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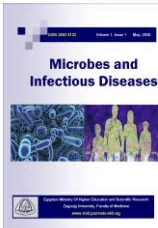

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Original article

Time-kill kinetics and antibacterial activity of ethanolic extract of *Allium sativum*

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ABSTRACT

Background: *Allium sativum (A. sativum)* has been known to possess various medicinal properties, including antibacterial activity. **Aim:** This study was designed to evaluate and quantify killing kinetics of *A. sativum*. **Methods:** *Allium sativum in vitro* time-kill kinetics antibacterial investigation was evaluated by plate count technique and analyzed by percentage and log reduction against *Pseudomonas aeruginosa and Staphylococcus aureus.* An ethanolic extract of *A. sativum* was prepared and tested against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The kinetics of killing and re-growth of the bacteria were then assessed as functions of both time and the extract concentration. **Results:** The time kill-kinetics of *Allium sativum* ethanol extract against the test organisms showed that the extract achieved 100% killing at 1 mg/ml against the two test organisms after 12 h of contact. All test organisms were susceptible to ethanolic extract. Average log reductions in viable cell counts for the extract ranged between 0.02log10 and 1.20log10 cfu/ml for *P. aeruginosa* and 0.03log10 and 0.97log10 cfu/ml for *S. aureus* after 10 h interaction at 0.5mg/ml and 1mg/ml. The extract was rapidly bactericidal at 1mg/ml achieving a complete elimination of the two test organisms within 12 h exposure. **Conclusion:** Overall, this quantified information on time-kill kinetics may provide an initial step towards understanding in vitro pharmacodynamics of antibacterial activity of *A. sativum*.

Introduction

The limitation of the potency of current drugs by the emergence of antimicrobial resistant bacterial strains has consistently been on the rise [1] and has significantly caused failure of treating infections [2] globally. This entails that the potencies of prevalent antibiotics are decreasing steadily [3]. Hence, there is a need to develop novel antibiotics to combat pathogenic microorganisms that have developed widespread microbial resistance to the current antibiotics [4]. Since the resistance of microorganisms to multiple antimicrobial drugs is a major medical concern, the search for new antimicrobial agents by the screening of natural products becomes a necessity [5].

Natural compounds derived from plants have gained widespread interest in the search to identify the

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alternatives for microbial control [6]. The chemicals are widely accepted due to public perceptions that they are safe and have a long history of use in folk medicine for disease prevention and treatment [7]. These natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds, many of which have been the basis for the development of new lead chemicals for pharmaceuticals.

The prevalence of multi-drug resistant (MDR) pathogens is a major pitfall in combating infectious diseases and therefore results in global medical predicament with high rate of morbidity and mortality. Prolonged abuse of antibiotics both in clinical practices and agricultural feeds has been reported from different studies as the cause of MDR [8]. Resistance to antibiotics is a limiting factor in the war against infectious diseases in addition to significant increment in the costs and side effects of newer drugs. As resistant strains of bacteria continue to increase there is no significantly different newer drugs to remedy this problem [9].

Plant derived bioactive compounds are widely being used in most pharmaceutical industries due to their therapeutic efficacy and there are several indications from ethno-botanical records pointing to the fact that potent medicinal plants may be a source of affordable drugs that may be readily available across varying societal classes [10,11]. *Allium sativum* (garlic) is a strong antibacterial agent and acts as an inhibitor on both Gram-positive and Gram-negative bacteria including such species as *Escherichia, Salmonella*, *Streptococcus mutans, Porphyromonas gingivalis, Staphylococcus*, *Klebsiella*, *Proteus* and *Helicobacter pylori* [12]. The main antimicrobial constituent of garlic has been identified as the oxygenated sulphur compound, thio-2- propene-1-sulfinic acid S-allyl ester, which is usually referred to as allicin When garlic cloves are crushed and the enzyme allinase (alliin lyase E.C. 4.4.1.4) of the bundle sheath cells reacts with its substrate, alliin, which is released from mesophyll cells, allicin is generated catalytically [13]. Garlic's capacity to suppress the growth of both gram-positive and gram-negative bacteria demonstrates that it has a broad spectrum of activity and can be utilised in the development of novel broad spectrum antibacterial compounds [14].

However, no study has elucidated time-kill kinetics of antibacterial activity of *Allium sativum (A.sativum)*. The purpose of this study was, thus, to quantify the antimicrobial activities and time-kill kinetics of *A. sativum* ethanolic extract against *Staphylococcus aureus* (*S. aureus)* and *Pseudomonas aeruginosa (P. aeruginosa).*

Material and Methods

Collection and identification of plant material

The bulbs of the plant *A.sativum* (garlic) were randomly purchased from Ubaani Main Market in Umuahia metropolis, South East Nigeria. The identification of the plant material was done in the Forestry Department of Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.

Preparation of plant materials

The garlic bulbs were air-dried at room temperature for three days and ground to a fine powder in an electric blender (SONIK R, Japan).

Preparation of ethanol extracts

The ethanol extract was prepared using the modified maceration technique [15]. The ground plant material (50 g) was extracted with 150 mL of the solvent in a foiled-sealed flask for 3 h with occasional shaking. Thereafter, the extracts were vacuum filtered using Whatman (No. 1) filter paper in a Buchner funnel. The residues were extracted twice with 100 mL and 50 mL of the solvent. The filtrates were concentrated in an oven at 50°C, and then transferred to a glass petri dish for drying at 60°C for 24 h in a vacuum oven. The extracts were weighed to obtain percentage yields and stored in sealed vials at 4°C.

Preparation of cultures

Typed bacterial strains of *S.aureus* and *P.aeruginosa* were obtained from the Diagnostic Centre of National Veterinary Research Institute, Vom, Plateau State, Nigeria. The strains were *S. aureus* ATCC 12600 and *P. aeruginosa* ATCC 10325. Glycerol stock cultures of each organism were prepared and kept at -4 ^{o}C prior to use. The strains were revived onto sterile Tryptone Soy Agar (Oxoid, UK) and incubated at 37°C for 18 h. Selective media were used to confirm their identity. Following incubation, the organisms were inoculated into sterile Tryptone Soy Broth (TSB) and incubated at 37°C overnight. The overnight culture was standardized to a concentration of 1.0×10^{6} CFU/mL. This was done by diluting the overnight cultures with TSB to obtain an absorbance (OD590 nm) of 0.02 for all bacteria [6].

Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) of the extract was determined using the tetrazolium

microplate assay as described by [16] with slight modifications. The assay was performed using the round bottomed polystyrene 96-well clear microtitre plates with standard plate layout as proposed by [17]. The extract was dissolved in 1% dimethyl sulfoxide (DMSO) and identical two-fold serial dilutions were made to form 0.03125 – 4.0 mg/mL. Following the serial dilutions, 100 µL of the standard culture (1.0 × 106 CFU/ mL) was then added to all the wells. The plates were sterile-sealed and incubated at 37 ºC for 24 h. The MIC was detected following the addition of 50 µL of 0.2 mg/mL of INT (2-4-iodophenyl-3-4-nitrophenyl-5-phenyl2H-tetrazolium chloride) in all the wells and incubating for further 30 min at 37ºC. Bacterial growth was determined by observing the colour change of INT in the microplate wells. Biologically active bacterial cells reduce the colourless tetrazolium salt which act as an electron acceptor to a red-coloured formazan product [18]. The inhibition of bacterial growth was seen when the solution in the well became clear after incubation with INT. MIC is defined as the lowest extract concentration that completely inhibits the growth of microorganisms and it is indicated by the first clear well in the column. For the determination of minimum bactericidal concentration (MBC), 20 µL of culture medium from the microtitre plate wells that showed no changes in colour were re-inoculated on Mueller Hinton (MH) agar plates. After 24 h of incubation at 37ºC, MBC was determined as the lowest concentration that showed no bacterial growth on MH agar plates. The MIC and MBC determination were performed in duplicate. The positive and negative controls are ciprofloxacin (V.S. International Pvt Ltd. India) and TSB (Oxoid, UK) respectively.

Determination of time kill-kinetics of the plant extracts

Kinetics of antibacterial activity of the ethanolic extract was performed based on the method of [19, 20] with slight modifications. Selection of the extract concentrations was guided by the MIC endpoints. Each 70 µL of the plant extract at various concentrations (MIC, ½ MIC, and ¼ MIC) was added to each well, and 70 µl/well of the bacterial inoculum with the density of 1.5×10^8 cfu/ml was also added. The controls were included the same as for the antibacterial activity. All experiments were performed in triplicate and the plates were incubated at 37°C. Photographic observations and absorbance readings were performed at 30- minute intervals for the first two hours, followed by two-hour intervals for the later 10 hours. Quantification of readings and determination of antibacterial activity was performed as explained before.

Time kill determination was performed to assess the killing kinetics of *A. sativum* ethanolic extract against *S. aureus* and *P. aeruginosa* ie its killing rate within a given contact time. This was done according to standard guide for assessment of antimicrobial activity using time kill-kinetics procedure of Antimicrobial Susceptibility Testing Method, (2008). Selection of the extract concentrations was guided by the MIC endpoints. Microbial population at the initiation and completion was determined by plate count methods at interval of 2 h. One hundred microliter (100 µL) of the plant extract at various concentrations (MIC, 2MIC, and 4MIC) was added to 100 µL of inoculums suspensions of test organisms (*S. aureus* and *P. aeruginosa*) of $1x10^6$ cfu/mL and incubated at 37ºC for 24 h. For surviving organism count, an aliquot of each dilution (1 mL) was transferred and plated on 20 mL Tryptone Soy agar at interval of 2 h for 10 h.

Plates were incubated at 37ºC for 24 h. Number of viable organisms was counted as cfu/plates. Average counts were multiplied by the dilution factor to arrive at cfu/mL and the population of organisms determined [21].

Statistical analysis

Data were expressed as means ± standard error of means of three replicates and were statistically analyzed using one-way analysis of variance (ANOVA). Differences were considered statistically significant at P<0.05. The means were separated using the Duncan's New Multiple Range Test. The statistical variables were evaluated using the International Business Machines (IBM) Statistical Package for Social Sciences (SPSS) 16.0 (IBM, USA) for windows software.

Results

Antimicrobial screening of plant extracts

Minimum inhibitory concentration and minimum bactericidal concentration

The extract was assessed on growth inhibitory ability against planktonic cells of *S. aureus* and *P. aeruginosa*. The extract was active against the test organisms with MIC values of 0.1875 and 0.10 mg/mL against *S. aureus* and *P. aeruginosa* respectively. *Pseudomonas aeruginosa* was a bit more susceptible to the extract than *S. aureus.* The positive control (ciprofloxacin) showed inhibitory activity with MIC values of 0.0625 and 0.05 against *S. aureus* and *P. aeruginosa* respectively.

The extract showed moderate bactericidal action with MBC values of 0.5 mg/mL for both *S. aureus* and *P. aeruginosa* as demonstrated **in table (1)**.

Time kill-kinetics

The time kill-kinetics of *Allium sativum* ethanol extract was carried out at concentrations of 0.5mg/ml, 1mg/ml and 2mg/ml against *S. aureus* and *P. aeruginosa*. The extract exhibited bacteriostatic activity at a concentration of 0.25 mg/ml. Bactericidal action of the extract against the two organisms was observed at a concentration of 1 mg/ml with complete lethality occurring after 12 h of contact. Also, a concentration of 0.5 mg/ml of the extract exhibited bactericidal action against the two organisms but only after 24 h of contact **(Tables 2 & 3)**.

The cell count of surviving microorganisms in the extract was determined by plate count method at sampling time and enumerated. To express the change (reduction or increase) in the microbial population compared to a starting inoculum, the percentage decrease and log reduction from initial microbial population for each time point were determined. The change was determined as follows:

$$\% \text{ Reduction} = \frac{\text{Initial count} - \text{Count at x interval}}{\text{Initial count}} \times 100$$

(ASTME, 2008)

The Log reduction was calculated as follows:

Log10 (initial count) – Log10 (× time interval) = Log10 reduction

In the time-kill kinetics study against *P. aeruginosa* shown in table 2, a significant decrease ($p \leq 0.05$) in the population of test organisms was observed at each interval when tested at a concentration of 1mg/ml and 0.5mg/ml. The average log reduction in viable cells ranged between 0. 09 log10 to 1.20 log10 after 10 hrs of interaction at a concentration of 1mg/ml, and between 0. 02 log10 to 0.52 log10 after 12 hrs of interaction at a concentration of 0.5mg/ml. Percentage reduction in viable cell count ranged from ≥18.35 to ≥99.9 and from ≥5.30 to ≥99.9% between 2 to 24 h of interaction at a concentration of 1mg/ml and 0.5mg/ml respectively. Concentration of 0.25mg/ml showed the least reduction in viable cell count as there was a significant decrease ($p \leq 0.05$) in reduction of viable cell count only after the first 2 hrs of interaction and then an increase in viable cell count. For *Staphylococcus aureus* as demonstrated in table 3 shows significant decrease ($p \leq 0.05$) in the population of test organisms was equally observed at each interval when tested at a concentration of 1mg/ml and 0.5mg/ml. The average log reduction in viable cells ranged between 0. 09 log10 to 0.97 log10 after 10 hrs of interaction at a concentration of 1mg/ml, and between 0. 03 log10 to 0.47 log10 after 12 hrs of interaction at a concentration of 0.5mg/ml. Percentage reduction in viable cell count ranged from ≥18.44 to ≥99.9 and from ≥6.00 to ≥99.9% between 2 to 24 h of interaction at a concentration of 1mg/ml and 0.5mg/ml respectively. Concentration of 0.25mg/ml showed a degree of inconsistency in the reduction/increase in viable cell count as there was a significant decrease ($p \leq 0.05$) in reduction of viable cell count only after the first 4 hrs. of interaction, which is followed by a slow increase in viable cell count.

Table 1. Antimicrobial activity of *Allium sativum* against *S. aureus* and *P. aeruginosa.*

	MIC		**MBC**	
Solvent	***S. aureus***	***P. aeruginosa***	***S. aureus***	***P. aeruginosa***
Ethanol	0.1875	0.09	0.5	0.5
Control	0.0625	0.05	0.10	0.10

Values are means of duplicate experiments.
Antimicrobial control used was ciprofloxacin

Table 2. Time kill-kinetics antibacterial study of *Allium sativum* ethanol against *P. aeruginosa.*

	Bacterial count (cfu/ml)			**Bacterial reduction (%)**			**Bacterial Log reduction**		
Initiation time	**1 mg/ml**	**0.5 mg/ml**	**0.25 mg/ml**	**1mg/ml**	**0.5 mg/ml**	**0.25 mg/ml**	**1mg/mg**	**0.5mg/ml**	**0.25mg/ml**
0	$3.16 \times 10^6 \pm 1.5^a$	$3.02 \times 10^6 \pm 1.5^a$	$2.68 \times 10^6 \pm 2.7$						
2	$2.58 \times 10^6 \pm 2.1^b$	$2.86 \times 10^6 \pm 2.1^b$	$2.50 \times 10^6 \pm 0.6$	18.35	5.30	6.72	0. 09	0. 02	0. 03
4	$2.05 \times 10^6 \pm 0.6^c$	$2.41 \times 10^6 \pm 1.5^c$	$2.47 \times 10^6 \pm 4.2$	35.13	20.20	7.84	0.19	0.10	0. 04
6	$1.41 \times 10^6 \pm 0.6^d$	$2.00 \times 10^6 \pm 1.2^d$	$2.54 \times 10^6 \pm 1.0$	55.38	33.77	5.22	0.35	0.18	0. 02
8	$6.9 \times 10^5 \pm 5.0^e$	$1.64 \times 10^6 \pm 2.1^e$	$2.65 \times 10^6 \pm 2.9$	78.16	45.70	1.12	0.66	0.26	0. 01
10	$2.0 \times 10^5 \pm 1.5^f$	$1.25 \times 10^6 \pm 0.6^f$	$2.69 \times 10^6 \pm 1.5$	93.67	58.61	-0.37	1.20	0.38	-0. 00
12	0	$9.2 \times 10^5 \pm 1.0^g$	$2.80 \times 10^6 \pm 1.5$	100	69.54	-4.48		0.52	-0. 02
24	0	0	$3.18 \times 10^6 \pm 4.9$	100	100	-18.66			-0. 07

Values are means ±standard error of means of three replicates. Values in each column followed by different superscripts within each column are significantly different at $p \leq 0.05$.

Table 3. Time kill-kinetics antibacterial study of *Allium sativum* ethanol extract against *S. aureus.*

	Bacterial count (cfu/ml)			**Bacterial reduction (%)**			**Bacterial Log reduction**		
Initiation Time	1 mg/ml	0.5 mg/ml	0.25 mg/ml	1 mg/ml	0.5 mg/ml	0.25 mg/ml	1mg/ml	0.5mg/ml	0.25mg/ml
0	$3.20 \times 10^6 \pm 2.9^a$	$3.00 \times 10^6 \pm 3.6^a$	$2.70 \times 10^6 \pm 1.5^b$						
2	$2.61 \times 10^6 \pm 2.1^b$	$2.82 \times 10^6 \pm 1.0^b$	$2.61 \times 10^6 \pm 2.7^c$	18.44	6.00	3.33	0. 09	0. 03	0. 01
4	$2.10 \times 10^6 \pm 2.5^c$	$2.33 \times 10^6 \pm 3.5^c$	$2.49 \times 10^6 \pm 2.1^d$	34.36	22.33	7.78	0.18	0.11	0. 04
6	$1.40 \times 10^6 \pm 2.1^d$	$2.04 \times 10^6 \pm 2.3^d$	$2.52 \times 10^6 \pm 4.0^d$	56.25	32.00	6.67	0.36	0.17	0. 03
8	$8.6 \times 10^5 \pm 2.1^e$	$1.72 \times 10^6 \pm 2.7^e$	$2.60 \times 10^6 \pm 1.2^c$	73.13	42.67	3.70	0.57	0.24	0. 02
10	$3.4 \times 10^5 \pm 1.7^f$	$1.30 \times 10^6 \pm 1.5^f$	$2.62 \times 10^6 \pm 0.6^c$	89.38	56.67	2.96	0.97	0.36	0. 01
12	0	$1.02 \times 10^6 \pm 2.5^g$	$2.72 \times 10^6 \pm 1.5^b$	0	66.00	-0.74	0.47	-0. 01	
24	0	0	$3.10 \times 10^6 \pm 1.5^a$	0	0	-14.81	-0. 06		

Values are means±standard error of means of three replicates. Values in each column followed by different superscripts within each column are significantly different at $p \leq 0.05$.

Discussion

This study explored the time-kill kinetics of antibacterial activity of ethanol extract of *Allium sativum* against *S. aureus* and *P. aeruginosa*. The test organisms used in this study are responsible for many diseases in Nigeria, including bronchopulmonary disorders and chronic otitis media by *P. aeruginosa,* [22,23] nosocomial infections and bacteremia due to multidrug- resistant staphylococcal infections, [24, 25]. The kinetics of killing and regrowth of the tested bacterial strains were assessed over a course of 24 hrs. post inoculation. Time-kill studies provide comprehensive information about pharmacodynamics of a putative antibacterial agent

unlike endpoints such as MIC [19]. Hence, time-kill assays are required to quantitate pharmacodynamics of a putative antibacterial agent by quantifying the decrease in bacterial growth as a function of time and drug concentration [19, 26]. We monitored time-kill kinetics at 24 hours of incubation at different concentrations to investigate the possibility of cfu rebound due to extract instability or volatilization of bioactive agents in garlic, culminating in significantly increased cfu. This expectation was observed in the two test organisms at concentration of 0.25mg/ml.

A simple comparison of the dose-response values observed in this study showed gram negative bacteria being more sensitive to ethanolic extract of *Allium sativum* compared to gram positive bacteria. However, our findings might contradict others [27], where Gram positive bacteria are often found to be more susceptible to plant extracts than the Gram negative bacteria. It is well known that the outer membrane present only in the Gram negative bacteria play an important role as an effective barrier. However, in this study, *S. aureus* was less susceptible to the extracts compared to *P. aeruginosa* possibly because of its thicker cell wall consisting of few peptidoglycan layers which acts as a functional barrier thus hindering the penetration of antimicrobial compound into the bacterial cell [28]. It can be concluded that ethanol extracts of the plants exhibited broad spectrum antimicrobial activity as they were active against the two microorganisms.

The bactericidal kinetics of *Allium sativum* ethanol extracts at three different concentrations indicated mild bactericidal effects against the two test organisms. At a concentration of 1 mg/ml, *Allium sativum* ethanol killed all the tested bacteria within 12 h of contact. This result is similar to that of *Acacia nilotica* which killed *S. aureus* and *P.aeruginosa* cells within 6 h of contact [29]. *Allium sativum* and *Acacia nilotica* contain different bioactive compounds. Therefore, *Acacia nilotica* may have bioactive compounds with stronger antimicrobial activity than *Allium sativum.*

The time-kill findings in this study displayed levels of time-dependent bacterial inhibition, that were different among the tested bacteria and the concentrations, irrespective of whether the organism is Gram positive or Gram negative. For example, the two test organisms displayed similar time-kill patterns at all concentrations. These findings might suggest that kinetics of responding of bacterial strains to the *Allium sativum* during the 24 h of incubation does not necessarily depend on being Gram negative or Gram positive.

The present study has further demonstrated the antimicrobial potency of *Allium sativum* against local multidrug-resistant bacteria from Nigeria. The susceptibility of the test organisms to ethanol extract of *Allium sativum* also implies that the intrinsic biosubstances in this extract are naive to the various drug resistance factors of the isolates, which include beta-lactamase expression, increased pyrrolidonylarylamidase activity, aminoglycoside-modifying enzymes, and altered ribosomal binding [30, 31]. Meanwhile, the antimicrobial potency of garlic has been attributed to its ability to inhibit toxin production and expression of enzymes for pathogenesis [32, 33]. The variable quantities of individually and synergistically active biosubstances in garlic preparations, as well as their interactions with sulfhydryl agents in culture media, have been attributed to the antibacterial potency differential of garlic [34]. This phenomenon has been used to explain the stronger antimicrobial effect of allicin than garlic oil disulfides [35].

Also, the dose and time dependent manner in which the ethanol extract of *Allium sativum* elicited its antimicrobial action on the test organisms producing distinct time-kill kinetics as observed in this study suggests variations in the growth inhibitory responses of the tested isolates to the extract. Similar responses have been observed in antibiotic-resistant *E. coli, Enterobacter cloacae,* and *Citrobacter freundi* [36].

Conclusion

Finally, the findings of this study have offered scientific support for the use of garlic extract in health products and herbal treatments in Nigeria for the treatment of MDR bacterial infections and candidiasis. As a result, complementary and alternative medicine methods involving plant extracts, such as garlic, as a means of minimizing medication resistance and illness management costs would be of clinical and public health value in this country.

No evidence of recurrence or growth was observed between the 2 tested organisms at concentration of 1mg/ml and 0.5mg/ml which imply that the extract is bactericidal at this concentration by completely killing the test organisms in 2-4 hpi with an ~ 2 to 2.5 log reduction in the inoculum. **Nidadavolu et al.** (2012) observed ~ 7 log

reduction in *A. baumannii* biofilms, ~ 8 log reduction in *S. aureus* biofilms, and ~ 2 log reduction in *Enterococcus faecalis* biofilms after treatment with garlic oil.

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Conflicts of interest

The authors declare no conflict of interest.

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Authors contribution

All authors conceptualize the study. CGO, ACN, EGI, ESO were responsible for the methodology, analysis, data curation and manuscript preparation. Review and editing of the manuscript were done by CGO and ACN, but all the authors agreed on the content before submission.

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