Full Length Research Paper

Time kill-kinetics antibacterial study of *Acacia nilotica*

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In vitro time-kill kinetics antibacterial study of *Acacia nilotica* was assessed against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* determined by plate count technique and analyzed by percentage and log reduction. All test organisms were susceptible to the aqueous methanolic extract. The minimum inhibitory concentration ranged between 0.5 and 1 mg, while minimum bactericidal concentration ranged between ≥1 and ≥2 mg/ml. Average log reductions in viable cell counts for the extract ranged between $0.18\log_{10}$ and $0.35\log_{10}$ cfu/ml for *P. aeruginosa*, $0.27\log_{10}$ and $1.95\log_{10}$ cfu/ml for *S. aureus* and $0.27\log_{10}$ and $0.45\log_{10}$ cfu/ml for *E. coli* after 10 h interaction at 0.5x MIC and 1x MIC. Most of the extracts were rapidly bactericidal at 2x MIC achieving a complete elimination of most of the test organisms within 12 h exposure. A good correlation was found between the killing curves and the MIC of *A. nilotica* against the test organisms.

Key words: Time-kill kinetics, antibacteria, *Acacia nilotica*, percentage reduction.

INTRODUCTION

Medicinal plants are sources of enormous quantities of chemical substances which are able to initiate different biological activities including those useful in the treatment of human diseases (Kew Gardens, 2013). Most of the synthetic antibiotics now available in market have major setbacks due to the accompanying side effects on patients and the multiple resistances developed by pathogenic microorganisms to them (Nkomo, 2010). Hence, there is a justifiable need to explore for new and more potent antimicrobial compounds of natural origin to combat these pathogens. *Acacia nilotica* (L.) Willd. ex Delile is a plant used ethnomedically in the traditional treatment of tuberculosis in Northern Nigeria (Oladosu et al., 2010). It is reported to be bactericidal against wide range of microorganisms such as *Mycobacterium tuberculosis* and other HIV/AIDS opportunistic infections such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *C. albicans* (Oladosu, 2012). It is commonly called Gum Arabic tree, Egyptian mimosa, Egyptian thorn, red thorn, Babool, babul in India (Kew Gardens, 2013).

Geographically, *A. nilotica* is widely spread in sub-tropical and tropical Africa from Egypt to Mauritania southwards to South Africa, and in Asia eastwards to Pakistan and India. It has been introduced in China, the Northern Territory and Queensland in Australia (where it is considered to be a pest plant of national importance), in the Caribbean, Indian Ocean islands, Mauritius, United States, Central America, South America and the Galápagos Islands. It has naturalized in several countries where it has been introduced as a medicinal, forage and fuel wood plant (Bennison and Paterson, 1994, Kew Gardens, 2013). Its principal constituents are gallic acid, (+)-catechin and methyl gallate (Oladosu, 2012). It has been reported to contain l-arabinose, catechol, galactan, galactoaraban, galactose, N-acetyldjenkolic acid, N-acetyldjenkolic acid and sulphoxidespentosan. Seeds contain crude protein (18.6%), ether extract (4.4%), fiber (10.1%), nitrogen-free extract (61.2%), ash (5.7%) and silica (0.44%); phosphorus (0.29%) and calcium (0.90%) of DM (Pande et al., 1981). From phytochemical analysis of stem bark of *A. nilotica*, it showed the presence of

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carbohydrates, saponins,annins and cardiac glycosides (Ogbadoyi et al., 2011). Toxicologically, *A. nilotica*, at 2 and 8% levels, has a low toxicity potential (Al-Mustafa et al., 2000; Oladosu et al., 2012). In a survey of potentially allergenic plants in Pondicherry, it was reported likely to cause pollen allergy (Anonymous, 1998). *A. nilotica* has a wealth of medicinal uses. It is used for stomach upset and pain, the bark is chewed to protect against scurvy, an infusion is taken for dysentery and diarrhea (Kew Gardens, 2013). In Nigeria, it is one of the standard drugs for treating diarrhea and it has been authenticated to have an antidiarrhoeal property (Misar et al., 2008). Saba et al. (2011) reported the antibacterial activity of *A. nilotica* lysates against neuropathogenic *E. coli* K1, MRSA and *K. pneumonia.* Time kill kinetics study is a way of determining the spectrum and kill rate of an anti-bacterium.

The aim of the study is to determine the speed of cidal activity of *A. nilotica* within a given contact time.

MATERIALS AND METHODS

Plant extraction

The fruit pulp of *A. nilotica* was prepared according to NIPRD/NIH Standard Operating Procedure No. 1 (2006). The plant was air dried to constant weight at room temperature (35±2°C) and pulverized using a crushing machine (Trapp metallurgical, Trapp Ltd, Brazil). Seventy percent (70%) aqueous methanolic extraction was done so as to extract both polar and non polar components of the plants.

Organism preparation

The test organisms viz: *S. aureus*, *P. aeruginos* and *E. coli* were prepared as described by Dominiguez et al. (2001). Two to three colonies of 20 h growth on Mueller- Hinton Agar of the organisms to be studied were suspended on 50 ml prewarmed (37°C) Mueller-Hinton broth. The suspension was incubated overnight at 37°C, diluted 1/2500 in the same prewarmed medium and incubated in waterbath with agitation (50 rpm). The absorbance of the culture was monitored with a spectrophotometer (6405 Jenway, Barloworld Scientific Ltd. Dunmow, Essex CMB 3LB), using a wavelength 450 nm and 19 mm diameter spectrophotometer tubes until absorbance of 0.1 was reached and a plotted standard curve (equivalent 2.5 - 3.0 × 10^7 cfu/ml for *E. coli* and *P. aeruginosa* and 1.8 - 2.0 × 10^7 cfu/ml for *S. aureus*).

Procedure

The time- kill kinetics antibacterial study of the fruit extract of *A. nil*otica was carried out to assess the killing rate of the extract within a given contact time. This study was done according to standard guide for assessment of antimicrobial activity using time-kill kinetics procedure of (Antimicrobial Susceptibility Testing Method, 2008). Microbial population at the initiation and completion was determined by spectrometric and plate count methods at interval of 2 h. To 200 ml of Mueller- Hinton broth in three conical flasks was added 400, 200 and 100 mg of extract to obtain 2, 1 and 0.5 mg/ml extract, respectively. The solutions were centrifuged at 13,226 x g for 15 min to remove impurities. The extract solutions in flasks were inoculated with 100 µl of inoculum suspensions of test organisms

(*S. aureus*, *E. coli* and *P. aeruginosa*) of 10^6 cfu/ml and incubated at 37°C for 24 h. The optical density of each dilution was recorded on uv/spectrophotometer at 540 nm (Jenway, 6405) at initiation time (0 h) and every 2 h for 10 h. For surviving organism count, an aliquot of each dilution (1 ml) was transferred and plated on 20 ml Tryptic Soy agar at interval of 2 h.

Plates were incubated at 37°C for 24 h. Number of viable organisms was counted as cfu/plates. Average duplicate (2 plates from each replicate dilution) counts were multiplied by the dilution factor to arrive at cfu/ml. The results were analyzed using ANOVA with software SSPS version 16. Differences in the mean values of $p < 0.05$ were considered to be statistically significant and were separated using Duncan new multiple range test.

RESULTS

The time-kill kinetics profile of the test bacteria by *A. nilotica* is shown in Tables 1 to 3. This study unlike an MBC/MIC assay, allows the determination of the speed of cidal activity of the extract (Aiyegoro et al., 2009). The extract exhibited bactericidal effect at 2 mg/ml concentration against all the test bacteria. The number of surviving microorganisms in the extract was determined by plate count method at sampling time and enumerated. The percentage reduction and log reduction from initial microbial population for each time point was calculated to express the change (reduction or increase) of the microbial population relative to a starting inoculum. The change was determined as follows:

% Reduction = $\frac{\text{Initial count} - \text{count at x interval}}{\text{initial count}}$ X 100 (ASTM E, 2008)

The Log reduction was calculated as follows:

Log_{10} (initial count) – Log_{10} (× time interval) = Log_{10} reduction

A significant decrease ($p<0.05$) in population of test organisms was observed at each interval. In the time-kill kinetics antibacterial study against *P. aeruginosa,* Table 1 shows average log reduction in viable cell count ranged between 0.32 Log_{10} to 6.63 Log_{10} cfu/ml after 6 h of interaction, and between 6.61Log_{10} and 6.63 Log_{10} cfu/ml after 24 h of interaction in 1 and 2 x MIC. Percentage reduction in viable cell count was observed to be from 52.2 to 99% between 2 to 24 h of interaction. In the time-kill kinetics antibacteria study of *A. nilotica* extract against *S. aureus* in Table 2, average log reduction in viable cell count ranged between 0.32 Log_{10} to 1.95 Log_{10} cfu/ml, 2.14 log_{10} to 6.61 cfu/ml and 6.63 Log_{10} cfu/ml after 24 h of interaction in 0.5, 1 and 2 x MIC. Percentage reduction in viable cell count (99.99%) at 2 and 1 mg/ml respectively throughout the period of exposure indicated a very high significant decrease ($p<0.05$) as compared to 0.5 mg/ml (52.2 to 70.7%). In the time kill kinetics antibacterial study of *A. nilotica* against *E. coli*, there was a very high significant ($p<0.05$) percentage reduction of viable

Table 1. Time-kill kinetics antibacterial study of *A. nilotica* against *P. aeruginosa.*

Initiation time (h)	Population of organism (cfu/ml)			Percentage (%) reduction			log reduction		
	0.5 mg/ml	1 mg/ml	2 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml
0	$3.6 \times 10^6 \pm 0.3$	$4.1 \times 10^6 \pm 0.3$	4.3×10^6	N/A (%)	N/A (%)	N/A (%)	N/A	N/A	N/A
2	$2.4 \times 10^6 \pm 0.2$	$3 \times 10^6 \pm 0.3$	0	≥52.2	≥99.2	≥99.9	0.32	2.14	6.63
4	$1.6 \times 10^6 \pm 0.1$	$2.4 \times 10^6 \pm 0.2$	0	≥60.8	≥99.4	≥99.9	0.41	2.23	6.63
6	$1.7 \times 10^6 \pm 0.11$	$2.0 \times 10^6 \pm 0.2$	0	≥70.1	≥99.5	≥99.9	1.95	2.31	6.63
8	$1.7 \times 10^6 \pm 0.11$	0	0	≥80.0	≥99.9	≥99.9	2.01	6.61	6.63
10	$1.8 \times 10^6 \pm 0.1$	0	0	≥71.8	≥99.9	≥99.9	1.95	6.61	6.63
12	$2.0 \times 10^6 \pm 0.1$	0	0	≥70.7	≥99.9	≥99.9	1.95	6.61	6.63
24	$2.0 \times 10^6 \pm 0.1$	0	0	≥70.7	≥99.9	≥99.9	1.95	6.61	6.63

Values are means ± S.E.D.

Table 2. Time-kill kinetics antibacterial study of *A. nilotica* against *S. aureus.*

Initiation time (h)	Population of organism (cfu/ml)			% reduction			log reduction		
	0.5 mg/ml	1 mg/ml	2 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml	0.5 mg/ml	1 mg/ml	2 mg/ml
0	$2.0 \times 10^6 \pm 0.1$	$4.1 \times 10^6 \pm 0.3$	4.3×10^6	N/A (%)	N/A (%)	N/A (%)	N/A	N/A	N/A
2	$1.0 \times 10^6 \pm 0.07$	$3.0 \times 10^6 \pm 0.2$	0	≥52.2	≥99.2	≥99.9	0.32	2.14	6.66
4	$9.4 \times 10^6 \pm 0.14$	$2.4 \times 10^4 \pm 0.2$	0	≥60.8	≥99.4	≥99.9	0.41	2.23	6.66
6	$6.4 \times 10^6 \pm 0.3$	$2.1 \times 10^4 \pm 0.3$	0	≥70.1	≥99.5	≥99.9	1.95	2.31	6.66
8	$5.2 \times 10^6 \pm 0.14$	0	0	≥80.0	≥99.9	≥99.9	2.01	6.61	6.66
10	$4.5 \times 10^6 \pm 0.2$	0	0	≥71.8	≥99.9	≥99.9	1.95	6.61	6.66
12	$3.4 \times 10^6 \pm 0.14$	0	0	≥70.7	≥99.9	≥99.9	1.95	6.61	6.66
24	$4.4 \times 10^6 \pm 0.3$	0	0	≥70.7	≥99.9	≥99.9	1.95	6.61	6.66

Values are means ± S.E.D.

cell count of *E. coli* (92.0%) in 2 mg/ml than 1 mg/ml (45.74 to 60.99%) within 2 h of exposure as seen in the result in Table 3. Total bacteria elimination (≥99.99%) observed at the highest concentration (2 mg/ml) at the 6 h of exposure to the extract of *A. nilotica* was a minimal bacteriocidal effect.

DISCUSSION

Time-kill kinetics antibacterial study has been used to investigate numerous antimicrobial agents and they are also often used as the basis for *in vitro* investigations for pharmacodynamic drug interaction (Ogunwonyi et al., 2010). The time kill antibacterial assay of the extract of *A. nilotica* gave variable kinetics against susceptible bacteria tested as seen in Tables 1 to 3. The extract demonstrated both bacteriostatic and bactericidal effects as it shows a concentration-dependent killing. The bactericidal concentration of the extract was 2 mg/ml against *P. aeruginosa* and *E. coli*

Table 3. Time-kill kinetics antibacterial study of extract of *A. nilotica* against *E. coli.*

Expo time	Plate count (cfu/ml)		Percentage (%) reduction		Log reduction	
	1 mg/ml	2 mg/ml	1 mg/ml	2 mg/ml	1 mg/ml	2 mg/ml
0	$2.1 \times 10^6 \pm 0.3$	$3.1 \times 10^6 \pm 0.2$	N/A (%)	N/A (%)	N/A	N/A
2	$1.1 \times 10^6 \pm 0.1$	$2.4 \times 10^6 \pm 0.2$	≥45.74	≥92.0	0.27	1.10
4	$9.9 \times 10^6 \pm 0.63$	$1.0 \times 10^6 \pm 0.02$	≥51.73	≥96.67	0.32	1.48
6	$7.4 \times 10^6 \pm 0.14$	0	≥64.16	≥99.99	0.45	6.48
8	$8.6 \times 10^6 \pm 0.1$	0	≥58.07	≥99.99	0.38	6.48
10	$8.7 \times 10^6 \pm 0.3$	0	≥ 57.07	≥ 99.99	0.37	6.48
12	$8.8 \times 10^6 \pm 0.3$	0	≥57.09	≥99.99	0.37	6.48
24	$8.0 \times 10^7 \pm 0.01$	0	≥60.99	≥99.99	0.41	6.48

Values are means ± S.E.D.

and this is not surprising as Pseudomonas species have been reported to be resistant to many antimicrobial agents (Orishadipe et al., 2005). However, a bacteriostatic effect was observed at a lower concentration (1 mg/ml); only *S. aureus* was susceptible to the extract at 0.5 mg/ml. A complete elimination of *P. aeruginosa* was achieved after 10 h of exposure, while complete elimination after 2 h of exposure was observed in *S. aureus* and *E. coli.* In the overall study, the trend of cidal activities is also time and dose dependent. At higher concentration and longer duration of time (12 h), more bacteria were killed. Inhibitory levels of the crude extract could be bacteriostatic and bactericidal independent of Gram position of test organisms. This study revealed that the extract was rapidly bactericidal at 2 x MIC achieving complete elimination of test organisms after 12 h exposure. The result obtained in this study corroborates the previous study of Aiyegoro et al. (2008) in the time-kill study of *Helichrysum pedunculatum* that the time-kill is time and concentration dependent. A concentration of 1 mg/ml activity agrees with the findings of Saib et al. (2011) that 1 mg/ml concentration of the pod of *A. nilotica* exhibited >90% bactericidal activity. Furthermore, Suma et al. (2012) in their study of antimicrobial activity of *A. nilotica* against *Xanthomonas malvacearum* bacteria indicated the effect of the extract at 500 µg/ml, which is similar to our result against *S. aureus.*

Overall, this time-kill study corroborates the reported efficacies of preliminary antibacterial study of selected plant extracts as reported by Oladosu et al. (2010) and this support the folkloric uses of these plants in treatment of different ailments among the traditional people. In this study, the 70% aqueous methanolic extracts of the test plant exhibited a broadspectrum antibacterial activity at the test concentration of 2, 1 and 0.5 mg/ml and the rate of killing of the extract appear to be time and concentration dependent. The antibacterial activities exhibited by this plant suggest it as a potential candidate in bioprospecting for antimicrobial drugs and the isolation and the identification of the active principles of the plant will be a step forward in drug discovery.

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