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Investigation of the antibacterial activities of methanolic and water extracts of *Gongronema latifolium*, *Psidium guajava* and *Aspilia africana*

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Abstract

The epidemiological consequences of co-infections by enteric, wound, opportunistic, and drug-resistant pathogens, underscore the need for alternative and multi-target therapeutic approaches. Phytochemical properties of some plants are widely known but their potential utility as cheaper multi-target therapeutic options, have rarely been investigated. In the current work, *Gongronema latifolium*, *Psidium guajava*, and *Aspilia africana* extracts were studied for their potential utility as multi-target therapeutic alternatives. The plants were screened for phytochemical constituents. Based on the CLSI disc diffusion assay, the sensitivities of *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* to the plants' extracts were tested. The minimum inhibitory concentration of the extracts against susceptible bacteria was determined by CLSI broth microdilution protocols. The major families of plant bioactive compounds were detected, with alkaloids, terpenoids, and flavonoids common to the three plants investigated. The plant extracts showed variable activity against the test bacteria (activity index, AI range = 0.43 – 2.59), while the highest activity based on the MIC was recorded for *P. guajava* (MIC range = 25- 100mg/ml). These results provide the basis of the plants' therapeutic uses in folk medicine. Their activity index suggests the need for further investigation of their phytochemical components for potential medicinal application. The *P. guajava* methanolic extract with consistent activity across the test bacteria suggests its potentials in the formulation of multi-target antibiotic therapies.

Keywords: *Gongronema latifolium*, *Psidium guajava*, *Aspilia africana*, drug-resistance, co-infection, phytochemical.

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INTRODUCTION

The enteric bacteria including *Enterococcus faecalis*, *Escherichia coli*, and *Klebsiella pneumoniae* constitute important components of the human intestinal microbiota, which ecological niches in the gastrointestinal tract (GIT) are not often detrimental to the host (Koboziev *et al.*, 2014). However, given that some of the commensal organisms in the GIT are opportunistic pathogens and pathobionts, exposure to distressed microenvironmental conditions such as antibiotic stress often precipitates increased virulence (Poole, 2012; Khatoun *et al.*, 2018). Also, nosocomial infections by *E. faecalis* and *Pseudomonas aeruginosa* could pose a significant threat in the clinical setting due to their increased potential to form biofilm on both biotic and abiotic surfaces including the infection of vulnerable patients and medical devices respectively (Khatoun *et al.*, 2018).

Since the past decades, there has been an upward trend in the prevalence of immunosuppressive conditions (Thibault *et al.*, 2016; Arem and Lofffield, 2017; Magliano *et al.*, 2019.). This was further complicated with the increased emergence of antibiotic-resistant bacterial strains, and evidently, this ugly trend has remained unmitigated with improved therapeutic approaches (Ventola, 2015). Also, targeting a pathogenic enteric species in a case of mixed infection could enhance survival of the co-infecting bacteria, which proliferation in the intestine potentially worsens the treatment outcome of gastrointestinal disorders or enterotoxicity (Kim *et al.*, 2017). Plants can present sufficient natural antimicrobial compounds, and their medicinal potentials have historically been utilized in some edible plants as extra-nutritional values (Chidebelu *et al.*, 2019). *Psidium guajava* is a plant which its fruit, both ripe and unripe, are taken as nutraceutical for general health improvement including as an external source of ascorbic acid, and cardiovascular health (Gutiérrez *et al.*, 2008). The plant's leaf, bark, and root decoctions or infusions have traditionally been used for the treatment of microbial, parasitic, and viral infections, gastrointestinal diseases, blood stream and circulatory infections, hormonal and metabolic disorders, and as antitumor agent (Gutiérrez *et al.*, 2008; Diaz-de-Cerio *et al.*, 2017). In addition to its antigenotoxic, hepatoprotective, and antimutagenic therapeutic uses, *P. guajava* has also been shown to function as anti-plaque and anti-biofilm agent, thereby making it potentially

applicable in the treatment of periodontal diseases (Ravi and Divyashree, 2014), and other biofilm-induced resistance infections. Extracts of *A. africana* are popular for their wound healing effects and based on its anti-bleeding quality, the plant has been metaphorically described as haemorrhage plant (Akuodor *et al.*, 2012). Notably, the wound healing effect was related to the combinatorial actions of the component antimicrobial, anti-inflammatory, and antioxidant pharmacological properties of the plant (Komakech *et al.*, 2019). Earlier study (Okoli *et al.*, 2007), had shown that the plant's ability to block bleeding in fresh wounds is largely enhanced by the terpenoids and tannin components which mediate anti-inflammatory and haemostatic activities respectively. *Gongrenema latifolium* is an edible herbaceous shrub commonly used in many parts of Nigeria as vegetable and drug for management of certain health conditions and has been described as a good candidate for production of anti-diabetic drugs based on the presence of minerals and essential oils, and other phytochemical components, which were identified as mediators of the plant's hypoglycemic activity according to an *in vivo* study (Chime *et al.*, 2014). Modulation of specific gene expressions resulting in hypoglycemia was evident after *in vivo* administration of the plant's aqueous leaf extracts in rats (Ajiboye *et al.*, 2019).

Although bioactive compounds are usually extracted with different kinds of polar organic and aqueous solvents, the choice of a suitable solvent is paramount for the optimal recovery of these compounds. For some plants, and depending on the relative polarity of the solute, high polar solvents appear optimal, and for others, weak polar solvents are efficient (Truong *et al.*, 2019). Comparable extraction efficiency is also obtainable between solvents like methanol and water because of their close polarities (Silva *et al.*, 2016; Altemimi *et al.*, 2017). Most works on these plants had little focus on the link between extraction solvent and antimicrobial efficiency. Also, the selection of test microbial species was rarely based on their common ecological sites within the host, as well as their clinical prevalence. There is a need for multi-target therapeutic options for the control of infections due to enteric, wound, opportunistic, and antibiotic-resistant pathogens. In this study, the aqueous and methanolic leaf extracts of *G. latifolium*, *P. guajava*, and *A. africana* were compared for *in vitro* antimicrobial efficiencies.

MATERIALS AND METHODS

Preparation of plant samples

All the plant samples were collected within Nsukka (6°51'24"N;7°23'45"E) Enugu State, Nigeria. The leaves of *G. latifolium* were bought from sellers at Ogige local market, Nsukka while the leaves of *Aspilia africana* were collected from farmland at Ibagwa Nsukka. Also, *Psidium guajava* leaves were collected from the University of Nigeria, Botanical Garden. The plants were taken in sealed plastic baskets for identification by a plant taxonomist, in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

Plant Extraction

The collected plant materials were washed with clean tap waters and air-dried at room temperature. Dried and pulverised leaves of the plants under study were soaked in respective methanol and water solvents to a 20% concentration (g/vol), at 25°C for a maximum of 1 week, based on the method described by Truong *et al* (2019). Each plant's preparation was filtered using Whatman filter paper (no 1), and the resulting filtrates were then concentrated in a rotary vacuum evaporator at 45°C. The resulting crude methanolic and aqueous leaf extracts were then collected in sterile capped bottles and preserved at refrigeration temperature, ready for use.

Phytochemical Analysis

Phytochemical components of the plant's crude extracts were analysed qualitatively, and quantitatively. Briefly, each of the phytochemical components were tested qualitatively based on specific chemical reactions to determine the presence of the plants' bioactive compounds as described by Biswas *et al.* (2013), while the quantification of the components involved application of chemical, heating, and spectrophotometric procedures (Gupta *et al.*, 2013).

The test Bacterial strains

Stock cultures of *Escherichia coli* (NCTC13353), *Klebsiella pneumoniae* (NCTC13368), *Enterococcus faecalis* (NCTC12697), and *Pseudomonas aeruginosa* (ATCC27853), obtained from Water and Public

Health Research Laboratory, University of Nigeria, Nsukka, were rejuvenated by streaking on respective nutrient agar plates and incubated for 24 hours at 37°C. Pure colonies on each plate were inoculated into sterile normal saline test tubes and adjusted to 0.5 McFarland standard.

Antimicrobial Susceptibility

Dissolution of the crude methanolic and aqueous plant extracts was made in 10% DMSO to achieve a stock concentration of 200mg /ml. From the stock solution, a separate dilution was made to obtain each of the working concentrations of 150, 100, 50, and 25 (mg/ml). Sterile filter paper discs (6mm) were then aseptically impregnated with the varying extract concentrations. Based on the CLSI disc diffusion method (CLSI, 2007), Mueller Hinton agar plates were inoculated with respective standardized bacterial cultures using sterile swab sticks. The plant extracts impregnated discs were then placed on the inoculated plates in duplicates, and incubated for about 18 hours at 37°C. The minimum inhibitory concentration was determined by broth microdilution method. The standard (0.5 MacFarland) cultures were adjusted to 1.0×10^6 cfu/ml. Into Eppendorf tubes containing 0.1ml concentration gradients of the plants extracts, equal volumes of the inocula were introduced. Tetracycline antibiotic disc (30µg) and sterile water were respectively used as positive and negative controls, for the disc diffusion test, while uninoculated broth with test extract and extract-free inoculated broth served as positive and negative controls respectively for the broth microdilution assay. After about 18 hours of incubation at 37°C, the results were read and recorded. The activity index of the extracts was evaluated as:

Activity index (AI) =

Inhibition zone diameter of the extract

Inhibition zone diameter of tetracycline

Where: AI = 0: no activity; < 0.1 - 0.49: low activity; 0.5 – 0.79: moderate activity; >0.8: high activity.

Statistical analysis

The quantitative phytochemical screening results were presented in tables as percent (%) concentration of the bioactive compounds. Inhibition zone diameters were expressed as standard errors of the mean values. The antimicrobial activities of *P. guajava* methanolic and aqueous extracts were compared based on unpaired t-test, with the statistical significance evaluated at $p < 0.05$.

RESULTS

The highest alkaloid and flavonoid contents were found in *A. Africana* and *G. latifolium*, respectively. Although terpenoids, alkaloids, and flavonoids are common to all the plants investigated, only *P. guajava* and *A. africana* are positive for tannins. While only *G. latifolium* and *A. africana* were positive for saponins, only *P. guajava* was positive for glycosides. The present study shows that these plants are rich reservoirs of bioactive compounds such as alkaloids, glycosides, terpenoids, tannins, saponins, flavonoids, and steroids (Table 1).

Inhibition zone diameter was not observed in all the bacteria tested with the aqueous extract of *A. africana*, and except for its low activity against *P. aeruginosa* (AI = 0.43), no other bacteria was sensitive to the plant's methanolic extract (Table 2). Only one of the organisms (*E.*

coli) was sensitive to the *G. latifolium* methanolic extract, on the other hand, its aqueous extract showed activity against 50% of the test bacteria (Table 2). Evidence from the activity index indicated high activity of aqueous extracts of *G. latifolium* against *E. faecalis* (AI = 1.07) and *K. pneumoniae* (AI = 2.59), while its methanolic extract also had moderate activity (AI = 0.45) against *E. coli* (Table 2). Relatively, *P. guajava* extracts activities were consistent across the test bacteria. While the aqueous extracts of *P. guajava* were active against 50% of the test bacteria, all the test bacteria (100%) were sensitive to the plant's methanolic extract (Table 3). The minimum inhibitory concentrations, MIC, (mg/ml), of the extracts were determined for the sensitive bacteria following the disc diffusion test (Table 4). The MIC of *G. latifolium* aqueous extracts could not be determined against *E. faecalis* and *K. pneumoniae* at the highest extract concentration (200mg/ml). Although both *G. latifolium* and *A. africana* methanolic extracts MIC was 100mg/ml against *E. coli* and *P. aeruginosa* respectively, only the *P. guajava* methanolic extract MIC was determined for all the test bacteria (MIC range, 25 - 100mg/ml), with *E. faecalis* and *P. aeruginosa* the most sensitive (25mg/ml). The MIC of the aqueous extracts of *P. guajava* was recorded against *E. coli* (100mg/ml) and *P. aeruginosa* (50mg/ml). There was however, no significant difference in activity between its aqueous and methanolic extracts (unpaired t -test, $p = 0.1340$).

Table 1: Quantitative phytochemical contents of the medicinal plants

Plants	Alkaloids (%)	Flavonoids (%)	Terpenoids (%)	Tannins (%)	Glycosides (%)	Saponins (%)	Steroids (%)
<i>Gongronema latifolium</i>	3.5	10.0	5.5	ND	ND	12.0	4.5
<i>Psidium guajava</i>	5.5	9.0	7.0	0.018	2.05	ND	ND
<i>Aspilia africana</i>	7.0	9.0	7.0	0.02	ND	2.0	ND

Key: ND = Not detected.

Table 2: Diameter of Zone of inhibition (mm) of the aqueous and methanolic extracts of *A. africana* and *G. latifolium*

Extract	Organism	Mean IZD Tet	Mean IZD aq	AI aq	Mean IZD Met	AI Met
<i>Aspilia africana</i>	<i>E. coli</i>	31.00 ± 0.20	ND	ND	ND	ND
	<i>E. faecalis</i>	30.00 ± 0.00	ND	ND	ND	ND
	<i>K. pneumoniae</i>	11.00 ± 0.00	ND	ND	ND	ND
	<i>P. aeruginosa</i>	23.00 ± 0.00	ND	ND	10.00 ± 2.00	0.43
<i>Gongronema latifolium</i>	<i>E. coli</i>	25.00 ± 0.00	ND	ND	11.25 ± 1.11	0.45
	<i>E. faecalis</i>	30.00 ± 0.00	32.50 ± 3.50	1.07	ND	ND
	<i>K. pneumoniae</i>	11.00 ± 0.00	28.50 ± 4.50	2.59	ND	ND
	<i>P. aeruginosa</i>	23.00 ± 0.00	ND	ND	ND	ND

Key: Tet = tetracycline, aq= aqueous, met= methanol, AI= activity index, ND = no inhibition detected.

Table 3: Antimicrobial activities (Inhibition zone diameter in mm) of the aqueous and methanolic extracts of *Psidium guajava*.

Organism	Mean IZD Tet	Mean IZD aq	AI aq	Mean IZD Met	AI Met
<i>Escherichia. coli</i>	23.50 ± 1.50	11.00 ± 1.00	0.47	15.25 ± 2.14	0.65
<i>Enterococcus faecalis</i>	30.00 ± 0.00	ND	ND	17.75 ± 2.32	0.59
<i>Klebsiella pneumoniae</i>	22.50 ± 2.50	ND	ND	11.50 ± 1.04	0.51
<i>Pseudomonas aeruginosa</i>	24.50 ± 1.50	15.00 ± 3.00	0.61	17.25 ± 2.56	0.70

Key: Tet = tetracycline, aq= aqueous, met= methanol, AI= activity index, ND = no inhibition detected.

Table 4: Minimum inhibitory concentrations (mg/ml) of the plant extracts

Type of extract	Plant name	<i>E. coli</i>	<i>E. faecalis</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>
Aqueous	<i>Gongronema latifolium</i>	NT	>200	>200	NT
	<i>Psidium guajava</i>	100	NT	NT	50
Methanol	<i>Gongronema latifolium</i>	100	NT	NT	NT
	<i>Psidium guajava</i>	50	25	50	25
	<i>Aspilia africana</i>	NT	NT	NT	100

Key: >200= not detected at the highest test extract concentration (200mg/ml); NT = not tested.

DISCUSSION

The evidential importance of medicinal plants as natural sources of antimicrobial compounds remains inexhaustible. The present observation demonstrated the antibacterial potency of the plants and their relevance in the traditional medicine. Among the extracts, *A. africana*'s methanolic extract was the most active against *P. aeruginosa*. Considering the etiologic roles of *P. aeruginosa* in the wound and biofilm-associated infections, which risk especially increases with the prolonged use of in dwelling prosthetic and catheter devices (Dosler and Kraaslan 2014; Sharma *et al.*, 2014; Obioma *et al.*, 2017), the result suggests prospect of *A. africana*-based formulations for wound and resistance infection treatment. A similar observation of the high activity of the plant against *P. aeruginosa* was also, made in another study (Ezeigbo *et al.*, 2016). Unlike the methanolic extract with good antibacterial activity, the effect of the aqueous extract of *A. Africana* is scantily evident, as found in the present study, and as reported earlier (Obioma *et al.*, 2017). No activity was recorded when the aqueous extract was used against the test bacteria. This outcome contradicts the usually observed increase of activity with polarity (Kidaha *et al.*, 2013) and may reflect variable solubility of some active compounds in different solvents (Adeleye *et al.*, 2011).

The effect of methanolic extract of *P. guajava* was higher than its aqueous counterpart. There are corroborating reports on the high antibacterial activity especially, of the plant's methanolic extract (Bisi-Johnson *et al.*, 2017; Naseer *et al.*, 2018). Although only one Gram-positive bacterium was included for the susceptibility study, the *P. guajava* extract showed comparable activity against both the gram positive and Gram-negative bacteria. This was also indicated in a similar recent report (Patel *et al.*, 2019). In another related study, the methanolic leaf extracts of the plant were more effective against gram positive bacteria (Biswas *et al.*, 2013). Similarly, *P. guajava*'s activity against gram negative bacteria especially the multidrug-resistant enteric bacteria were demonstrated by Bisi – Johnson *et al.* (2017), using the plant's acetone extract. Due to its good activity against *E. coli* in the present report, *P. guajava* could be relevant in the control of food-borne diarrheagenic pathogens thereby ensuring food safety (Ibrahim *et al.*, 2011; Farhana *et al.*, 2017), besides its potential clinical application in the treatment of enteric bacterial infections. In folk medicine, *P. guajava* is used for maintaining oral hygiene

and other health benefits (Kabir *et al.*, 2017), and thus, following the current observation, the combination of its high antibacterial activity and high anti-plaque mediating flavonoid concentration (Naseer *et al.*, 2018), could specifically result in interference with the biofilm formation activity of *P. aeruginosa* in the oral cavity. In this study, the MIC result also, indicated that both the *P. guajava* methanolic and aqueous extracts inhibited at least 50% of the organisms. Specifically, its methanolic extract which inhibited all the bacteria at the test concentration range (25 – 100mg/ml) suggested its potential use as multi-target therapy against wound, enteric, and multidrug-resistant infections. Similarly, its activity against Gram-negative bacteria presented in this study could provide further evidence for its broad-spectrum antibacterial activity (Dhiaman *et al.*, 2011; Naseer *et al.*, 2018), and complements other reports on its high activity against Gram-positive bacteria (Biswas *et al.*, 2013; Anand *et al.*, 2016).

There is still little information on the antimicrobial activity of methanolic extract of *G. latifolium*. Although the aqueous extract showed a good inhibitory effect against *E. faecalis* and *K. pneumoniae*, its minimum inhibitory concentration could not be determined against the organisms at the highest concentration tested (200mg/ml). However, the methanolic extract activity against *E. coli* was evident at 100mg/ml MIC. In concordance with the current observation, an earlier finding associated this potential with saponin fractions present in the methanolic extract of the plant (Morebise, 2015). The lower activity of its aqueous leaf extract is a very common observation when compared with the methanolic leaf extract (Eleyinmi, 2007). Minimal or absence of activity of the aqueous extract against some test strains as presented, was also, reported in other studies (Nwinyi *et al.*, 2008; Nduche *et al.*, 2018). Investigating the variation in bioactivity potentials of some plants, a possible association between secretion of phytochemical components which drives bioactivity, and geographical differences in soil properties and other natural environmental factors affecting plant growth including seasonal variations was deducible (Anyanwu and Okoye, 2017).

Analytical procedures suggest that antimicrobial activities of medicinal plants are the physiological functions of their phytochemical constituents. Identification and quantification of these bioactive components as reported in the current work authenticated the

reliability of the applied methods. The phytochemical components common to the extracts include alkaloids, flavonoids, and terpenoids. These represent the major family of plant secondary metabolites (Forni *et al.*, 2019). Due to their ability to modulate antibiotic resistance, some metabolites like alkaloids are the model structure for most synthetic antibiotics' analogues used in the treatment of human and animal infections (Othman *et al.*, 2019). The present observation showed that all the investigated plants possess alkaloids which may have provided the bases for their antimicrobial effects. Similarly, both terpenoids and flavonoids are common to all the extracts. Following their anti-quorum sensing effects, which reportedly suppress the development of multidrug-resistance by pathogens, terpenoids could enhance the effectiveness of the extracts especially against the test bacteria associated with wound infections (Mahizan *et al.*, 2019). Flavonoid's antibacterial mechanisms consist in nucleic acid synthesis inhibition, disruption of biofilm formation and cell attachment, interference with the bacterial energy metabolism, etc.

CONCLUSION

The bioactivity of *P. guajava*, *A. africana*, and *G. latifolium* presented in this study provided conventional bases and insights on their therapeutic uses in folk medicine for treatment of bacterial infection. Their activity index against the test bacteria could serve as a guide to further investigation of their phytochemical components for potential medicinal applications. Although the three plants demonstrated relatively good activity against the pathogens investigated, the *P. guajava* methanolic extract specifically, showed consistent antimicrobial effects, and thus, suggests its potential use in the formulation of multi-target antibiotic therapies. More studies are therefore recommended especially on the chemical structures and functions of the plants' phytochemical components.

Conflict of Interest

The authors have no conflict of interest to declare.

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