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Phytochemical and antibacterial properties of *Diodia scandens* and *Phyllanthus amarus* on staphylococci isolated from patients in tertiary hospitals in Nigeria

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Background: The rapidly growing use of herbal drugs or supplements in complementary and alternative medicine as substitute for orthodox medicine both in developed and developing countries is fast gaining ground.

Aim: This study evaluated both qualitative and quantitative phytochemical constituents of *Diodia scandens* and *Phyllanthus amarus* vis-à-vis their synergistic effects on clinically isolated staphylococci.

Methods: A total of 200 wounds and burns samples were obtained from patients in the accident and emergency unit of different tertiary hospitals. Staphylococci were isolated and characterised using standard microbiological procedures. Whole plants of *D. scandens* and *P. amarus* were Soxhlet extracted with absolute ethanol. The phytochemical analysis was carried out using standard methods. Also, the minimum inhibitory concentration and bactericidal effect of the combined extracts were determined.

Results: The phytochemicals present in *D. scandens* include saponin (6.58%), tannin (2.27 mg/100g), alkaloids (10.53%) and phytin phosphorus (1.80 mg/g), while phytochemicals in *P. amarus* include saponin (9.99%), tannin (5.82 mg/100g), alkaloids (9.67%) and phytin phosphorus (2.44 mg/g), revealing their antibacterial properties and phytonutrients. The combination study showed that a synergistic effect exists between the two plants on the isolates tested compared with individual extracts alone at the concentrations used.

Conclusion: It is noteworthy that the traditional use of these plants was not only confirmed but the combination of *D. scandens* and *P. amarus* also proved more effective as antibacterial agent compared with a previous study on the same plants using single determination.

Introduction

The Current and universally recognised effective health care delivery includes primary, secondary and tertiary health care systems. The Primary health care recognises the importance of alternative, complementary and traditional medicines both in developed and developing countries (WHO 2003).

Diversity, flexibility, easy accessibility, broad continuing acceptance in developing countries and increasing popularity in developed countries, relative low cost, low levels of technological input, relative low side effects and growing economic importance are some of the positive features of traditional medicine (WHO 2002).

Herbal medicine is gradually gaining ground especially on the African continent because of recurrent antibiotic-resistant strains of bacteria that are prevalent within the hospital environment and the community owing to cross-infection (Ojo, Ogodo & Esumeh 2013a). The primary benefits of using plant-derived medicines are that they are safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment with minimal or no side effects in the treatment of infectious diseases (Robbers, Speedie & Tyler 1996).

Diodia scandens belong to the family Rubiaceae, a straggling herb with slender angular stems of 1–3 m in length and scabrid leaves. It is commonly dispersed in tropical Africa, tropical Asia and America. Studies showed its use as a vermifuge for children, in treating pregnant women and as an afterbirth treatment to clear the womb, antidotes (venomous stings, bites, etc.), as a pain killer, in treating venereal diseases, arthritis, rheumatism, cutaneous and subcutaneous parasitic

infection, diarrhoea and dysentery, anti-aborificant treatment of dropsy, swellings, oedema and gout and as a lactation stimulant (including veterinary). The sap of the plant is also used for ear treatment, paralysis, epilepsy, convulsions, spasm, pulmonary troubles, wounds, eczema and ring worm (Ojo et al. 2010).

Phyllanthus amarus (Schum & Thonn) is a herb belonging to the family Euphorbiaceae and is mostly used in central and southern India, China, Philippines, Cuba, Nigeria and Guam. Its traditional uses range from the treatment of malaria-related symptoms, jaundice, constipation, diarrhoea, diabetes, flu, kidney ailments, chronic dysentery, frequent menstruation, ringworm, ulcers, genito-urinary tract infections, haemorrhoids, gonorrhoea, hepatic and urolitic diseases, nervous debility, epilepsy and dropsy. It has been reported to possess antiviral, anti-cancer, anti-tumour, antioxidant, anti-inflammatory and diuretic activity (Hanumanthacar & Milind 2007; Lim & Murtijaya 2007; Oluwafemi & Debiri, 2008).

Plants offer a large range of natural compounds belonging to different classes of phytochemicals. These molecules possess interesting biological activities which have attracted several researchers to their elucidation to provide knowledge that will lead to advancement in medicine (Zabri et al. 2008). The phytochemical analysis of plants has revealed various bioactive ingredients and other phytonutrients such as saponin, alkaloid, tannin, phytin phosphorus, polyphenol (flavonoids), phytate, oxalate and others in varying quantities, thus conferring antimicrobial properties on plants and producing definite physiological action on the human body. Most of the phytochemicals are rich in antioxidant activity. Studies have shown that many of these compounds possess anti-inflammatory, anti-atherosclerotic, anti-tumour, anti-mutagenic, anti-carcinogenic, antibacterial, and antiviral activities (Cowan 1999; De Britto, Gracelin & Rathna Kumar 2013).

Traditional healing practitioners are known to combine different medicinal plants in the treatment of infectious diseases and physiological sickness, thus increasing the efficacy or potency of the herbal decoction. This, however, implies that synergistic interaction exists between or among the various medicinal plants employed in patients' treatment.

D. scandens and *P. amarus* showed great potential as antibacterial agents and their rate of killing on multidrug-resistant staphylococci has been previously reported with continuous average logarithm reduction in viable cell count at 6-hour time interval (Ojo, Ejims-Enukwe & Esumeh 2013b).

This study was then designed to elucidate the essential bioavailable constituents present in *D. scandens* and *P. amarus* and the possibility of their synergism against clinical isolates (staphylococci), which could be incorporated into staphylococcal wound treatment in orthodox medicine.

Materials and methods

Study site

Fresh whole plants of *D. scandens* and *P. amarus* were harvested from Novena University, Ogume (Amai campus), Delta State, and its environs and subsequently identified at the Herbarium section of Delta State University, Abraka, Delta State, Nigeria.

Collection of clinical isolates

A total of 200 samples from infected sites of wounds and burns of patients were obtained from different tertiary hospitals within Delta State with sterile cotton swabs and transported to the laboratory. The cotton swabs were applied to freshly prepared slants of nutrient agar and mannitol salt agar (oxoid) and were incubated at 37°C for 24 h.

Isolation and identification

Colonies growing on slants were streaked on top of freshly prepared plates of mannitol salt agar and incubated again at 35°C. Primary characterisation of isolates was based on Gram stain, morphological and cultural characteristics, growth on nutrient agar and DNase agar and fermentation on mannitol salt agar, catalase and coagulase tests and other biochemical tests. β-lactamase assay was performed using the method as described by Ako-Nai et al. (2005).

β-lactamase assay

Strips of starch paper measuring 4 cm × 7 cm were cut and sterilised with 70% ethanol. These were then soaked for 10 min in a solution of 0.1 g benzyl penicillin dissolved in a sufficient amount of phosphate buffer (pH 7.0, 0.067 mol/L) to make up 100 mL. They were spread evenly onto sterile Petri dishes. A culture of 24-h-old test isolates grown on nutrient agar was then inoculated on the surface of the paper and spread over an area of 2–3 mm. Each test paper was then used to test the staphylococcal isolates at a time with the inocula placed at least 1.5 cm apart. The Petri dishes were then inoculated for 30 min at 37°C, after which the plate was flooded with Gram's iodine solution, which was immediately drained off. This caused the starch paper to turn uniformly black within 30 s of application. Colonies with decolourised zones were indicative of β-lactamase production. Results were read within 5 min, as black background tends to decolourise, making interpretations more difficult (Ako-Nai et al. 2005).

Processing of plants

Fresh whole plants of *D. scandens* and *P. amarus* were properly washed in tap water and then rinsed in sterile distilled water and left to air-dry for several weeks. The whole plants were micronised to powdered form using an electric blender (Magic Blender – Nakai Japan; Model number – SG-KIPN). The pulverised plants were stored in air-tight containers until required.

Sample preparation and extraction

The extraction of the two plants was carried out using absolute ethanol as extracting solvent. The extraction of the active ingredients of the plants was performed using the method described by Harbone (1994) with slight modification. Seventy-five grams of the pulverised plant was Soxhlet extracted using 500 mL of the absolute ethanol. The volatile oil obtained was purified by filtration through Whatman No.1 filter paper (Atata, Sani & Ajewole 2003), and further sterilised by filtration through Millipore membrane filter of 0.45 µm pore size (Sule & Agbabiaka 2008) and then concentrated by evaporation using water bath at 100°C. The sterile extracts obtained were stored in sterile capped bottles and refrigerated at 4°C until required for use. The extracts were re-dissolved in 5% dimethyl sulphoxide to achieve different concentrations of 400 µg/mL, 200 µg/mL and 100 µg/mL.

Microbe-free proof of the extracts

The extracts were tested for presence or absence of turbidity using Millipore filtration technique by introducing 2 mL of these extracts into 10 mL of sterile Mueller–Hinton broth and incubating at 37°C for 24 h. A microbe-free extract was indicated by the absence of turbidity or clearness of the broth after the incubation period (Sule & Agbabiaka 2008).

Standardisation of microbial cell suspension

Each of the 24-h-old pure culture was prepared to McFarland standard using the method described by Clinical Laboratory Standards Institute (CLSI 2008). This was performed as follows: 0.5 mL of 1.0% (w/vol) anhydrous $BaCl_2$ was added to 99.5 mL of 1% (vol/vol) H_2SO_4 solution, which was stirred to maintain a suspension and thoroughly mixed immediately before the next step; 5 mL of the 0.5 McFarland Standard was distributed into screw-cap tubes. The diameter of these tubes was the same as those used for adjusting the density of culture suspensions prior to inoculation. When these standards are thoroughly shaken, the turbidity equals that of a culture containing about 1.5×10^8 cells. The tubes containing the 0.5 McFarland standards were stored in the dark at room temperature.

Antibacterial susceptibility test

The samples obtained were cultured on Mueller–Hinton agar supplemented with 2% of NaCl and incubated at 35°C for pure isolates. The antibacterial susceptibility profiles of the isolates were determined using broth dilution method as described by the CLSI (2008), Wayne, PA, USA. Reference-type strains of *Staphylococcus aureus* ATCC 25923 were included as positive control.

Combination studies (synergistic tests)

The checkerboard technique was performed using different antibacterial combinations: *D. scandens* whole plant with *P. amarus* whole plant at various minimum inhibitory concentrations (MICs). Stock solutions were prepared according to CLSI (2008) standards. Synergy tests were performed in 96-well microtiter plate containing the combined extracts of different concentrations of *D. scandens* (200 µg/mL, 100, 50, 25, 12.5 … 1.56 and 0 µg/mL) on the top horizontal row (from highest to lowest) and *P. amarus* (100 µg/mL, 50, 25, 12.5 … 1.56 and 0 µg/mL) in the vertical row (from highest to lowest) dispensed in a checkerboard fashion on the day of the assay. Each well contains 0.1 mL of the individual extract in combinations and 0.3 mL of Mueller–Hinton broth. A 1 µL suspension with turbidities equivalent to that of a 0.5 McFarland standard was prepared to yield a final inoculum of 5×10^8 cfu/mL and dispensed into each well. MICs were determined as the lowest concentration with inhibitory effect (i.e. no turbidity) after overnight incubation at 35°C. Positive and negative controls were included in each plate. The Fractional Inhibitory Concentration Index (FICI) was determined using the formula described by Mounyr, Moulay & Saad (2016) (Table 1). Synergy was defined by FICI ≤ 0.5, antagonism by FICI > 4, addition by FICI between 0.5 and 1 and indifference between 1 and 4 (Mounyr, Moulay & Saad 2016).

Phytochemical analysis

Quantitative determinations were carried out on the extracts and screened for the presence of tannins, alkaloids, saponins, polyphenol, phytate, phytin phosphorus and oxalate as described by various authors (Bohm & Kocipai-Abyazan 1994; Day & Underwood 1986; Harborne 1973; Obadoni & Ochuko 2001; Van-Burden & Robinson 1981; Young & Greaves 1940).

Results

The rate of isolation of staphylococci from 200 wounds and burns sources revealed a high prevalence of *S. aureus* (85%) and coagulase-negative staphylococci (CoNS) (15%). DNase characterisation showed 74% DNase-positive staphylococci and 26% DNase-negative staphylococci. The β-lactamase assay produces 30% β-lactamase-producing *S. aureus* and β-lactamase-producing CoNS (17%) (Table 2).

TABLE 1: Antibacterial susceptibilities and synergistic effects (µg/mL) of *Diodia scandens* and *Phyllanthus amarus* on selected staphylococci using microdilution method.

Isolate	MIC (DS)	FIC (DS)	MIC (PA)	FIC (PA)	FICI	Remark
S. aureus	200	0.1563	100	0.3125	0.4688	Synergistic
CoNS	200	0.1563	100	0.3125	0.4688	Synergistic
S. aureus	200	0.1563	100	0.3125	0.4688	Synergistic
ATCC25923						

MIC (DS), minimum inhibitory concentration of D. *scandens*; MIC (PA), minimum inhibitory concentration of P. *amarus*; FIC (DS), fractional inhibitory concentration of D. *scandens*; FIC (PA), fractional inhibitory concentration of P. *amarus*; FICI, fractional inhibitory concentration index.

TABLE 2: Summary of biochemical assay on clinical isolates.

Biochemical characterisation	No. of isolates (%)
Coagulase positive	33 (85)
Coagulase negative	6 (15)
DNase positive	29 (74)
DNase negative	10 (26)
β-lactamase-producing *S. aureus*	10 (30)
Non β-lactamase-producing *S. aureus*	23 (70)
β-lactamase-producing CoNS	1 (17)
Non β-lactamase-producing CoNS	5 (83)
Mannitol fermenters	12 (31)
Non-mannitol fermenters	27 (69)

CoNS, coagulase-negative staphylococcus.

The MIC value for *P. amarus* was 100 µg/mL, while for *D. scandens* it was 200 µg/mL (Table 3). The combined plant extracts revealed synergism with fractional inhibitory concentration (FIC) of *D. scandens* at 0.1563 and *P. amarus* at 0.3125, showing no turbidity (no cell growth) in all the isolates tested (Table 1). FICI was 0.4688 (≤ 0.5). It was observed from this study that the FIC has a lower value indicating synergism when the extracts were combined at lower concentrations. Table 4 shows the quantified phytochemical constituents present in *P. amarus* as saponin (9.99 ± 0.30%), alkaloids (9.67 ± 0.01), oxalate (4.73 ± 0.06), phytin phosphorus (2.44 ± 0.16), phytate (20.08 ± 1.35), polyphenol (3.44 ± 0.04) and tannin (5.82 ± 0.03), while *D. scandens* contains saponin (6.58 ± 0.03%), alkaloids (10.53 ± 0.04), oxalate (5.36 ± 0.06), phytin phosphorus (1.80 ± 0.08), phytate (14.82 ± 0.67), polyphenol (1.59 ± 0.04) and tannin (2.27 ± 0.06).

Discussion

The high prevalence of pathogenic *S. aureus* and emerging infectious CoNS from clinical patients within the tertiary hospitals in Nigeria and around the world are becoming a life-threatening condition, which calls for urgent medical and research attention if the life expectancy of the entire populace is to be attained. This research which was a follow-up study to those conducted by Ojo et al. (2013a) and Ojo, Sargin and Esumeh (2014) has corroborated the work of other researchers who also isolated high *S. aureus* and CoNS from hospitals and communities (Akinjogunla & Enabulele 2010; Akinkunmi & Lamikanra 2010; Asma, Talata & Nayar 2007; Ombui, Kimotho & Nduhiu 2000; Yahmeen et al. 2010).

The MIC and minimum bactericidal concentration (MBC) values recorded in this study were in consonance with an earlier research by the same author (Ojo et al. 2010), while Tamil et al. (2010) reported an MIC value of 100 µg/mL for *P. amarus* α-amylase inhibitory activity. However, Akinjogunla, Balab and Sylvestera (2012) reported an MIC and MBC value for *P. amarus* at 80 mg/mL, and Ojo et al. (2013b) reported an MIC and MBC value of 128 µg/mL and 256 µg/mL for *P. amarus* and *D. scandens*.

The combination studies of *D. scandens* and *P. amarus* have further proven the traditional medical system of combining two or more medicinal plants for the treatment of staphylococcal infections. This study showed synergism on the combined concentrations of the plant extracts under study at a lower concentration, with FICI ≤ 0.5, which was in consonance with an earlier report (though not FIC determined) by the same author (Ojo et al. 2013a) (see Figure 1).

Cowan (1999) enumerated and analysed various phytochemical constituents found in medicinal plants that are used as antibacterial, antiviral, antifungal or antiparasitic and they include: quinones, phenols, flavonoids, tannins, terpenoids, alkaloids, polyphenols and others. Phytochemical analysis of the two medicinal

TABLE 3: Determination of minimum inhibitory concentration and minimum bactericidal concentration of *Diodia scandens* and *Phyllanthus amarus* extracts on clinically isolated staphylococci from wounds and burns patients.

Sample I.D	MIC PA (µg/mL)	MBC PA (µg/mL)	MIC DS (µg/mL)	MBC DS (µg/mL)	isolate
W275	100	NG	200	G	CoNS
W344	100	NG	100	NG	*S. aureus*
W380	100	NG	200	G	CoNS
W345	100	G	100	G	*S. aureus*
W285	100	NG	200	NG	*S. aureus*
W271	100	NG	200	NG	*S. aureus*
W327	100	G	200	G	*S. aureus*
W248	100	NG	200	NG	CoNS
W297	200	NG	200	NG	*S. aureus*
W302	200	NG	200	NG	*S. aureus*
W349	200	G	200	NG	*S. aureus*
W337	200	NG	200	NG	CoNS
W388	200	G	200	NG	*S. aureus*
W235	200	NG	200	G	*S. aureus*
W394	100	NG	200	NG	*S. aureus*
B383	200	NG	200	NG	*S. aureus*
W300	100	NG	200	NG	*S. aureus*
W237	100	NG	200	G	CoNS
W252	100	NG	100	NG	*S. aureus*
W295	100	NG	200	G	*S. aureus*
W318	100	G	100	G	*S. aureus*
W320	100	NG	200	NG	*S. aureus*
W307	100	NG	200	NG	*S. aureus*
W241	200	NG	200	NG	*S. aureus*
W392	200	NG	200	NG	*S. aureus*
W263	200	G	200	NG	*S. aureus*
W310	200	NG	200	NG	*S. aureus*
W296	200	G	200	NG	*S. aureus*
W357	200	NG	200	G	*S. aureus*
W291	100	NG	200	NG	*S. aureus*
W395	200	NG	200	NG	*S. aureus*
W273	100	NG	200	NG	*S. aureus*
W314	100	NG	200	NG	*S. aureus*
W262	100	NG	200	NG	*S. aureus*
W306	200	NG	200	NG	*S. aureus*
W250	200	NG	100	G	*S. aureus*
W279	100	NG	200	NG	*S. aureus*
W387	100	NG	200	NG	*S. aureus*
W339	100	NG	200	NG	*S. aureus*
ATCC 25923	100	NG	200	NG	*S. aureus* Ref. strain

G, Growth; NG , No Growth

CoNS, coagulase-negative staphylococcus; DS, *Diodia scandens*; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; PA, *Phyllanthus amarus*.

TABLE 4: Quantitative analysis on the phytochemical constituents of *Diodia scandens* and *Phyllanthus amarus*.

Phytochemical constituents	Mean ± SD	
	Diodia scandens	*Phyllanthus amarus*
Tannin (mg/100 g)	2.27 ± 0.06	5.82 ± 0.03
Polyphenol (mg/100 g)	1.59 ± 0.04	3.44 ± 0.04
Phytate (mg/g)	14.82 ± 0.67	20.08 ± 1.35
Phytin phosphorus (mg/g)	1.80 ± 0.08	2.44 ± 0.16
Oxalate (mg/g)	5.36 ± 0.06	4.73 ± 0.06
Saponin (%)	6.58 ± 0.03	9.99 ± 0.03
Alkaloids (%)	10.53 ± 0.04	9.67 ± 0.01

SD, standard deviation

$$\text{FIC (DS)} = \frac{\text{MIC (DS) in combination}}{\text{MIC (DS) alone}} = \frac{6.25 + 25}{200} = 0.1563$$

$$\text{FIC (PA)} = \frac{\text{MIC (DS) in combination}}{\text{MIC (DS) alone}} = \frac{25 + 6.25}{100} = 0.3125$$

Therefore: $\sum$FICI = FIC (DS) + FIC (PA) = 0.1563 + 0.3125 = 0.4688 (Synergism ≤ 0.5)

Source: Ojo et al. 2013

FIGURE 1: Combination studies of *D. scandens* and *P. amarus*.

plants under investigation revealed the presence of tannins, saponins, polyphenols (flavonoids), alkaloids, phytate, phytin phosphorus, oxalate and cyanidin. The results obtained by Akinjogunla et al. (2012) on the preliminary phytochemical analysis of the ethanolic leaf extracts of *P. amarus* revealed the presence of phytoconstituents (qualitative) including: alkaloids (+++), tannins (+++), saponins (++), flavonoids (++), cardiac glycoside (+), free anthraquinones (++), deoxy-sugar test (+) and phlobatannins (+), while combined anthraquinones were not detected.

Several authors have reported varying concentrations of the phytonutrients in different medicinal plants (De Britto et al. 2013; Tamil et al. 2010; Van-Burden & Robinson 1981; Young & Greaves 1940; Zabri et al. 2008) as in this study. By implication, the combination of these medicinal plants produces a greater efficacy in the treatment of staphylococcal wound infection as regularly experienced in orthodox medicines. This is because the anti-nutrients are equally present in all fruits, which have been found to have antioxidant and radical-scavenging properties alongside antimicrobials.

Conclusion

Research in ethnobotanical uses of medicinal plants in combined form has validated its claim in complementary alternative medicine owing to the presence of some bioactive constituents, which have similar mechanism of action with antibiotics. Therefore, the prophylactic and curative treatment of these plants should be determined for further study.

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Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Authors' contributions

S.K.S.O. and F.I.E. conceptualised and designed the experiment; S.K.S.O., S.A.O. and T.O.J. were involved in sample collection and processing. All the authors were involved in the bench work and report writing. S.K.S.O. did the final editing, and it was proofread by F.I.E.

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