Nig. J. Biotech. Vol. 41(1): 157-164 (June 2024)
ISSN: 0189 1731
Available online at
http://www.ajol.info/index.php/njb/index
and https://bsn.org.ng
DOI: https://dx.doi.org/10.4314/njb.v41i1.17

NIGERIAN JOURNAL OF BIOTECHNOLOGY

In vitro susceptibility of drug-resistant fungi and urinary bacteria to *Psidium guajava* L. leaf extract

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Abstract

Drug-resistant pathogens are responsible for high mortality and morbidity rates. Plant remedies are known to treat microbial infections. This research was aimed to determine the susceptibility of drug-resistant fungi (*Candida albicans*) and bacteria (*Staphylococcus aureus* and *Escherichia coli*) responsible for candidiasis and urinary tract infection to *Psidium guajava* L. leaf extracts. The *P. guajava* leaves were subjected to extraction using two solvents (methanol and ethanol) with varied polarity and concentrated with a rotary evaporator. Qualitative phytochemical screening of the extracts was done. Antimicrobial susceptibility testing was done to identify the resistant pathogens. The antimicrobial effect of the extracts was established using the agar-well diffusion method at a given concentration. The methanol and ethanol extracts had both phenols but tannins were absent rather. Flavonoids were detected in the ethanol extract. Multi-drug resistance was observed among the pathogens particularly, *E.coli*. The antibiotics with 100% resistance by the bacterial pathogens were Amoxicillin and Erythromycin whereas *C. albicans* showed resistance to Ketoconazole. The extracts of *P. guajava* L. leaves had an inhibitory effects against drug-resistant *E. coli* and *C. albicans*, whereas *S. aureus* was resistant to the extracts. The methanol extract had a higher antibacterial activity with mean±SD zone of inhibition 28.0±1.56 mm and 35.5±2.25 mm against *E.coli* and *C. albicans* respectively. In an in-vitro study with such high susceptibility to methanol extract; it is paramount to consider *P. guajava* L. leaf as a good candidate with natural therapeutics against drug-resistant urinary tract infection and candidiasis, an outcome that could necessities further pharmacological elucidation.

Keywords: *Psidium guajava* L. leaves, Drug resistant bacteria, *Candida albicans*, Antimicrobial activity, Phytochemical
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Introduction

Globally, the use of antimicrobials has had a significant impact on the mortality and morbidity of microbial infections (bacterial and fungal) in humans with an emphasis on developing countries like Nigeria (Antimicrobial Resistance Collaborators, 2022). Antimicrobial resistance may arise from the overuse and/or misuse of antimicrobials, increasing in drug-resistant strains circulating the hospitals and communities. There have been increasing report of multidrug-resistant *Staphylococcus aureus* (Akujobi *et al.*, 2013; Agbakoba *et al.*, 2020; Ezeanya-Bakpa *et al.*, 2023), *Escherichia coli* (Akujobi *et al.*, 2012; Akujobi and Ezeanya, 2013; Ezeanya *et al.*, 2017) and *C. albicans* (Ejike *et al.*, 2018) in Nigeria. These pathogens are responsible for public health important infections such as urinary tract infections, gastrointestinal infections, vaginal and disseminated infections (Whaley *et al.*, 2017). More recently, antimicrobial resistance has become a major international health threat while

exploration of antimicrobials from natural sources (medicinal plants) has become a current trend.

The genus, *Psidium*, a member of the family Myrtaceae, is reported to readily grow in tropical areas like Africa. The species *P. guajava* L. (guava) is most commonly cultivated. There have been reports on the presence of bioactive constituents and essential oil in the leaves with antimicrobial activities against pathogens (Begum *et al*., 2004). The leaves are rich in phytochemicals like flavonoids, tannins, phenol (eugenol, cineol,), triterpenes, and resin (Ncube *et al*., 2008; Okareh *et al*., 2019). Phytochemicals are natural compounds of medicinal value formed by plants. A few phytochemicals in *P. guajava* with active antibacterial values and nutrients are triterpenoids (guavanoic and guavacoumaric acid) and flavonoids (Arima *et al*., 2002; Begum *et al*., 2002) with varied mechanism of actions. Specifically, disruption of cell wall integrity leads to cell content leakage and increasing cell membrane permeability (Juven *et al*., 1994; Burt, 2004).

The leaves of *P. guajava* have been reported to show antibacterial activity against both Gram positive and Gram negative bacteria using solvent extraction of essential oils (Biswas *et al*., 2013). The leaves have been evaluated for antibacterial activity against *S. aureus* using ethanol extract. Sanches *et al*. (2005), Vieira *et al*. (2001) and Gnan and Demello (1999) reported remarkable antibacterial activity of *P. guajava* leaves against *S. aureus* of clinical sources. Other authorities like Biswas *et al* (2013), Das and Goswami (2019) reported significantly low resistance antibacterial activity of various *P. guajava* leaf extracts against *E.coli.* Furthermore, Bezerra *et al* (2018) reported a significant antifungal activity of *P. guajava* leaves against *C. albicans* and other non-albicans species via fungal growth inhibition. Padron-Marquez *et al* (2012) also reported a significant antifungal activity of hexane extract against *C. albicans* unlike other extracts (methanol and acetone).

Although *P. guajava* L. is an important plant used in Phytomedicine; yet there is a paucity of information on the susceptibility of drug-resistant bacterial and fungal pathogens. In Nigeria, drug-resistant *S. aureus* and *E.coli* have been tested against other medicinal plants and their phytochemicals demonstrated high efficacy (Ezeanya and Daniel, 2012; Akharaiyi *et al*., 2021; Ezeanya-Bakpa *et al*., 2021). This work aimed to evaluate the in vitro susceptibility of drug-resistant clinical isolates of fungus (*C. albicans*) and bacteria (*S. aureus, E. coli*) responsible for urinary tract infection and candidiasis to *P. guajava* leaves extracts, using organic solvents (ethanol and methanol).

MATERIALS AND METHODS

Collection and Preparation of P. guajava L. *leaves*

The fresh leaf samples were collected from the vegetable market in Ikorodu, Lagos. The leaves were collected at random and placed in a sterile collection bag with proper labelling and transported immediately to the laboratory for preparation and extraction. The fresh guava leaves were further weighed and hot air-dried in an oven at 55^{o}C for 72 hours.

Extraction Methods for P. guajava leaves

The dried leaf samples were ground into powder using a laboratory blender. Two solvents were used for the extraction: methanol (>95%) and ethanol (>99.5%), using maceration procedure. The leaf powder of 50g was mixed with each solvent of 100ml at a ratio of 1:2. The mixtures were placed in a sterile flask for 3 days in a cool and dark cupboard to prevent light exposure at room temperature. The mixtures were placed on a shaker at 70 rpm. After shaking for 3 days in the solvent, the mixtures were filtered. The filtrate were concentrated using a rotary evaporator at 50°C and stored at 4°C for further analysis.

Phytochemical Analysis

The concentrated extracts were subjected to qualitative phytochemical analysis using the standard procedure as previously described (Huang *et al*., 2009; Yadav and Agarawala, 2011). For each test, 0.5 mL of the leaf extract was used.

For Phenols and Tannins, the extracts were mixed with a 100% solution of ferric chloride ($FeCl_3$) and sodium chloride ($NaCl_2$) solution containing 1% gelatin respectively.

Test for Flavonoids, concentrated hydrochloric acid was added in drops to the extract using methods by Moreno *et al*. (2000).

Antibiotic and antifungal sensitivity testing for test organisms

The test organisms were inoculated onto four media plates (Oxoid, Cambridge, UK): Potato dextrose agar, Mannitol salt agar, Mac Conkey agar and Eosin methylene blue agar. For bacteria and fungi, aerobic incubation for 24 and 48 hours respectively at 37 °C was done. After incubation, the isolates were further identified using standard confirmatory microbiological tests such as Gram staining, Biochemical tests and Cultural morphology. Before identification, all pathogens were collected from urine and vaginal samples at the Medical Laboratory of Isalu Hospital, Lagos.

For the antibiotics and antifungal sensitivity testing, the antibiotics tested included: Amoxicillin (30 µg), Streptomycin (15 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Erythromycin (15 µg), Cotrimoxazole (50 µg); antifungal agent: Ketoconazole (20 µg/mL) (Liofilchem s.r.l., Italy).

Test organisms were prepared in suspension with turbidity equivalent to 1.5×10^8 CFU/mL (0.5 McFarland standard solution). Subsequently, antibacterial and antifungal sensitivity testing was done using the modified Kirby-Bauer Disc Diffusion method on Muller Hinton agar (Oxoid, Cambridge, UK) and the sensitivity result was interpreted according to the clinical and laboratory standards (CLSI, 2020). The degree of susceptibility of the test organisms was presented as a zone of inhibition with a diameter measured in millimeters using a calibrator.

Antimicrobial Activity of P. guajava L. leaf extract

An antimicrobial activity assays were done using the agar-well diffusion method. 100 μL aliquots of each extract were tested against the test organisms in triplicates on Mueller Hinton agar plates (bacteria) and potato dextrose agar (fungi). Before a sterile cork borer was used to bore 6 mm wells 2 inches apart on the already inoculated Mueller Hinton agar plates with the test organisms. Plates were inoculated with test organisms prepared to 0.5 McFarland standard. The bacterial inoculated plates were incubated at 37°C for 24 hours. The fungi inoculated plates were incubated at 25±2 °C for 72 hours. Afterwards, zones of inhibition were observed and measured in millimeters (mm). The extraction solvent served as the control.

Statistical analysis

Descriptive statistics were done. Mean ± standard deviation (SD) was used to represent the diameter of the zone of inhibition. Results were also presented in percentage.

RESULTS AND DISCUSSION

Qualitative Phytochemical Analysis of extract

The screening of phytochemicals present in *P. guajava* L. leaf extract on a qualitative basis is presented in Table 1. A blue-black, white-coloured and bright yellow precipitate was indicative of phenols, tannins and flavonoids respectively. The extracts (methanol and ethanol) were positive for phenols but had no tannins. Ethanol extract had flavonoids, unlike the methanol extract.

Table 1: Screening of *Psidium guajava* L. leaves for phytochemicals

Extracts	Flavonoids	Phenols	Tannins
Ethanol	+	+	-
Methanol	-	+	-

KEY: (+): Positive; (-): Negative

Oncho *et al.* (2021) reported the absence of flavonoid and the presence of tannin in the ethanol leaf extract of *P. guajava* L. in Ethiopia. This study showed a degree of difference in phytochemicals' constituents with this work which may be due to geographical location although methods of extraction and solvents were the same. In Nigeria, Engwa *et al*. (2013) used methanol as an extraction solvent for *P. guajava* L. leaves. In our study, phenols were present. This justifies previous reports on phenolic acids, flavonoids, and tannins as chemical compounds extracted from medicinal plants (Ansari *et al*., 2013).

Antibacterial Activity of Psidium guajava L. leaves extract

The isolated bacteria and fungus were subjected to antibiotic and antifungal susceptibility testing against 6 antibiotics and 1 antifungal. The susceptibility testing showed *S. aureus* resistant to erythromycin, whereas *E. coli* was sensitive to only cotrimoxazole (Table 2). All bacteria isolates were 100% resistant to amoxicillin. Furthermore, *C. albicans* was resistant to ketoconazole. Antibiotics and antifungal tests are commonly administered antimicrobials by clinicians in Nigeria (Ejike *et al.,* 2017).

Table 2: Susceptibility of Test organisms to Antibiotics and Antifungal agents.

Test bacteria	CPX	STR	AM	CN	ERY	COT	KET
S. aureus	S	S	R	S	R	S	ND
E.coli	R	R	R	R	R	S	ND
C. albicans	ND	ND	ND	ND	ND	ND	R

KEY- S: Susceptible; R: Resistant; ND: Not done; CPX: Ciprofloxacin; STR: Streptomycin; AM: Amoxicillin; CN: Gentamicin; ERY: Erythromycin; COT: Cotrimoxazole; KET: Ketoconazole

In this study, methanol and ethanol extract demonstrated antifungal activity against drug-resistant *C. albicans* with a mean zone of inhibition of 35.5±2.25 mm and 20.0±1.27 mm respectively. For the drug-resistant bacteria, both extracts had no antibacterial activity against the Gram positive (*S. aureus*). The mean ± standard deviation of the zone of inhibition is presented in Table 3. At 100 µL, the methanol extract had a relatively higher antibacterial activity with mean zones of inhibition 28.0±1.56 mm when compared with the ethanol extract mean zones of inhibition 23.0±1.46 mm against drug-resistant *E.coli*. The Gram-negative bacteria, drug-resistant *E.coli* was found to be susceptible to both extracts.

Table 3: Mean diameter (mm) values of inhibition zone of *Psidium guajava* L. extracts

Plant Extracts	*S. aureus*	*E. coli*	*C. albicans*

Ethanol	0.00	23.0±1.46	20.0±1.27
Methanol	0.00	28.0±1.56	35.5±2.25

Three replicates of each test organism per extract were used for this study

A similar susceptibility study of methanol extract of *P. guajava* L. leaf against drug-resistant pathogenic bacteria responsible for urinary tract infection has been reported in India (Gilford *et al*., 2019). In contrast, no antibacterial activity of methanol and ethanol extract of *P. guajava* L. leaf was documented for drug-resistant *E.coli* in the United States of America (Biswas *et al*., 2013) though the test isolates were responsible for food spoilage. Another study on the antibacterial activity of *P. guajava* L. leaf methanol extract on drug-resistant bacteria responsible for urinary tract infection has been reported on carbapenem-resistant *Klebsiella pneumoniae* (Hackam *et al*., 2020). Carbapenem-resistant *K. pneumoniae* are well-known multi-drug resistant bacterium notorious for an array of hospital-acquired infections (Akujobi and Ezeanya, 2013). Interestingly, our study addressed the lack of information on the susceptibility of multi-drug-resistant *E.coli* isolated from urine samples to methanol and ethanol extract of *P. guajava* L. leaf.

The ketoconazole-resistant *C. albicans* were more susceptible to methanol leaf extract. Ketoconazole-resistant *C. albicans* are responsible for infections ranging from superficial infections of the vaginal and oral mucosa to deep-tissue and/or disseminated bloodstream infections with a prevalence of 32% in Nigeria (Ejike *et al*., 2018). This is concordant with previous studies in Brazil (Padron-Marquez *et al*., 2012) attributed this to presence of flavonoids in the extract. Morais-Braga *et al* (2017) reported on the ethnomedicinal value and therapeutic versatilities of *P. guajava* L. for the treatment of *C. albicans*-associated infections.

The resistance demonstrated by Gram-positive bacteria to the extract could be due to the thickness of their cell wall. They are known to have a thick peptidoglycan layer in their rigid cell wall. This could play a role in the impermeability of the bacteria to the plant extracts. Contrarily, there are reports of Gram-negative bacteria demonstrating resistance to plant extracts (Bansode and Chavan, 2014). Their susceptibility to the plant extracts is owed to their thin cell wall-like structure (Akharaiyi *et al*., 2021). The antifungal efficacy of the extracts is seen in the susceptibility of *C. albicans* despite their double-layered cell wall structure.

The presence of different phytochemicals is known to contribute to the antibacterial and antifungal activity of *P. guajava* L. (Sagbo *et al*., 2017). The plant extract was positive for flavonoids and phenols. These phytochemicals have well-known inhibitory properties. Flavonoids are hydroxylated polyphenolic compounds of secondary metabolites by plants in response to stress or microbial infections. They form complexes which could inhibit bacterial cell wall synthesis (Xie *et al*., 2015). Phenols affects the dimorphic transition and inhibit ABC transport proteins in *C. albicans* (Manayi *et al*., 2013). Consequently, the qualitative phytochemical analysis of the methanol and ethanol extract supports the presence of chemical compounds with both antifungal and antibacterial properties, which could contribute to the in vitro susceptibility of the test isolates.

CONCLUSION

P. guajava L. leaf extracts had antifungal and antibacterial activities against drug-resistant *C. albicans* and *E.coli* respectively. These findings substantiate this plant as a natural therapeutics against drug-resistant urinary tract infection and candidiasis thus; could serve as an alternative mode of treatment to the conventional antimicrobials.

CONFLICT OF INTEREST

There are no conflict of interest to declare.

ACKNOWLEDGEMENT

We acknowledge the management of Caleb University, Lagos for the facility for laboratory work.

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