Preliminary phytochemistry and in-vitro antibacterial efficacy of hydro-ethanolic leaf extracts of Psidium guajava on common urinary tract bacterial pathogens.

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Akinjogunla OJ, Etok CA, Oshoma CE

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Abstract

The aim of this study was to determine the preliminary phytochemistry and antibacterial potentiality of hydro-ethanolic leaf extracts of Psidium guajava on Gram positive bacteria (Staphylococcus aureus, Coagulase negative Staphylococcus spp, Streptococcus pyogenes and Enterococcus faecalis) and Gram negative bacteria (Serratia marcescens, Pseudomonas aeruginosa, Escherichia coli. Proteus mirabilis, Klebsiella spp., Enterobacter spp and Citrobacter freundii) isolated from mid-stream urine of males and females between August and October, 2009 using standard microbiological and agar disc diffusion techniques. The results of the preliminary phytochemical constituents revealed that hydro-ethanolic leaf extracts of Psidium guajava contained secondary and primary metabolites such as saponins (++), alkaloids (++), tannins (++), cardiac-glycosides (±), terpenes (+), flavonoids (+) and sterols (+). The results also showed that both Gram positive and Gram negative bacteria were susceptible to hydro-ethanolic extracts of Psidium guajava at different graded concentrations (5 mg ml-1, 10 mg ml-1, 20 mg ml-1, 40 mg ml-1 and 80 mg ml-1) with the Mean ± SD zones of inhibition among the Gram positive bacteria ranging from 8.0 ± 1.5mm in Streptococcus pyogenes to 25.0 ± 3.0mm in Staphylococcus aureus while among the Gram negative bacteria, it ranged from 8.3 ± 0.5mm in E coli to 27.0± 3.0mm in Serratia marcescens. However, growth of Enterococcus faecalis was not inhibited by the tested leaf extracts at (5 mg mL-1). This depicts that the inhibitory activity was both organism and concentration dependent. The presence of the phytochemical constituents and inhibitory activities of hydro-ethanolic leaf extracts of Psidium guajava signify the potential of Psidium guajava as a source of therapeutic agents combating infections caused by common urinary tract pathogens.

Introduction

Traditional or herbal medicine has been in practice as far back as 1500BC and the knowledge on use of its use in curing certain diseases has dramatically increased over the decades[30]. The World Health Organization (WHO) estimates that approximately 80% of the world's inhabitants rely on traditional medicines for their primary health care and plants have long formed the basis of sophisticated traditional medicine systems and purportedly provide excellent leads for new drug developments[24332]. The rediscovery of the connection between plants and health is responsible for the launching of a new generation of multi-component botanical drugs, dietary supplements and plant-produced recombinant proteins[25]. However, the increasing problems of multi-drug resistant (MDR) bacteria is of great concern to both the clinicians and pharmaceutical industries and this has made it significant to search for newer drugs that are highly effective, affordable, acceptable and available[203].

Psidium guajava belongs to the family Myrtaceae. It is a native of Central America but is now widely cultivated, distributed and the fruits enrich the diets of millions of people in the tropics of the world[2712]. It is a genus of about 100 species of tropical shrubs and a small tree of about 10 m high with spreading branches that thrives on all kinds of soils. It is one of the most gregarious fruit trees and is widely known by its common English name (guava). In Nigeria, it is called guava (Hausa), gurfa (Yoruba) and Gwaibwa (Igbo)[12]. *Psidium guajava* also known as the ‘’poor man's apple’’ of the tropics has a long history of traditional use, a good proportion of which have been validated by scientific research[7]. The ethno-medicinal uses include the crushing of the leaves and the application of the extract on wounds, boils, skin and soft tissue infectious site[6]. Extracts of roots, bark and leaves are also used to treat gastro-enteritis, vomiting, toothaches, coughs, sore throat, oral ulcers, inflamed gums, leucorrhea, stomach ache, epilepsy, convulsions and upper respiratory tract infections[18]. In some cultures, a decoction of the leaves is drunk for regulation of menstrual periods, expulsion of the placenta after child birth, tightening of vaginal walls after childbirth as well as a douche for vaginal discharges [718]. Its anti-amoebic and antimalarial effects have also been documented[22 32]. Guava fruit paste and cheese are popular dishes in Florida, the West Indies and parts of South America[10].

Urinary tract infection (UTI) is caused by pathogenic invasion of the urinary tract, which leads to an inflammation of the urothelium. Urinary tract infections (UTIs) are the most frequently diagnosed cases, having an estimated figure of 150 million per annum worldwide[31]. The clinical manifestations of UTI depend on the portion of the urinary tract involved, the etiologic organisms, the severity of the infection, and the patient's ability to mount an immune response to it. The prevalence and incidence of urinary tract infection is higher in women than in men, which is likely the result of several clinical factors including anatomic differences, hormonal effects, and behaviour patterns[16]. The leading causes of acute and uncomplicated UTIs in patients have been reported to be due to *Escherichia coli, Staphylococcus aureus, Proteus* spp, *Klebsiella* spp and *Pseudomonas aeruginosa*[1519528]. In Nigeria, *E. coli, Proteus* spp *and Klebsiella* spp have been isolated in 90% of UTI reposted cases[2314]. UTIs are associated with a high risk of morbidity and mortality and account for significant health care costs in spite of the availability and use of the antimicrobial drugs. Microbial resistance to nearly all classes of antibiotics continues to rise despite increasing awareness and concerns worldwide and this has necessitated the search for new antibacterial from medicinal plants with various chemical structures and novel mechanism of action for new and reemerging urinary tract infections. This study was carried out to determine the phytochemical components of leaf of *Psidium guajava* and its antibacterial potentiality on the urinary tract pathogens.

Materials and Methods

Collection of Plant materials and Identification

The leaves of *Psidium guajava* were collected in August, 2009 at Ikot Ekpene, Akwa Ibom State, Nigeria. The taxonomic identification of the plants was done at the Department of Botany and Ecological Studies, University of Uyo, Akwa Ibom State. The leaves were cleaned of extraneous matters and repeatedly rinsed thoroughly under running distilled water for further analysis.

Sterilization of Materials

The Petri dishes and pipettes packed into metal canisters were appropriately sterilized in the hot air oven at 180°C for 2hrs at each occasion. The culture media were autoclaved at 121°C for 15mins.

Bacterial Strains:

Bacterial species consisting both Gram positive and Gram negative were used in this study. The Gram positive species were. Staphylococcus aureus, Coagulase negative *Staphylococcus* spp, *Streptococcus pyogenes* and *Enterococcus faecalis*. While the Gram negative bacteria were: *Serratia marcescens, Pseudomonas aeruginosa, Escherichia coli. Proteus mirabilis, Klebsiella* spp., *Enterobacter s*pp and *Citrobacter freundii*. These species were freshly isolated from clinical specimens (Urine) and identified using colonial and microscopic morphology and various standard biochemical reactions. Stock cultures were maintained on nutrient agar slant at 4^{o}C until needed.

Preparation of plant extracts

A sample (50g) of the powder of the shade-dried leaves of *Psidium guajava* was macerated in 70% ethanol and 30% water for 72 hrs and filtered. The filtrate was concentrated under vacuum at 30^{o}C and the dry extract was weighed and preserved at 5ºC .The graded concentrations of the extracts were prepared and tested for antibacterial activity on urinary tract pathogens by the disc diffusion methodusing Oxoid- Mueller Hinton agar (Difco Laboratories, Detroit, Mich) supplemented with 2% NaCl. Sterile filter paper discs of 6 mm diameter were separately impregnated with leaf extracts of graded concentrations (5 mg ml^{-1}, 10 mg ml^{-1}, 20 mg ml^{-1}, 40 mg mL^{-1} and 80 mg ml^{-1}) and then applied onto the agar plates. Control experiments comprising Streptomycin were set up. The plates were incubated at 37^{o}C for 24 hrs.The diameters of the inhibitory zones were measured in millimeters. Assays were performed in triplicate and the data are presented as mean ± standard deviation (SD).

Preliminary Phytochemical Screening

The plant extracts of *Psidium guajava* were screened for the presence of bio-active components in the leaves and roots[13302].

Test for Tannins

About 1.0g of the hydro-ethanolic leaf extracts of *P. guajava* was weighed into a beaker and 10 ml of distilled water added. The mixture was boiled for five minutes. Two drops of 5% ferric chloride solution ($FeCl_3$) were then added. Development of dark green or deep blue colour indicated the presence of tannin.

Test for alkaloids

About 0.5 g of the hydro-ethanolic leaf extracts of *P. guajava* was stirred with 5 ml of 1% HCl on a steam bath. The solution was filtered and 1 ml of the filtrate was treated with two drops of Mayer's reagent. Development of turbidity on addition of Mayer's reagent was regarded as evidence for the presence of alkaloids in the extract

Test for saponins

About 0.5 g of hydro-ethanolic leaf extracts of *P. guajava* extract was introduced into a tube containing 5.0 ml of distilled water, the mixture was vigorously shaken for 2 min, and formation of froth indicated the presence of saponins.

Test for flavonoids (Shinoda Test)

A small piece of magnesium ribbon was added to hydro-ethanolic leaf extracts of *P. guajava* followed by drop wise addition of concentrated hydrochloric acid. Colours varying from orange to red indicated flavones, red to crimson indicated flavonoids, crimson to magenta demonstrated the presence of flavonoids.

Test for Terpenes

To 0.5g of hydro-ethanolic leaf extracts of *P. guajava*, 3.0ml chloroform were added and filtered, 10 drops of acetic anhydride and 2 drops of H_2SO_4 were added to the filtrate and the colour change from blue to green indicated the presence of terpenes.

Cardiac glycosides

About 0.5g of hydro-ethanolic leaf extracts of *P. guajava* was dissolved in 2ml of acetic anhydride and cooled in ice bath. Concentrated H_2SO_4 was carefully added drop by drop. A colour change from violet to blue to green indicated the presence cardiac glycoside. Also 0.5g of the plant extract was dissolved in 2ml of chloroform, Concentrated H_2SO_4 was carefully added drop by drop to form a lower layer. A reddish- brown colour at the interface indicated the presence of cardiac glycoside.

Anthraquinones

About 0.5g of hydro-ethanolic leaf extracts of *P. guajava* was shaken with 10ml of benzene and filtered and 5ml of 10% ammonia was added to the filtrate. The mixture was shaken and the presence of pink, red or violet colour indicated the presence of anthraquinones.

Test for Sterols

10 mg of hydro-ethanolic leaf extracts of *P. guajava* was dissolved in 2 ml of chloroform and 2ml of concentrated sulphuric acid was added from the side of the test tube. Test tube was shaken for few minutes. The development of red color in chloroform layer indicated the presence of sterols.

Results

The eleven genera consisting both Gram positive bacteria (*Staphylococcus aureus, Coagulase negative Staphylococcus* spp, *Streptococcus pyogenes, Enterococcus faecalis*) and Gram negative bacteria (*Serratia marcescens, Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Klebsiella* spp., *Enterobacter* spp and *Citrobacter freundii*) were isolated from 50 mid stream urine samples using their morphological and biochemical characteristics (Table 1).

	ISOLATES										
PARAMETERS	a	b	c	d	e	f	g	h	i	j	k
Grams reaction	+/cocci	+/cocci	+/cocci	-/rod	-/rod	-/rod	-/rod	-/rod	+/cocci	-/ rod	-/rod
Catalase test	-	+	+	-	-	-	-	-	-	-	-
Citrate test	-	-	-	+	-	-	+	-	-	+	+
Oxidase test	-	-	-	-	+	-	-	-	-	-	-
Coagulase test	-	+	-	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	+	+	-	-	-	-	-
Urease activity	-	-	-	-	-	-	+	-	-	-	+
Glucose	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	+	-	+	-	+	-	-	+
Sucrose	-	-	-	-	+	-	+	-	-	+	+
Mannitol	+	+	+	+	+	+	-	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	-

Table 1: Morphological and Biochemical Characteristics of Urinary Tract Bacteria.

Keys a: *Streptococcus* spp.; b: *Staphylococcus aureus*; c: CON *Staphylococcus* spp.: d: *Citrobacter freundii*; e: *Pseudomonas aeruginosa*; f: *Escherichia coli*; g: *Proteus mirabilis*; h: *Enterobacter* spp.; i: *Enterococcus faecalis*; j: *Serratia marcescens*; k: *Klebsiella* spp.

The in vitro antibacterial activities of crude hydro-ethanolic leaf extract of *Psidium guajava* on the uropathogenic bacteria employed was assessed both qualitatively and quantitatively by the presence or absence of inhibition zones and zone diameters. The crude hydro-ethanolic leaf extracts of *P. guajava* showed variable degrees of antibacterial efficacy on the uropathogens tested at the graded concentrations (5 mg ml^{-1}, 10 mg ml^{-1}, 20 mg ml^{-1}, 40 mg ml^{-1} and 80 mg ml^{-1}) with hydro-ethanolic leaf extracts of the *P. guajava* showing the highest inhibitory activity on both the Gram positive and Gram negative bacteria at a concentration of 80 mg ml^{-1} (Tables 2 and 3). The Mean ± SD zones of inhibition ranged from 8.0 ± 1.5mm in Streptococcus pyogenes to 25.0 ± 3.0mm in Staphylococcus aureus among the Gram positive bacteria while among the Gram negative bacteria, it ranged from 8.3 ± 0.5mm in E coli to 27.0± 3.0mm in *Serratia marcescens* (Tables 2 and 3). Among the uropathogens tested, *Enterococcus feacalis* did not show any sensitivity to the hydro-ethanolic leaf extract of the tested plant at 5 mg ml^{-1}. Comparisons of the antibacterial activities of hydro-ethanol leaf extract of *P. guajava* were done using standard streptomycin as positive control.

Concentration	*S. aureus* (n=20)	CONS.spp (n=12)	*S. pyogenes* (n=8)	*E. faecalis.* (n=3)
	Mean ± SD zones of inhibition of extracts on isolates			
Streptomycin (30ug)	24.2 ± 2.5	19.5±1.5	20.0 ± 3.0	19.3 ±1.5
5 mg mL^{-1}	8.8 ± 1.5	12.8 ± 2.0	8.0 ±1.5	NZ
10 mg mL^{-1}	10.4 ± 2.0	14.8 ± 1.5	8.2 ± 2.5	10.3 ± 2.0
20 mg mL^{-1}	20.6 ± 2.5	18.6 ± 1.0	10.0 ± 1.0	13.6 ± 2.0
40 mg mL^{-1}	22.0 ± 1.0	21.0 ± 1.5	13.6 ± 1.0	16.0 ± 3.0
80 mg mL^{-1}	25.2 ± 3.0	21.0 ± 2.0	17.0 ± 2.0	16.3 ± 1.0

Table 2: Antibacterial Efficacy of Hydro-ethanolic Leaf extracts of *Psidium guajava* on Gram Positive Bacteria isolated from Mid-stream Urines.

Each inhibitory zone includes 6 mm diameter of the disc.

NZ: No zone of inhibition observed

S. aureus: Staphylococcus aureus ; CONS.spp.: Coagulase negative *Staphylococcus* spp

S. pyogenes : Streptococcus pyogenes ; *E. faecalis: Enterococcus faecalis.*

Concentration	*S. marcescens* (n=10)	*Pseu aeruginosa.* (n=15)	*E. coli.* (n=29)	*P. mirabilis* (n=4)	*Kleb.* spp. (n=8)	*Enter* spp. (n=8)	*C. freundii.* (n=3)
	Mean ± SD zones of inhibition of extracts on isolates						
Streptomycin (30ug)	21.3 ± 2.3	19.7±1.5	20.5 ± 3.0	26.5 ± 1.0	21.6 ± 1.0	23.0 ± 1.0	17.0 ± 1.0
5 mg mL^{-1}	8.9±1.0	12.4 ± 2.0	8.3±0.5	10.5± 0.5	9.2 ± 0.5	8.8±0.5	8.4 ± 0.5
10 mg mL^{-1}	10.8 ± 2.0	13.7 ± 1.5	8.8 ± 0.5	12.8 ± 2.0	10.6 ± 1.0	10.0±0.5	8.4 ± 1.0
20 mg mL^{-1}	20.4 ± 2.5	18.0 ± 1.0	10.3 ± 1.0	15.8 ± 2.0	13.4 ± 2.0	12.6 ± 0.5	11.2 ± 2.0
40 mg mL^{-1}	25.6 ± 1.0	18.6 ± 1.5	15.2 ± 1.0	16.2 ± 3.0	18.0 ± 1.0	16.0 ± 1.0	13.0 ± 1.0
80 mg mL^{-1}	27.0 ± 3.0	21.5 ± 2.0	20.8 ± 2.0	20.0 ± 1.0	23.6 ± 1.5	19.5 ± 2.0	15.0 ± 1.0

Table 3: Antibacterial Efficacy of Hydro-ethanolic Leaf extracts of *Psidium guajava* on Gram Negative Bacteria isolated from Mid-stream Urine.

Each inhibitory zone includes 6 mm diameter of the disc.

S. marcescens: Serratia marcescens ; Pseu spp. *Pseudomonas aeruginosa ; E. coli: Escherichia coli*;

P. mirabilis: Proteus mirabilis Kleb. spp: *Klebsiella spp; Enter* spp *: Enterobacter* spp;

C. freundii. : Citrobacter freundii.

The results of phytochemical analysis showed that the crude hydro-ethanolic leaf extract of *P. guajava* have different classes of bioactive constituents such as saponins, alkaloids, tannins, cardiac-glycosides, terpenes, flavonoids and sterols . The results showed that saponins, tannins and alkaloids are present in high concentrations, followed by terpenes, flavonoids and sterols while small concentration of cardiac-glycosides was detected (Table 4).

Phytochemical Components	Saponins	Alkaloids	Tannins	Cardiac glycosides	Terpenes	Flavonoids	Anthraquinones	Sterols
Occurrence	++	++	++	±	+	+	-	+

Table 4: Phytochemical Constituents of Hydro-ethanolic Leaf Extract of Psidium guajava.

Keys: -: Not Detecte ±: Present in small concentration. +: Present in moderately high concentration ++: Present in high concentration.

Discussion

Urinary tract infections (UTIs) are associated with a high risk of morbidity and mortality and account for significant health care costs in spite of the availability and use of the antimicrobial drugs. Microbial resistance to nearly all classes of antibiotics continues to rise despite increasing awareness and concerns worldwide. It has been reported that nosocomial infectious bacteria exhibited least susceptibility to antibiotics and some of these bacteria out rightly developed multi drug resistance to these antibiotics[21].

The result of this research revealed the presence of the primary and secondary metabolites such as saponins, alkaloids, tannins, cardiac-glycosides, terpenes, flavonoids and sterols in the hydro-ethanolic leaf extract of *Psidium guajava*. This result agrees with the findings of Akinpelu and Onakoya[4], Kamath et al.,[17] who reported the presence of tannins and flavonoids in the leaf extract of *Psidium guajava*. The presence of the metabolites such as cardiac glycosides, saponins, tannins, alkaloids in *Psidium guajava* may be responsible for its potential use as a drug against pathogenic bacteria[29,3]. According to Ebana et al.,[11] and Cushnie and Lamb[9], both alkaloids and flavonoids had antimicrobial activities. Alkaloids are naturally and widely distributed chemical constituents in plants and the presence of alkaloids in the hydro-ethanolic leaf extract of *Psidium guajava* augments its use in pharmaceutical industries for medicinal purpose. Saponins possess biological properties such as antimicrobial and anti-inflammatory that make them useful as drugs. Flavonoids are phenolics structure containing one carbonyl group complexes with extra cellular and soluble protein and with bacterial cell wall[8], thus exhibits antibacterial activity through these complexes.

The results obtained showed that hydro-ethanolic extracts of *Psidium guajava* exhibited inhibitory activities on the uropathogenic bacteria with different degrees as demonstrated by measuring the diameters of inhibition zones and these results are in conformity with the results obtained by Abu-Zaida et al.,[1] and Akinjogunla et al.,[2]. The extracts were also found to be active at low concentration (5mg/ml), which is very essential for antimicrobial agents.

The Gram positive bacteria isolated from the mid-stream urine samples are more susceptible to the hydro-ethanolic leaf extracts of *Psidium guajava* than the Gram negative bacteria and this is in agreement with Ravikumar et al.,[26]. The degree of susceptibilities of the Gram positive and Gram negative bacteria may be as a result of the physical and chemical compositions of their cell walls.

In conclusion, the demonstration of antibacterial efficacy of hydro-ethanolic leaf extracts of *P. guajava* on both Gram-negative and Gram-positive bacteria isolated from mid stream urine samples was an indication that the plant is a potential source for production of drugs with a broad spectrum of activity and this supports the traditional use of the plant and also suggests that the plant extracts possess compounds that can be used as antibacterial agents in novel drugs for the treatment of urinary tract infections.

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