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IN-VITRO ANTIFUNGAL ACTIVITIES OF *PSIDIUM GUAJAVA* AND *ANACARDIUM OCCIDENTALE* LEAVES EXTRACTS AGAINST GASTROINTESTINAL FUNGAL ISOLATES

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Abstract: The present treatment of diarrhea and other gastrointestinal problems has been associated with a number of shortcomings such as antimicrobial resistance, hence increasing the need for developing alternative cost-effective approaches to treatment of diarrhea, one of which being screening of medicinal plants. The in-vitro antifungal activities of *Psidium guajava* (guava) and *Anacardium occidentalis* (cashew) ethanolic leaves extracts against fungal species of the gastrointestinal tract were studied.

Fresh tender leaves ethanolic extracts of *P. guajava* and *A. occidentalis* were prepared using the cold maceration method. Eight (8) fungal species isolated from stool specimens of gastroenteritis patients, and characterized using standard methods, were used for this study. They include: *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Lentinus squarrosulus*, *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida glabrata*. The antimicrobial activities of the plants leaves extracts against the fungal isolates were assayed using the agar-well diffusion method, and results were analyzed statistically using statistical package for social sciences (SPSS) version 23, and comparisons made using one-way Analysis of variance (ANOVA).

Psidium guajava leaves extract gave an inhibition zone diameter (IZD) of 6.50±0.71 mm against both *C. albicans* and *C. glabrata*, but exerted no activity (6.00±0.00) against other isolates (6.00±0.00 is equal to the diameter of the agar-well). *Anacardium occidentalis* leaves extract showed no activity against all isolates (6.00±0.00). All isolates were susceptible to clotrimazole (positive control), but resistant to the plants extracts, and the differences in their activities were statistically significant ($p<0.05$). Amphotericin B also exhibited significantly higher activities than the plants extracts ($p<0.05$).

Sequel to the low antifungal activities exhibited by these plants leaves extracts in this study, it is therefore recommended that further studies be carried out on the antimicrobial (especially bacteria and fungi) and other properties of leaves extracts of *Psidium guajava* and *Anacardium occidentalis* using different extraction solvents, and at varying concentrations, especially against infectious and non infectious gastroenteritis.

Index Terms: Antifungal activities, *Psidium guajava*, *Anacardium occidentale*, gastroenteritis, fungi

1.0 INTRODUCTION

Medicinal plants contain chemical substances that could be used either for direct treatment of infectious and non infectious diseases, or as ingredients for production of useful drugs (Sofowora, 2008). Nigeria has a unique and diverse botanical heritage with over 7,895 plant species of which over 3000 species are used therapeutically (Adeleye *et.al*., 2011). While some of these plants exhibit antimicrobial effect against specific microorganisms, others show broad spectrum action against both Gram positive bacteria, Gram negative bacteria, yeasts and moulds (Okwu and Nnamdi, 2011). Over 400 medicinal plants have been known to possess antidiarrheal properties (Gupta and Birdi, 2015). The use of *Psidium guajava* (Guava) and *Anacardium occidentale* (Cashew) in traditional medicine for gastrointestinal problems has been reported (Gutiérrez *et al*., 2008; Chabi *et al*., 2014). Hence, this study was focused on in-vitro assessment of the antifungal activities of *Psidium guajava* and *Anacardium occidentalis* ethanolic leaves extracts against fungal species of the gastrointestinal tract.

Psidium guajava, popularly known as guava, is a small tree belonging to the myrtle family (Myrtaceae). Native to tropical areas from southern Mexico to northern South America, guava trees have been grown by many other countries having tropical and subtropical climates, thus allowing production around the world (Salazar *et.al*., 2006). Traditionally, preparations of the leaves have been used in folk medicine in several countries, mainly as anti-diarrheal remedy. Depending upon the illness, the application of the remedy is either oral or topical. The consumption of decoction, infusion, and boiled preparations is the most common way to overcome several disorders, such as rheumatism, diarrhea, diabetes mellitus, and cough, in India, China, Pakistan, and Bangladesh, while in Southeast Asia the decoction is used as gargle for mouth ulcers and as anti-bactericidal in Nigeria (Morais-Braga *et.al*., 2016; Sanda *et.al*., 2011). Aqueous and organic extracts of guava leaves have been demonstrated to have antibacterial activity due to an inhibitory effect against antibiotics-resistant clinical isolates of *Staphylococcus aureus* strains (Milyani, 2012). A methanol extract exerted antibacterial effects, preventing the growth of different strains from several bacteria such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus* spp., and *Shigella* spp. (Chah *et.al*., 2006). Furthermore, different extracts of the leaves such as aqueous, acetone–water, methanolic, spray-dried extracts, and the essential oil, showed potential inhibitory activity against Gram-positive and Gram-negative bacteria and fungi (Fernandes *et.al*., 2014).

Anacardium occidentale (Cashew) is an important tropical nut tree that belongs to the family Anacardiaceae, which includes about 75 genera and 700 species among which the well economically known ones are mango and pistachio (Nakasone and Paul, 1998). The leaves and bark of cashew have bactericidal and germicidal activities (Olife *et al*., 2013). They also help to stop dry secretion, increases libido, and reduce fever, blood sugar and pressure (Akash *et al*., 2009). In western Nigeria young leaves are used for arthritis and other inflammatory conditions. In some parts of Nigeria, bark, roots and leaves are traditionally used for the treatment of numerous diseases such as; allergy, cough, stomach ache, diarrhea, skin infections and others (Chabi *et al*., 2014). In the Southeastern part of Nigeria, the leaf extracts is used to bath malaria patients (Ibe and Nwufo, 2005). An infusion of the stem bark and leaves of the plant is used as a remedy for tooth ache and sore gums while the astringent bark is given for severe diarrhea and thrush (Ayepola and Ishola, 2009).

Sequel to these numerous traditional uses of these plants, especially the common use of their tender leaves against stomach ache and diarrhea in most communities, more scientific facts are therefore needed to establish their antimicrobial activities especially against gastrointestinal fungi, so as to better understand their role in treatment of infectious gastroenteritis.

2.0 RESEARCH METHODOLOGY

2.1 Collection and Preparation of plant leaves

Fresh tender leaves of guava and cashew plants used for this study were collected from Ogidi metropolis in Anambra State, Nigeria, and authenticated in the Department of Botany, Nnamdi Azikiwe University Awka, Anambra State, as *Psidium guajava* and *Anacardium occidentalis*, with herbarium numbers, NAUH – 03^{A} and NAUH – 27^{A} respectively.

The leaves were washed with distilled water, and air-dried for seven days under shed, at room temperature. They were ground to powder using a hand milling machine (mechanical grinder), and the powdered samples were stored in an air-tight container. Forty grams (40 g) of the leaves powder was each extracted by cold maceration method in 400 mL of ethanol for 48 h. The extracts were filtered using muslin

cloth and evaporated to dryness at 50 ^{o}C using water bath (Selvamohan and Ramadas, 2012; Anagor *et.al*., 2019).

2.2 Collection and Confirmation of Isolates

Eight (8) fungal species were used for this study, which include: *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Lentinus squarrosulus*, *Candida albicans*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida glabrata*. All isolates were gotten from stool specimens of gastroenteritis patients attending Iyi-Enu mission hospital Ogidi, Anambra state, Nigeria, and confirmed both by macroscopic, microscopic, and molecular methods.

2.3 Antifungal Activities Assay

Sterilized Sabouraud dextrose agar (20 mL) was poured into sterile petri dishes, and allowed to solidify. The entire surface of the solidified culture plates were seeded each with fresh suspension of the fungal isolates adjusted to 0.5 McFarland Standard. Wells of 6.00 mm diameter were aseptically punched on the agar plates, and 30 uL of the crude extracts (reconstituted in dimethyl sulfuroxide) were added to the wells, and allowed to stand for 30 minutes. They were incubated at 37 ^{0}C for 24 h. Positive controls (clotrimazole and amphotericin B) and negative controls (ethanol, distilled water and DMSO) were set up similarly, and incubated. After the incubation period, zones of inhibition for duplicate analyses were measured in millimeters, and recorded as mean± standard deviation. Results were interpreted as sensitive, intermediate or resistant using the National Committee for Clinical Laboratory Standards (NCCLS) interpretative breakpoints as adopted by Magaldi *et al*. (2004).

2.4 Statistical Analysis

All data were analyzed using statistical package for social sciences (SPSS) version 23. Antifungal activities of the plants leaves extracts against each isolate were compared using one-way Analysis of variance (ANOVA).

3.0 RESULTS AND DISCUSSION

3.1 Antifungal activities of leaves extracts of *P. guajava* and *A. occidentalis*

Table 3.1 Antifungal activities of leaves extracts of *P. guajava* and *A. occidentalis*

Extracts	*C. albicans*	*C. parapsilosis*	*C. tropicalis*	*C. glabrata*	*A. niger*	*A. flavus*	*A. fumigatus*	*L. squarrosulus*	p-value
P. guajava	6.50^{R} ±0.71	6.00^{R} ±0.00	6.00^{R} ±0.00	6.50^{R} ±0.71	6.00^{R} ±0.00	6.00^{R} ±0.00	6.00^{R} ±0.00	6.00^{R} ±0.00	0.574
A.occidentalis	6.00^{R} ±0.00	6.00^{R} ±0.00	6.00^{R} ±0.00	6.00^{R} ±0.00	6.00^{R} ±0.00	6.00^{R} ±0.00	6.00^{R} ±0.00	6.00^{R} ±0.00	NA
p-value	0.423	NA	NA	0.423	NA	NA	NA	NA	

Values show mean zone of inhibition (mm) for duplicate analysis ± standard deviation
Keys: R= Resistant; NA= Not Available.
Notes:

- The diameter of agar well is 6.00 mm; hence, zone of inhibition of 6.00±0.00 mm signifies no activity.
- There are no significant p-values both down the columns and across the rows (p>0.05)
- NCCLS interpretive breakpoints for antifungal agents (Magaldi *et al*, 2004) are as follows:
  -Susceptible = ≥19 mm for azoles; ≥15 mm for non azoles
  -Intermediate= 18 – 13 mm for azoles; 14 – 10 mm for non azoles
  -Resistant= ≤12 mm for azoles; ≤9 mm for non azoles

Table 3.1 presents the antifungal activities of leaves extracts of *P. guajava* and *A. occidentalis* against the test isolates. *Psidium guajava* leaves extract gave an inhibition zone diameter (IZD) of 6.50±0.71 mm against both *C. albicans* and *C. glabrata*, but exerted no activity against other isolates (note: 6.00±0.00 = well diameter). *Anacardium occidentalis* leaves extract showed no activity against all isolates. Based on NCCLS interpretive breakpoints, all isolates were resistant to both plant extracts.

Although some authors have recorded the antibacterial and antifungal activities of *P. guajava* and *A. occidentalis* leaves extracts (Akash *et al*., 2009; Fernandes *et al*., 2014; Tafinta *et al*., 2020), Dhiman *et al* (2011) noted that *P. guajava* leaves extract exhibited less inhibition to fungi compared to bacteria; and for *Aspergillus* spp., no activity was found (Nair and Chanda, 2007). This supports the findings of this study, as the plants extracts exhibited no activities against most of the isolates. On the other hand, Tafinta *et al* (2020) recorded higher activity of aqueous leaves extract of *A. occidentalis* at varying concentrations against *A. niger*, than the ethanol extract. Hence, extraction method or solvent, as well as concentration of extract may affect result. This may explain the low activities observed with the crude (undiluted) ethanolic extracts used in this study.

3.2 Antifungal Activity Control Tests

Table 3.2 Antifungal Activity Control Results

Fungal Isolates	Positive Controls		Negative Controls		
	CLO	AMB	Ethanol	Water	DMSO
Candida albicans	30.00^{S}±2.83	8.50^{R}±0.71	6.00^{R}±0.00	6.00^{R}±0.00	6.00^{R}±0.00
Candida parapsilosis	30.00^{S}±0.00	9.00^{R}±0.00	6.00^{R}±0.00	6.00^{R}±0.00	6.00^{R}±0.00
Candida tropicalis	25.00^{S}±2.83	16.00^{S}±1.41	6.00^{R}±0.00	6.00^{R}±0.00	6.00^{R}±0.00
Candida glabrata	30.00^{S}±1.41	10.00^{I}±1.41	6.00^{R}±0.00	6.00^{R}±0.00	6.00^{R}±0.00
Aspergillus niger	29.00^{S}±1.41	10.00^{I}±0.00	6.00^{R}±0.00	6.00^{R}±0.00	6.00^{R}±0.00
Aspergillus flavus	30.00^{S}±0.00	10.00^{I}±0.00	6.00^{R}±0.00	6.00^{R}±0.00	6.00^{R}±0.00
Aspergillus fumigatus	38.00^{S}±2.83	15.00^{S}±1.41	6.00^{R}±0.00	6.00^{R}±0.00	6.00^{R}±0.00
Lentinus squarrosulus	38.00^{S}±0.00	18.50^{S}±2.12	6.00^{R}±0.00	6.00^{R}±0.00	6.00^{R}±0.00

Values show mean zone of inhibition (mm) for duplicate analysis ± standard deviation
Keys: CLO= Clotrimazole; AMB= Amphotericin B; DMSO = Dimethyl sulphoxide; S= Susceptible; I= Intermediate; R= Resistant;
Notes: The diameter of agar well is 6.00 mm; hence, zone of inhibition of 6.00 mm signifies no activity.
NCCLS interpretive breakpoints for antifungal agents (Magaldi *et al*, 2004) are as follows:
-Susceptible = ≥19 mm for azoles; ≥15 mm for non azoles
-Intermediate= 18 – 13 mm for azoles; 14 – 10 mm for non azoles
-Resistant= ≤12 mm for azoles; ≤9 mm for non azoles

Table 3.2 represents the results of the controls used for the study. The positive controls, clotrimazole and amphotericin B showed highest IZD of 38.00±2.83 and 18.50±2.12 against *Aspergillus fumigatus and Lentinus squarrosulus* respectively. All negative controls exerted no activities (note: 6.00±0.00 = well diameter) against all the isolates.

3.3 Comparison of Antifungal activities of the plants extracts with clotrimazole control

Table 3.3 Comparison of Antifungal activities of the plants extracts and clotrimazole control

Fungal Isolates	Clotrimazole	*P. guajava*	*A. occidentalis*	p-value
Candida albicans	30.00^{S}±2.83	6.50^{R}±0.71	6.00^{R}±0.00	0.001
C. parapsilosis	30.00^{S}±0.00	6.00^{R}±0.00	6.00^{R}±0.00	-
Candida tropicalis	25.00^{S}±2.83	6.00^{R}±0.00	6.00^{R}±0.00	0.002
Candida glabrata	30.00^{S}±1.41	6.50^{R}±0.71	6.00^{R}±0.00	0.001
Aspergillus niger	29.00^{S}±1.41	6.00^{R}±0.00	6.00^{R}±0.00	0.001
Aspergillus flavus	30.00^{S}±0.00	6.00^{R}±0.00	6.00^{R}±0.00	-
A. fumigatus	38.00^{S}±2.83	6.00^{R}±0.00	6.00^{R}±0.00	0.001
L. squarrosulus	38.00^{S}±0.00	6.00^{R}±0.00	6.00^{R}±0.00	-

Values show mean zone of inhibition (mm) for duplicate analysis ± standard deviation
Note: NCCLS interpretive breakpoints for antifungal agents (Magaldi *et al*, 2004) are as follows:
-Susceptible (S) = ≥19 mm for azoles; ≥15 mm for non azoles
-Intermediate (I) = 18 – 13 mm for azoles; 14 – 10 mm for non azoles
-Resistant (R) = ≤12 mm for azoles; ≤9 mm for non azoles
* p values are significant ($p<0.05$)

Table 3.3 Compares the antifungal activities of the plants extracts with clotrimazole control. All isolates were susceptible to clotrimazole but resistant to the plant extracts, and the differences were statistically significant ($p<0.05$).

3.4 Comparison of Antifungal activities of the plants extracts with amphotericin B control

Table 3.4 Comparison of Antifungal activities of the plants extracts and amphotericin B control

Fungal Isolates	Amphotericin B	*P. guajava*	*A. occidentalis*	p-value
Candida albicans	8.50^{R}±0.71	6.50^{R}±0.71	6.00^{R}±0.00	0.044
C. parapsilosis	9.00^{R}±0.00	6.00^{R}±0.00	6.00^{R}±0.00	-
Candida tropicalis	16.00^{S}±1.41	6.00^{R}±0.00	6.00^{R}±0.00	0.002
Candida glabrata	10.00^{I}±1.41	6.50^{R}±0.71	6.00^{R}±0.00	0.033
Aspergillus niger	10.00^{I}±0.00	6.00^{R}±0.00	6.00^{R}±0.00	-
Aspergillus flavus	10.00^{I}±0.00	6.00^{R}±0.00	6.00^{R}±0.00	-
A. fumigatus	15.00^{S}±1.41	6.00^{R}±0.00	6.00^{R}±0.00	0.002
L. squarrosulus	18.50^{S}±2.12	6.00^{R}±0.00	6.00^{R}±0.00	0.003

Values show mean zone of inhibition (mm) for duplicate analysis ± standard deviation
Note: NCCLS interpretive breakpoints for antifungal agents (Magaldi *et al*, 2004) are as follows:
-Susceptible (S) = ≥19 mm for azoles; ≥15 mm for non azoles
-Intermediate (I) = 18 – 13 mm for azoles; 14 – 10 mm for non azoles
-Resistant (R) = ≤12 mm for azoles; ≤9 mm for non azoles
* p values are significant ($p<0.05$)

Table 3.4 Compares the antifungal activities of the plants extracts with amphotericin B control. Although *Candida albicans* and *Candida parapsilosis* were resistant to amphotericin B as they also were to the plant extracts, there were significant differences in the activities of amphotericin B and plant extracts against the fungal isolates.

The commercial antifungal agents (clotrimazole and amphotericin B) used as positive controls, exhibited significantly higher activities against the fungal isolates than both plants leaves extracts, with clotrimazole showing the highest activities; hence, all isolates were susceptible to clotrimazole. Several antifungal agents have been in use for the treatment of fungal infections, including the azoles such as clotrimazole, and the non azoles such as amphotericin B (Benitez *et al*., 2019). Most antifungal drugs interfere with biosynthesis or integrity of ergosterol, the major sterol in the fungal cell membrane. Others cause disruption of the fungal cell wall (Chen and Sorrell, 2007). Azoles are the most widely used antifungal drugs due to their high activities, as also observed in this study. They act primarily by inhibiting the fungal cytochrome P450 enzyme, 14α-demethylase. Resistance to several antifungal agents is increasing, especially among *Candida* species and *Aspergillus* species (Pinto *et al*., 2018) as also observed in this study, with most of the isolates showing resistance to the non azole amphotericin B and plant extracts.

4.0 CONCLUSIONS

Crude ethanolic leaves extract of *Psidium guajava* (Guava) and *Anacardium occidentalis* (Cashew) exhibited generally very low antimicrobial activities against selected gastrointestinal fungal isolates used in this study, especially when compared with commercially available antifungal agents.

This outcome may suggest that the common traditional use of the tender leaves of these plants in treating gastrointestinal problems, such as stomach ache and diarrhea, may be a function of their antimicrobial activities against bacteria or viral-caused gastroenteritis, or other properties against non infectious gastroenteritis, and not necessarily against fungi.

Therefore, we recommend further studies on the antimicrobial (especially bacteria and fungi) and other properties of leaves extracts of *Psidium guajava* and *Anacardium occidentalis* using different extraction solvents, and at varying concentrations, especially against infectious and non infectious gastroenteritis.

LIST OF ABBREVIATIONS

DMSO: Dimethyl sulfuroxide
NCCLS: National Committee for Clinical Laboratory Standards
SPSS: Statistical package for social sciences
ANOVA: Analysis of variance
IZD: Inhibition zone diameter

DECLARATIONS

Ethics approval and consent to participate

Ethical approval was obtained from the Health Research and Ethics Committee, Chukwuemeka Odumegwu Ojukwu University Teaching Hospital (COOUTH) Amaku Awka, Anambra state, Nigeria, with reference number, COOUTH/CMAC/ETH.C/Vol.1/FN: 04/270.

Consent for publication

Not applicable

Availability of data and materials

The data generated during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CUU contributed to the conception and design of the work. CMO performed the laboratory work, generated, analyzed and interpreted the data, and drafted the manuscript. ISA revised the manuscript substantively. All the authors read and approved the final manuscript before submission.

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