-related disorders.

Keywords: *Phyllanthus amarus*, Flavonoid, Kidney stone, *Escherichia coli*

Introduction

Nephrolithiasis (kidney calculi or stones) is urinary tract stones formation, which begins in kidney and may enlarge in a ureter or the bladder. Nephrolithiasis affect 10-12% of the population in industrialized countries thus the incidence has been increasing while the age of onset is decreasing over the last years (Mirian, 2010).

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Genetic, metabolic, environmental and nutritional factors aggravate the crystallization of salts inside the renal tubules leading to retention and accumulation of crystal salts which causes kidney stone. Under certain circumstances crystal salts bind to each other due to chemical and electrical forces triggering the process of aggregation; thus the crystals attach to the epithelium which eventual leads to formation of stone (Khaskhali, 2009). General risk factors include disorders that increase urinary salt concentration, either by increased excretion of calcium or uric acid salts, or by decreased excretion of urinary citrate (Bartoletti, 2007).

Uric acid calculi usually develop due to increased urine acidity (urine pH < 5.5), or rarely with severe hyperuricosuria (urinary uric acid > 1500 mg/day [> 9 mmol/day]), which crystallizes undissociated uric acid while magnesium ammonium phosphate calculi (struvite, infection calculi) formation is due to urinary tract infection caused by urea-splitting bacteria (e.g, *Proteus* species, *Klebsiella* species) (Glenn, 2020). Urease-producing bacteria play a prominent role in the formation of infection-induced urinary stones. The most common bacteria responsible for kidney infection are *Escherichia coli*, accounts for close to 80% of cases of kidney and urinary tract infections, *Klebsiella*, *Proteus*, *Pseudomonas*, *Enterococcus*, and *Staphylococcus saprophyticus* (Vasudevan, 2014).

Hypocitruria (urinary citrate < 350 mg/day [1820 micromol/day]) existing in almost 40 to 50% of calcium calculi–formers promotes calcium calculi formation because citrate usually binds urinary calcium and inhibits the crystallization of calcium salts (Glenn, 2020). Hyperoxaluria (urinary oxalate > 40 mg/day [> 440 micromol/day]) is caused by excess ingestion of oxalate-containing foods (eg, rhubarb, spinach, cocoa, nuts, pepper, tea) or by excess oxalate absorption due to various enteric diseases (eg, bacterial overgrowth syndromes, chronic pancreatic or biliary disease) or ileojejunal (eg, bariatric) surgery(Glenn, 2020).

Materials and Methodology

Chemicals and Growth Media

Chemical reagents, crystal salts (calcium oxalate, calcium phosphate and cysteine) and growth media were procured from Sigma while phenolic acid standards were purchased from Aldrich Chemical Co., Milwaukee.

Collection and Processing of *Phyllanthus amarus* Leaves

Phyllanthus amarus plant was collected from Fountain University, Osogbo, Nigeria during the flowering periods. Fresh *Phyllanthus amarus* leaves were separated from other parts of the plant and unwanted materials. The residual soil on the plant materials were cleaned, air dried at 30°C processed into powdery form, and sieved through a 24-mesh sieve to obtain a homogeneous powder. The smooth homogeneous powder sample was stored in an airtight container at 30ºC for further analysis.

Phyllanthus amarus Crude Extraction

Crude extract of *Phyllanthus amarus* leaf powder was prepared using hot percolation and cold percolation techniques.

Cold Percolation Technique

Phyllanthus amarus leaf powder (100 mg) was soaked in distilled water (10 mL) for 24 hours at 25°C; the solution was filtered and the filtrate was concentrated to obtain a crude extract (solid residue) using water bath at 40°C. The solid residue was weighed, reconstituted in Dimethyl sulfoxide (DMSO) and stored in a refrigerator at 4˚C for further analysis

Hot Percolation Technique

Phyllanthus amarus leaf powder (100 mg) was dissolved in distilled water (10 mL) and subjected to heating in a shaking water bath at 40°C for 24 hours. The mixture was filtered and the filtrate was concentrated to obtain a crude extract (solid residue) using water bath at 40°C. The crude extract was weighed, reconstituted in Dimethyl sulfoxide (DMSO) and stored in a refrigerator at 4°C for further analysis.

Proximate Analysis of *Phyllanthus amarus*

Proximate analysis of *Phyllanthus amarus* such as moisture content, ash content, crude protein, crude fibre, crude lipid and nitrogen-free extract were determined according to the methodology of Jimoh *et al.* (2018a).

Qualitative Analysis of Non-reducing Sugar in *Phyllanthus amarus*

Sugars in *Phyllanthus amarus* extract react with the anthrone reagent under acidic conditions to yield a blue-green colour. Variability in colours produced by the reaction of the crude extract and anthrone reagent were blue, light green and green respectively. Thus, variation in the blue-green colouration indicated the presences of non-reducing sugars such as sucrose which lack free anomeric carbon atom required for formation of the glycosidic bond (Jimoh *et al.*, 2018b).

Quantitative Analysis of Reducing Sugar Concentration in *Phyllanthus amarus* Extract

The reducing sugar concentration was determined by adding 1ml of 3, 5-dinitrosalicylic acid to 1ml of the crude extract and boiled for 5 minutes, the reducing sugar was determined at 540 nm using the spectrophotometer (Jimoh *et al.*, 2018b).

Antioxidant Activity of *Phyllanthus amarus* Extract

The antioxidant activity of *Phyllanthus amarus* extract was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay according to methodology of Jimoh *et al.* (2017a). The method is based on the reduction of methanolic DPPH solutions in the presence of a hydrogen donating antioxidant. One millimetre (1 mL) of 0.004% methanolic solution of DPPH was added to 1 mL of varying concentrations (0.2-1.0 mL) of each crude extract and ascorbic acid (standard reference). The solution was incubated in the dark at 28°C for 30 minutes and absorbance was read against a blank at 517 nm using a spectrophotometer. Thus percentage inhibition of antioxidant activity was calculated using the equation below;

$$\% \text{ inhibition} = \{\underline{Ab - Aa}\} \times 100 Ab$$

where Aa = absorbance of the test samples; Ab = absorbance of blank

Phytochemical Screening and Quantification of Flavonoid Compounds in *Phyllanthus amarus*

Quantitative analysis of flavonoids was done using two stage extraction procedures for the effective removal of flavonoids present in *Phyllanthus amarus* extract (Jimoh *et al.*, 2017b). Extraction was done by adding 5 mL of 1 M of NaOH to 50 mg of cell free-biotrasformation supernatant and incubating on a shaker at ambient temperatures for 16 h. The mixture was centrifuged (5000 rpm) twice, rinsed with water and the supernatant was heated at 90 °C for 2 h to release the conjugated phenolic compounds. The heated extract was cooled, titrated with 4 M HCL to pH<2.0, diluted to 10 mL with deionised water and centrifuged to remove the precipitate. The supernatant was stored for subsequent purification; residue was extracted further with 5 mL of 1 M of NaOH and heated to 160 °C in Teflon. After cooling, the supernatant was adjusted to pH <2.0 with 4 M HCL; filtered and the filtrate was purified by passing through a conditioned Varian (Varian Assoc., Harbor City, CA) Bond Elut PPL (3 mL size with 200 mg packing) solid phase extraction tube at 5 mL/min attached to a Visiprep (Supelco, Bellefonte, PA). The GC condition for the analysis include GC (HP 6890 Powered with HP ChemStation Rev. A 09.01 [1206]) software, injection temperature (split injection), split ratio (20:1), carrier gas (nitrogen), inlet temperature (250 °C), column type (HP- 1, capillary), column dimensions (30 m × 0.25 mm × 0.25 µm), oven program (initial temperature at 60 °C for 5 min; first rate at 15 °C/min for 15 min, maintained for 1 min; second ramping at 10°C/min for 4 min), detector (FID), hydrogen pressure (28 psi) and compressed air (32 psi).

In-vitro Analysis of *Phyllanthus amarus* Extract

The analysis was carried out using human urine, crystal salts (calcium oxalate, calcium phosphate and cysteine) that causes kidney stone infections and *Phyllanthus amarus* extract. Human urine sample was pre-incubated with *Phyllanthus amarus* extract (hot percolation and cold

percolation extract) for 24 hours to determine the reducing effect of the plant extract on the crystal salts. After incubation period, 3 g of each crystal salt was added separately and left on the working bench at 30°C for 30 minutes and the effectiveness of *Phyllanthus amarus* extract on the crystal salts were determined using the formula,

W1 = Weight of crystal salt before pre-incubation with *Phyllanthus amarus* extract

W 2 = Weight of crystal salt remaining after pre-incubation with *Phyllanthus amarus* extract

W3 = Weight of dissolved crystal salt during pre-incubation with *Phyllanthus amarus* extract i.e (W1 - W2)

% Effectiveness or dissolution= W3 x 100

Antibacterial Effect of *Phyllanthus amarus* Extract

Sensitivity pattern of previously characterized bacterial isolates (*Proteus mirabilis, Escherichia coli, Pseudomonas aeruginosa and Klebsiella pneumonia*) to the crude extract were determined using agar well diffusion technique according to the methodology of Jimoh *et al.*, (2017b). Antibiotics such as ciprofloxacin, streptomycin, chloramphenicol, augmentin and amoxycillin were used as standard reference.

Results and Discussion

Proximate Analysis of *Phyllanthus amarus* Extract

Portioning of compounds in *Phyllanthus amarus* into six categories based on the chemical properties of the compounds was determined through proximate analysis. Thus, moisture content, ash content (inorganic residue) crude protein (nitrogen content), crude fibre (quantity of digestible compounds), crude lipid (fat and oil content) and soluble carbohydrates (nitrogen-free) content of *Phyllanthus amarus* are as presented on table 1. The results are mean and standard deviation of triplicate analysis.

Quantitative Analysis of Reducing Sugar for *Phyllanthus amarus* Extract

The estimation of reducing sugar in *Phyllanthus amarus* was carried out using the dinitrosalicylic acid (DNS) method. The reducing sugar concentration increases concurrently with the *Phyllanthus amarus* extract concentration, although the cold percolation extract had higher concentration than the hot percolation extract (Figure 1).

Table 1: Proximate Composition of *Phyllanthus amarus* Extract

Parameters	Mean ± SD (%)
Moisture content	15.4 ± 0.09
Ash content	30.2 ± 0.17
Crude lipid	0.5 ± 0.03
Crude protein	27.5 ± 0.15
Crude fiber	22.7 ± 0.11
Nitrogen–Free Extract (Digestible carbohydrates)	3.7 ± 0.01

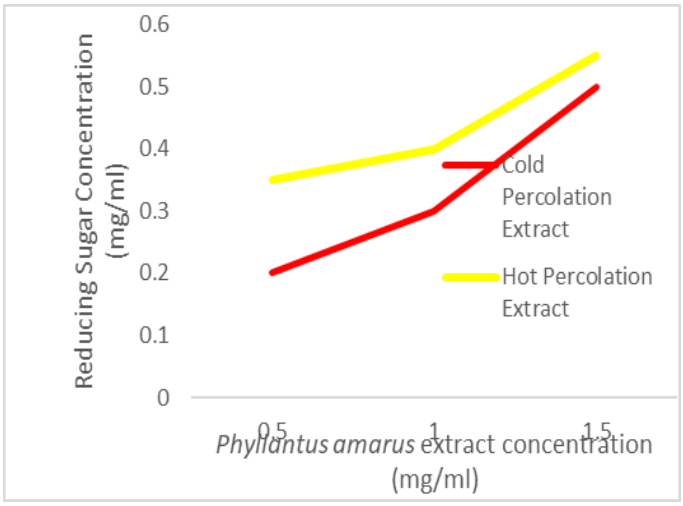

Figure 1: Reducing Sugar Concentration of *Phyllanthus amarus* Extract

Antioxidant Analysis of *Phyllanthus amarus* Extract

Reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radical anion, hydroxyl radical, alkylperoxyl radical, nitric oxide, and singlet oxygen are often associated with some physio-pathological states in human. Oxidative stress, caused by an imbalance between antioxidant systems and the production of oxidants, including ROS, is considered to contribute to a wide variety of degenerative processes and diseases such as atherosclerosis, kidney stone and urinary tract

infection. In this study, *Phyllanthus amarus* crude extract were investigated for potential free radical inhibitors and the degree of ROS inhibition of the extract was evaluated at a concentration of 100 µg/ml. The hot percolation crude extract (HP-CE) revealed higher scavenging activity compared to ascorbic acid and cold percolation crude extract (CP-CE) respectively (Figure 2). Varying percentage inhibition values exhibited by these extract depend on the varying concentrations of flavonoids obtained through GCFID because the configuration, substitution, and total number of hydroxyl groups available in each flavonoids substantially influence antioxidant activity mechanisms such as radical scavenging and metal ion chelation ability (Panday *et al.*, 2012).

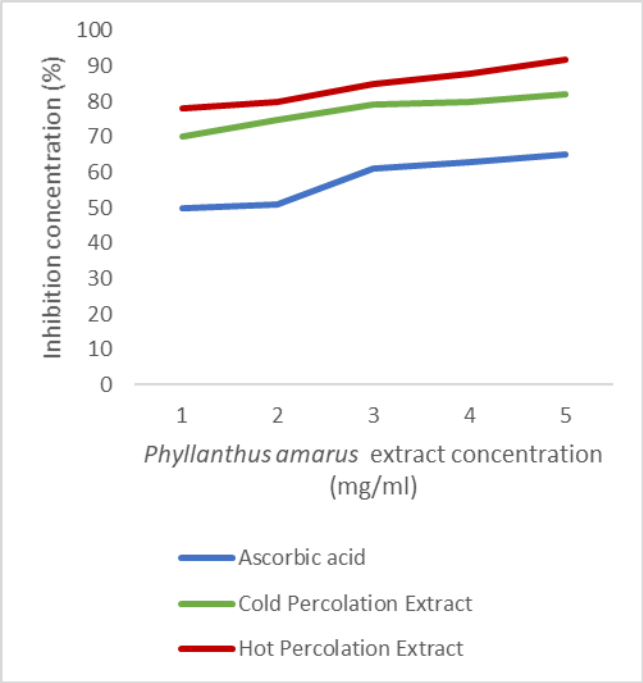

Figure 2: Antioxidant Effect of *Phyllanthus amarus* Extract

Phytochemical Screening of *Phyllanthus amarus* Extract

The potentially phytoactive compounds responsible for the ROS inhibitory activities investigated were present in both HP-CE and CP-CE *Phyllanthus amarus* crude extract except CP-CE extract that lacks alkaloids. (Table 2). Flavanoid compounds (15) detected in the hot and cold percolation extract of *Phyllanthus amarus* using GC analysis include Flavan-3-ols , Flavones, Flavonols, Flavanones and Isoflavanones (Table 3). High total flavonoid contents in the HP-CE was due to the presence of significant concentrations of quercetin, catechin, kaemferol , luteolin, apigenin, epicatechin, isorhamnetin, and rutin (Table 3). This result revealed the mechanism of antioxidant inhibitory activity of the extract because flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. These high pharmacological activities of the extract on crystal salts are linked to the antioxidant properties of flavonoids and the defensive effects are due to their capacity to suppress ROS formation by inhibiting some enzymes or chelating trace elements involved in free radical production, scavenge radical species and improve regulation of antioxidant defense (Hirano *et al.*, 2001).

Table 2: Phytochemical Analysis of *Phyllanthus amarus* Extract

Phytoactive compounds	**Hot Percolation Extract** (HP-CE)	**Cold Percolation Extract** (CP-CE)
Total Phenolics	+	+
Alkaloid	+	-
Volatile oil	+	+
Anthraquinones	+	+
Tannin	+	+
Phlobatannins	+	+
Steroids	+	+
Flavonoids	+	+
Terpenoids	+	+
Glycosides	+	+
Cardiac	+	+

Key - = Absence of phytochemicals
+ = Presence of phytochemicals

In-vitro Analysis of *Phyllanthus amarus* on Crystal Salts

Pre-incubation of human urine with hot percolation and cold percolation crude extract of *Phyllanthus amarus* to determine its effect on crystallization process of crystal salts yielded

excellent results on calcium phosphate (100% effect), calcium oxalate (97 % and 85.67 %) while cysteine (91% and 84.67%); which negate the report of Barros *et al.* (2003) which stated that the pre-incubation of human urine with *Phyllanthus*

Table 3: Flavonoid Compounds Extracted from *Phyllanthus amarus* Extract

Flavonoid compounds	Metabolites	Flavonoids Concentration (mg/100ml) Present in *Phyllanthus amarus* leaf	
		Hot Percolation Extract	Cold Percolation Extract
Flavan-3-ols	Catechin	16.32	12.42
	Epicatechin	4.13	3.88
	Epigallocathecin	0.74	0.63
	Epicatechin-3-gallate	0.35	0.31
	Epigallocatechin-3-gallate	4.21×10^{-4}	3.17×10^{-4}
Flavones	Luteolin	51.79	45.21
	Apigenin	1.33	0.72
Flavonols	Kaempferol	214.33	193.65
	Quercetin	282.50	276.00
	Isorhamnetin	5.49	4.52
	Myricetin	8.78×10^{-6}	8.98×10^{-6}
	Rutin	11.72	10.51
Flavanones	Naringenin	0.66	0.45
Isoflavanones	Genistein	1.46×10^{-5}	4.59×10^{-6}
	Daizein	7.03×10^{-6}	2.34×10^{-6}

amarus did not inhibit the precipitation of calcium oxalate particles and even more crystals were obtained in *Phyllanthus amarus*-containing urine. In this research, although the crystals of calcium oxalate and cysteine treated urine samples were sparsely dispersed but the weight of crystals decreased drastically after the analysis. The activity of *Phyllanthus amarus* crude extract due to its effectiveness will prevent proliferative response and decrease synthesis of fibrogenic substances which promotes additional stimulus for crystal growth exhibited by damaged cells. Based on this result, *Phyllanthus amarus* will prevent adhesion of calcium phosphate crystals which is the main constituent of human urinary calculi to the plasma membrane of epithelial cells; thus preventing endocytosis of the crystals which causes cell damage or death.

Sensitivity pattern of *Phyllanthus amarus* on Urea –Splitting Bacteria

The sensitivity pattern of *Phyllanthus amarus* leaf extract towards test organisms (*Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis and Klebsiella pneumonia*) are presented on table 4 below. The zones of inhibition were recorded to the nearest diameter in millimeters according to Jimoh *et al.,* (2017b) and each value is the mean of triplicate analysis. All test organisms were extremely sensitive to hot percolation extract of *Phyllanthus amarus* leaf compared to cold percolation extract and antibiotics used as standard reference. Hot percolation extract had inhibitory effect on the test organisms while cold percolation extract did not exhibit any inhibitory characteristics i.e. non- sensitive (Figure 4).

Table 4: Antimicrobial Activity of *Phyllanthus amarus* Extract

***Phyllanthus amarus* extract**	**Antibiotic**	**Zones of Inhibition of *Phyllanthus amarus* extract and Antibiotics (mm)**			
		Escherichia coli	***Pseudomonas aeruginosa***	***Proteus mirabilis***	***Klebsiella pneumonia***
Hot percolation extract (30 µg/ml)		32	28	30	26
Cold percolation extract (30 µg/ml)		8	6	4	4
	Chloramphenicol (30 µg)	19	8	6	4
	Augmentin (30 µg)	8	-	3	4
	Streptomycin (30 µg)	11	6	8	6
	Ciprofloxacin (10 µg)	10	9	10	8
	Amoxicillin (30 µg)	14	-	-	-

Result Interpretation

No zones of inhibition: --

Non sensitive: For total diameter lower than 8 mm

Sensitive: For total diameter between 9 – 14 mm

Very sensitive: For total diameter between 15 – 19 mm.

Extremely sensitive: For diameter higher than 20mm

Conclusion

Prevention of renal stone recurrence is a serious problem in human health but results obtained in this research shows that the *Phyllanthus amarus* leaf is a good source of effective crude inhibitors for crystal formation which can be used in the treatment of kidney stone and other ROS-related disorders. Presences of varying concentrations of flavonoid compounds and its high radical scavenging activity compared to ascorbic acid shows that the hot percolation crude extract will interfere with many stages of stone formation, reducing crystals aggregation, modifying their structure and composition as well as altering the interaction of the crystals with tubular cells leading to reduced subsequent endocytosis. However, further biological investigations are required, particularly using animal models to verify the reported inhibitory activities under *in vivo* conditions. Although farmers consider *Phyllanthus amarus* as a problematic weed but this research revealed that it contains potential agent to prevent and/or to treat kidney stone disease.

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