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In-Vitro Antibacterial Time-Kill Assay Of Phyllanthus Amarus And Diodia Scandens Crude Extracts On Staphylococci Isolated From Wounds And Burns Patients.

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ABSTRACT: Medicinal plants are sources of enormous quantities of phytochemical substances which are able to initiate different biological activities including those useful in the treatment of human diseases. This claim initiated the in vitro time-kill studies on the two plants using standard microbiological procedures. Crude extracts of *Phyllanthus amarus* and *Diodia scandens* were investigated for the rate of time-kill on staphylococci isolated from clinical samples. Biochemical test, β-lactamase assay, MIC and MBC were determined. The result yielded an MIC value of 128µg/ml and 256µg/ml while the MBCs were at 256µg/ml. The time-kill assay revealed a bactericidal effect on the isolated staphylococci ranging between $3.0\log_{10}$ and $5.2\log_{10}$cfu/ml for *P. amarus* crude extract while for *D. scandens* crude extract ranges between $3.0\log_{10}$ and $5.5\log_{10}$cfu/ml.

KEY WORDS: time kill assay, staphylococci, medicinal plants, crude extracts

I. INTRODUCTION

Scientific investigation of medicinal plants used in folklore remedies have attracted increased attention in the world of medicine, especially in a bid to finding lasting solutions to the problems multiple resistance to the existing conventional antimicrobials (Aiyegoro et al., 2008). The therapeutic efficacy of many indigenous plants for several disorders or ailments has been described by traditional medicine practitioners in India, Africa and other parts of the world, which predates the introduction of antibiotics and other modern drugs into African continent (Ojo et al., 2010; Shrivastava et al., 2009). Green medicine is safer and more dependable compared with costly synthetic base, many of which had side effects (Joseph and Raj, 2011). Although several studies have been conducted on *P. amarus*, there is still a paucity of information on the nature of bacterial inhibitions, while information on *D. scandens* has been scanty.

The emergence of resistant microorganisms in hospitals and the community is causing problems for both the treatment of patients and infection control. Organisms of particular concern include methicillin-resistant Staphylococcus aureus (MRSA), glycopeptides-resistant enterococci (GRE), gentamicin-resistant and extended-spectrum β-lactamase-producing Klebsiella and multi-resistant pseudomonads (May et al., 2000). A recent major review of antibiotic resistance emphasized the importance of hospital infection control, and the control of these organisms, and many authorities have reiterated the key role of hand-washing with appropriate disinfectants in this process (Larson, 1995).Time-kill assay involves the determination of the rate of kill on the plant extracts against tested organisms. Previous researchers have used MICs and MBCs as prediction tools for antimicrobial action of plant crude extracts, thus limiting the use of such data since it does not consider time-related antimicrobial effects, such as killing rate (Aiyegoro et al., 2009). The microbial killing potential of the crude extracts of *P. amarus* and *D. scandens* in terms of the kinetics of bacterial death have not been reported. This study will therefore evaluate the antibacterial and time-kill study of *P. amarus* and *D. scandens*, has part of the ongoing exploration of indigenous plants for novel antimicrobial compounds.

II. MATERIALS AND METHODS

2.1 Sample Collection

The whole plants of *P. amarus* and *D. scandens* were harvested from Amai Community, Ukwuani L.G.A in Delta State, Nigeria. It was rinsed in flowing tap water and air-dry for several weeks. The dried plants were pulverized in an electric blender and stored in air-tight plastic bag till required for use.

2.2 Isolation and Species Identification

Fifty (50) clinical samples were obtained from wounds and burns patients at the General Hospital, Kwale, Delta State and inoculated on freshly prepared nutrient agar and mannitol salt agar (Oxoid) slants using sterile swab sticks. Slants were incubated at 37^{o}C for 24hrs.

Colonies growing on slants were streaked on freshly prepared mannitol salt agar (MSA) plates and incubated at 35^{o}C. Primary characterization and identification of isolates was based on Gram stain, morphological and cultural characteristics on different media, fermentation on MSA, catalase and coagulase (tube) test.

2.3 Preparation of Extracts

The ethanolic extracts of the active ingredients of the plants were carried out using the method described by Harbone (1994). 25g of the pulverized powdered plants were soxhlet extracted using 250ml of the absolute ethanol. The extraction lasted for 6hrs and the volatile oil obtained was purified by filtration through Whatman No.1 filter paper (Atata *et al*., 2003) and further sterilized through Millipore membrane filter (0.45µm pore size) (Ronald, 1995). This was concentrated by evaporation using water bath at 50^{o}C until molten semi-solid extracts were obtained (Mbata and Saika, 2008).

2.4 Susceptibility Testing

MICs for the staphylococcal isolates were determined by the micro dilution method recommended by Clinical Laboratory Standards Institute (CLSI) (2008). Micro dilution plates were inoculated with 100µl of Mueller-Hinton (MH) broth containing the appropriate antimicrobial (plant extracts) concentrations and a final concentration of 10^{5}cfu/ml of the test isolates. After an incubation of 24h at 35^{o}C, plates were examined for turbidity, indicating growth. The MBCs were determined by plating out 0.1µl of the MIC on MH agar incubated at 35^{o}C for 24h. Reference type *S. aureus* strain (ATCC 29523) was used as positive control.

2.5 Preparation of inocula for time-kill assay

Inocula for the time-kill determination were prepared as described by May et al. (2000). Organisms were grown in Brain Heart Infusion (BHI) broth (Oxoid). The overnight broth was adjusted to a 0.5 McFarland standard as described by CLSI (2008). The tubes for all isolates were shaken at 150rpm for 90mins at 37^{o}C to ensure that organisms were out of their phases and into their logarithmic phases.

2.6 Time-kill (Bactericidal kinetic) assay

Bactericidal kinetic assay were performed in glass tubes containing 10ml of Mueller Hinton broth. The extracts of *P. amarus* and D. *scandens* were used at the MIC and 2xMIC concentrations. An inoculums containing approximately $5x10^{5}$cfu/ml was introduced into the Mueller Hinton broth containing various extracts and incubated at 37^{o}C. 500µl sample was removed from culture at 6, 12, 18 and 24h, diluted serially and 100µl of the diluted samples were inoculated on Mueller Hinton agar and incubated at 37^{o}C for 24h. Control include extract free Mueller Hinton broth seeded with the test inoculums, viable counts were calculated to give cfu/ml, and kill curves were plotted with time against logarithm of the viable count. Each experiment was performed in duplicate and mean variance obtained.

2.7 Determining time-kill endpoints

A bactericidal effect is defined as $3\log_{10}$ decrease in the cfu/ml or a 99.9% kill over a specified time. The definition of kill for this study was as described by May et al. (2000) with modification. A constant logarithmic rate of kill has been assumed during a time-kill. A 90% kill at 6h is equivalent to a 99% kill at 24h. In this study, the kill measurement was determined by the actual reduction in viable counts at 6h for each isolate and in comparison with 12, 18, and 24h time-kill period.

III RESULTS

Fifty (50) wounds and burns samples were analyzed in this study, obtaining 21 staphylococci isolates, with 3(14%) as Coagulase-negative staphylococci (CoNS) and 18(86%) Coagulase positive *S. aureus* (Table 1). The MICs of *P. amarus* crude extracts on various isolates ranged between 128µg/ml and 256µg/ml while MIC for *D. scandens* was 256µg/ml. The MBCs of *P. amarus* and *D. scandens* were as observed in MIC (Table 2). Table 3 showed the bactericidal activity of the crude extracts on staphylococci isolates at 6h intervals after incubation. The average logarithm reduction in viable cell count for *P. amarus* crude extract ranged between $2.6\log_{10}$cfu/ml and $4.0\log_{10}$cfu/ml after 6h of incubation, $2.7\log_{10}$cfu/ml and $3.8\log_{10}$cfu/ml after 12h, $2.7\log_{10}$cfu/ml and $4.3\log_{10}$cfu/ml after 18h and $0\log_{10}$cfu/ml and $5.2\log_{10}$cfu/ml after 24h in 1xMIC and 2xMIC. For *D. scandens* crude extract, it ranges between $2.6\log_{10}$cfu/ml and $4.0\log_{10}$cfu/ml after 6h of interaction, $2.6\log_{10}$cfu/ml and $4.4\log_{10}$cfu/ml after 12h, $2.7\log_{10}$cfu/ml and $4.4\log_{10}$cfu/ml after 18h, and $2.5\log_{10}$cfu/ml and $5.5\log_{10}$cfu/ml after 24h of interaction in 1Xmic and 2xMIC. The greatest reduction in cell

viability among the staphylococci isolates tested on *P. amarus* was 5.2log$_{10}$cfu/ml while for *D. scandens*, it was 5.5log$_{10}$cfu/ml. The S. aureus type ATCC 25923 tested on *P. amarus* had a higher cell reduction in density of 3.1log$_{10}$cfu/ml as compared to that tested on *D. scandens* with 3.0log$_{10}$cfu/ml.

Table1. Identification and biochemical characterization of wounds and burns isolates from hospital patients.

Biochemical test	No. of staphylococcal isolates (%)	
	Positive	Negative/others
Gram staining	21(42%)	29(58%)
Catalase	21	-
Coagulase	18(86%)	3(14%)
Mannitol fermentation	16(76%)	5(24%)

Table2. Determination of MIC and MBC of the staphylococci isolates on *P. amarus* and *D. scandens* crude extracts

Isolate	*P. amarus*		*D. scandens*	
No.	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
W44	128	NG	256	G
W39	128	NG	128	NG
W34GY	128	NG	256	G
B29	128	NG	128	G
B40M	128	NG	256	NG
W28	128	NG	256	NG
B18	128	NG	256	G
B7	128	NG	256	NG
B5	256	NG	256	NG
W42	256	NG	256	NG
W38	256	NG	256	NG
W37	256	NG	256	NG
W40GY	256	NG	256	NG
W25	256	NG	256	NG
W41	128	NG	256	G
B36	256	NG	256	NG
W33	128	NG	256	NG
W17	128	NG	256	NG
W10	256	NG	256	NG
B22	128	NG	256	NG
W19	128	NG	256	NG
S aureus (ATCC25923)	128	NG	256	NG

Key: GY- Golden Yellow, M- Milky, W- wound, B- burns, G- Growth, NG- No Growth

Table3. Nature of inhibition of crude extracts of P. amarus and D. scandens on wounds and burns staphylococci

Isolate	6h(log$_{10}$kill)		12h(log$_{10}$kill)		18h(log$_{10}$kill)		24h(log$_{10}$kill)	
No	MIC	2xMIC	MIC	2xMIC	MIC	2xMIC	MIC	2xMIC
PA 42	*3.1	*3.3	*3.1	*3.4	2.8	*3.1	*3.0	*4.2
PA 7	*3.4	*3.4	*3.5	*3.6	2.7	*3.0	0	0
PA 39	2.7	*3.2	2.9	*3.5	*3.4	*3.9	*3.3	*4.6
PA29	2.7	*3.2	2.8	*3.2	*4.3	*4.1	*4.4	*4.7
PA41	2.7	*3.1	*3.1	*3.1	*3.1	*3.3	*3.0	*4.1
PA40M	2.6	*3.1	*3.1	*3.4	*3.0	*3.1	2.8	2.8
PA5	2.9	*3.4	*3.1	*3.6	2.8	*3.1	2.7	*3.4
PA28	*3.0	*3.2	*3.2	*3.3	*3.2	*3.5	*3.1	*3.1
PA34GY	2.7	2.8	2.7	2.9	2.8	*3.3	2.9	*3.8
PA38	*3.0	*3.6	*3.4	*3.8	2.8	*3.2	2.9	*3.8
PA44	2.7	*3.0	*3.3	*3.7	*3.1	*4.3	*3.0	*4.4
PA37	2.8	*3.1	*3.0	*3.3	*3.2	*4.0	*3.4	*4.3
PA33	*3.0	*3.5	*3.0	*3.6	2.9	*3.8	2.8	*5.2
PA18	2.6	2.6	2.7	2.7	2.8	2.8	*3.0	*3.8
PA ATCC	*3.1	*3.1	2.9	2.9	*3.0	*3.0	2.6	2.6
DS29	*3.0	*3.0	*3.0	*3.0	*3.1	*3.5	2.9	2.9

DS36	2.6	2.6	2.9	2.9	*3.0	*3.3	2.5	2.7
DS5	2.6	2.8	2.9	2.9	2.7	*3.2	*3.0	*3.0
DS19	*3.0	*3.3	*3.0	*3.4	*3.1	*3.7	2.9	*5.1
DS34GY	*3.4	*4.0	*3.2	*4.4	*3.2	*3.7	*3.1	*5.5
DS40GY	2.7	*3.0	2.6	*3.0	2.9	2.9	2.8	*3.4
DS28	*3.0	*3.0	*3.0	*3.1	*3.1	*3.1	2.7	*3.7
DS39	2.7	2.7	2.9	*3.3	*3.0	*3.0	*3.0	*3.4
DS25	*3.0	*3.2	*3.1	*3.2	*3.1	*4.2	2.7	*3.5
DS38	2.6	*3.0	2.8	*3.1	2.9	2.9	*3.0	*3.0
DS7	2.9	*3.1	*3.0	*3.4	*3.1	*4.4	*3.0	*4.1
DS37	2.9	*3.0	*3.0	*3.0	2.8	*3.4	2.7	*5.5
DS33	*3.0	*3.6	*3.0	*3.8	2.9	*3.0	2.8	2.9
DS ATCC	2.6	2.6	2.9	2.9	2.7	2.7	*3.0	*3.0

Key: PA: isolates tested with *P. amarus*; DS: isolates tested with *D. scandens*; *: Bactericidal effect; PA ATCC: *S. aureus* ATCC 25923 tested with *P. amarus*; DS ATCC: *S. aureus* ATCC 25923 tested with *D. scandens*

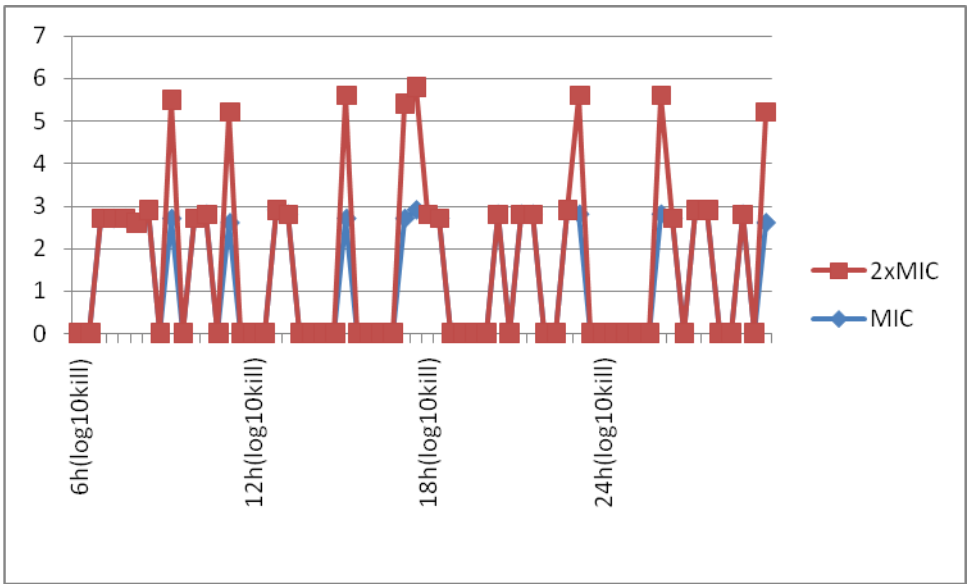

Fig.1. Time-kill curve of Staphylococci by *P. amarus* crude extracts

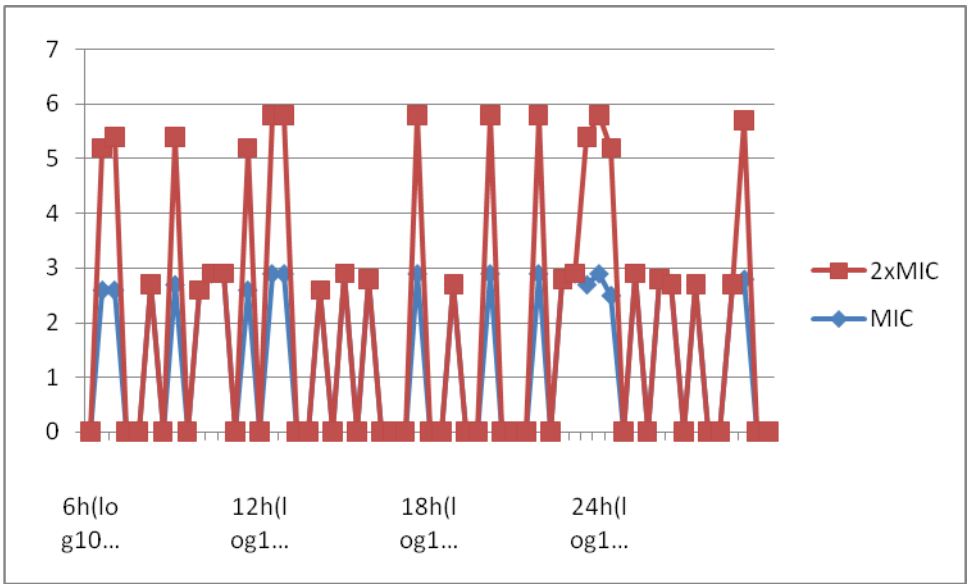

Fig.2. Time-kill curve of Staphylococci by *D. scandens* crude extracts

IV DISCUSSION

The incidence of multi-resistant *Staphylococcus spp.* to antibiotics of choice is increasing and is a problem of global concern, which needs an urgent attention especially in the development of herbal drugs. The results from this study evaluated the possible claim of *P. amarus* and *D. scandens* as having antimicrobial properties used in folkloric medicines for the treatment of various human infections. The rate of killing as demonstrated in this study with fixed concentration of the crude extracts has further proved the in vitro time-kill assay as a reliable method in determining tolerance, thus conforming to earlier studies by Aiyegoro et al. (2008 and 2009), and May et al. (2009).

The time-kill study on *P. amarus* and especially *D. scandens* was the first to be reported with reference to Aiyegoro et al. (2009), that the conventional bactericidal activity standard is a $3\log_{10}$cfu/ml or greater reduction in the viable colony number, which was achieved in this study. It was also observed in this study that the rate of killing was high in 2xMIC (Fig.1&2) for the various hours under study indicating a higher antimicrobial efficacy of the crude extracts against clinical staphylococci isolates. It could be ascertained from this study that an increase in the concentration of extracts with time dependence could yield a better result. This was similar to an earlier report by Aiyegoro et al. (2008) who worked on *H. pendunculatum* and observed a high killing effect on 2xMIC at longer duration of interaction (12h). Although May et al. (2009) and Shrivastava et al. (2009) concluded on 90% kill at 6h is equivalent to a 99.9% kill at 24h. However, this study which was corroborated by Aiyegoro et al. (2008, 2009), revealed a longer duration (time dependent) of kill at 12h of interaction.

V CONCLUSION

On the basis of the results reported in this study, we conclude that the crude extracts of *P. amarus* and *D. scandens* exhibit significant antibacterial activity against tested staphylococci. It also suggests that these plants are a potential candidate in bioprospecting for antimicrobial drugs. However, the rate of kill ascertained from this study should be compared with the rate of kill from conventional antibiotics.

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