Antimicrobial activities and the Bactericidal Kinetics of *Allium ascalonicum* Linn. (Whole plant) against standard and clinical strains of *Helicobacter pylori*: Support for Ethnomedical Use

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Abstract

Helicobacter pylori are the causative agents of gastroduodenal disorders, such as peptic ulcer, acute gastritis and even stomach cancer. It is believed to infect over half the world's population. Recently, there have been cases of chemotherapy – failures and emergence of resistant strains. This necessitated the search for more active drugs. Crude *n*-hexane and methanol extracts were used to screen *Helicobacter pylori* ATCC 43504 and forty-two other clinical isolates of *Helicobacter pylori*, using the agar diffusion method in Mueller-Hinton agar supplemented with 5% defibrinated horse blood. The strains were incubated in a microaerophilic incubator, and it was observed that the standard strain and twenty-eight out of the forty-two clinical strains were susceptible to the methanol extract with the diameter of zone of inhibition ranging from 10 $\pm$ 0.00 to 20 $\pm$ 0.00 mm at test concentrations of 100 mg/mL to 200 mg/mL. The minimum inhibitory concentrations of susceptible strains ranged between 100 mg/mL to 200 mg/mL. The kill kinetics showed that at 4×MIC, equivalent to the minimum bactericidal concentration (MBC), the methanol extract of *Allium ascalonicum* gave 96-98% and 100% kill of the organisms within the contact time of 6 and 24 hours respectively. The *n*-hexane fraction did not show any inhibitory potential against any of the strains. Phytochemical screening of the extract showed the presence of alkaloids, saponins, flavonoids, essential oil and cardiac glycosides. Anthraquinones, tannins and terpenoids were not detected. The presence of these secondary metabolites and the observed activity of *Allium ascalonicum* provide a scientific rationale for the use of this plant in folk medicine in Nigeria and other parts of the world.

Keywords: *Allium ascalonicum*, *Helicobacter pylori*, antimicrobial activities, bactericidal kinetics

1. Introduction

Helicobacter pylori is a helix-shaped (classified as a curved rod, not spirochaete), Gram-negative bacterium; about 3µm long with a diameter of about 0.5µm. It is microaerophilic, requiring oxygen only in much lower concentration than found in the atmosphere. It contains a hydrogenase which can be used to obtain energy by oxidizing molecular hydrogen (H_2) that is produced by intestinal bacteria (Olson and Maier, 2002). *H. pylori* produce oxidase, catalase, and urease. It is capable of forming biofilms (Stark *et al*., 1999), and can convert from spiral to a possibly viable but non-culturable coccoid form (Enroth *et al*., 1999); both forms likely to favour its survival and be factors in the epidemiology of the bacterium. The coccoid form can adhere to gastric epithelial cells *in vitro* (Liu *et al*., 2006).

Helicobacter pylori possess five major outer membrane proteins (OMP) families (Kusters *et al*, 2006). The largest family includes known and putative adhesins. The other four families include porins, iron transportes, flagellum-associated proteins and proteins of unknown functions. Like other typical Gram-negative bacteria, the outer membrane of *H. pylori* consists of phospholipids and Lipopolysaccharide (LPS). The O-antigen of LPS may be fucosylated and mimic Lewis blood group found on the gastric epithelium (Kusters *et al*., 2006). *H. pylori* consist of a large diversity of strains, and the genomes of three have been completely sequenced (Tomb *et al*., 1998; Oh *et al*., 2006). The study of the genome is geared towards understanding the organism's pathogenesis. And approximately 29% of the loci are in the "pathogenesis" category of the genome database.

Rising antibiotic resistance in *H. pylori* increases the need for a preventive strategy for the bacteria (Selgrad and Malfertheiner, 2011). This informed the drive for the present research. Crude *n*-hexane and methanol extracts of *Allium ascalonicum* Linn; were used to challenge standard strain *Helicobacter pylori* ATCC 43504, and forty-two other clinical strains of *Helicobacter pylori* for susceptibilities.

Allium ascalonicum is an herb used in folk medicine in Nigeria and other parts of the world in the treatment of various ailments. Methanol extract of the leaves of this herb has been reported to show therapeutic potential against *H. pylori* infection and gastroduodenal disorders (Adeniyi and Anyiam, 2004). Yin *et al*., (2003) also reported that *A. ascalonicum* oil significantly inhibited the growth of four food-borne bacteria. Adeniyi and Anyiam, (2003) reported that methanol extract of *A. ascalonicum* bulbs, not only inhibited the growth of *H. pylori*, but also significantly reduced the urease activity of the strains screened. This study aimed to establish the

antimicrobial and bactericidal activity of *A. ascalonicum,* against locally isolated multidrug resistant clinical strains and a standard strain of *Helicobacter pylori.*

2. Materials and Methods

2.1 Plant collection and preparation of extracts

The plant sample *Allium ascalonicum* bulbs was purchased fresh from Ogbomoso area of Oyo State, Southwest of Nigeria, identified and authenticated at the Department of Botany (Herbarium), University of Ibadan. Specimen sample was deposited at Herbarium for reference purposes, with voucher number UIH-22337. The sample was properly washed and air-dried, after which they were coarsely milled for extraction. 543 g of sample was successfully extracted using Soxhlet apparatus in a successive extraction process, for 24 hours, with *n*-hexane and methanol. Each fraction of the extracts was filtered, concentrated using rotary evaporator and stored at 4^oC until needed.

2.2 Microorganisms

Overnight culture of *Helicobacter pylori* ATCC 43504 and forty-two clinical strains of *H. pylori* subcultured from stored slopes were used in this study.

2.3 Media and Antimicrobial agents

The media used were Tryptic soy broth, Mueller-Hinton agar, and Columbia agar (OXOID). Laked or defibrinated horse blood was added to the agar as supplement. Ofloxacin was used as the positive control while 50% methanol and 20% DMSO were used as negative controls.

2.4 Phytochemical screening

The plant sample was analyzed quantitatively for the detection of secondary metabolites, such as tannins, alkaloids, saponins, anthraquinones, cardiac glycosides and flavonoids, using the methods described by Abo and Adeyemi (1999).

2.5 Antimicrobial screening of crude extract

The antimicrobial screening was carried out by the agar diffusion method (Aibinu, *et al*., 2007). Sterile molten Mueller-Hinton agar at 45^OC, supplemented with 5% defibrinated horse blood, was seeded with 0.2ml of 1:100 dilutions of fresh overnight culture of the *Helicobacter pylori* strains and thoroughly mixed. The mixture was aseptically poured into Petri dishes and allowed to set. A sterile cork borer with diameter 8mm was used to make equidistant wells in the seeded agar. A hundred microlitres (100 µL) of the reconstituted extracts were placed in the wells. The drug-positive control and the negative controls were equally placed in their respective wells.
The plates were allowed to stand for an hour, for pre-diffusion of the extracts and the controls, after which they were incubated in a microaerophilic incubator at 37^oC for 48 – 72 hours. The plates were observed for inhibition and the diameter zones of inhibition measured to the nearest millimeter. All tests were carried out in duplicates to ensure accuracy.

2.6 Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined using the agar dilution method as previously described (Adeniyi *et al.,* 2009). One millilitre (1mL) of each of the resuspended extracts, prepared to give concentrations of 400 mg/mL, 200 mg/mL, 100 mg/mL, 50 mg/mL, and 12.5 mg/mL was added to19 mL of molten Mueller-Hinton agar at 45^OC supplemented with 5% defibrinated horse blood. The well-mixed agar/extract was poured into sterile Petri dishes, allowed to solidify and the surface dried in sterile oven to remove moisture. An inoculating loopful of overnight culture of each test organism was streaked on the dried surface of the agar. The plates were appropriately incubated and after 48 to 72 hours, the plates were observed for growth. The lowest concentration that prevented the growth of the organisms was taken as the minimum inhibitory concentration. All tests were performed in duplicates.

2.7 Bactericidal kinetics

The concentrations of extracts used for the kill kinetics were those corresponding to MIC, 2 x MIC, 4 x MIC and 8 x MIC values, by a modification of methods described by Aibinu *et al.,* (2007). An overnight culture of each selected organism was subcultured for another 24 hours to maintain them in a sustained log-phase of growth. A 0.1ml of such culture was introduced into 3.9 mL of Tryptic Soy broth, containing 1mL of the extract. A known volume (0.1mL) of the mixture was withdrawn aseptically, serially diluted before plating out 100 µL of the final dilution (10^{-8}) on extract-free Mueller-Hinton agar supplemented with 5% defibrinated horse blood to give the zero minute (0 min) count. The inoculum was evenly dispersed using sterile glass spreader. Other samples were

withdrawn from the mixture at time-intervals of 0.5 hour, 1.0 hour, 2.0 hours, 4.0 hours, 6.0 hours and 24 hours. The process was repeated for the various concentrations of extracts used, and these ran simultaneously with the positive and negative controls. The plates were appropriately incubated and after 48 to 72 hours, numbers of colonies on each plate were counted using the Stuart scientific colony-counter. The number of survivors (cfu/mL) was calculated taking into consideration the final volume of inoculums plated out and the dilution factor. The results are the average of duplicate tests. The percentage survival of the organisms was determined hence, the rate of kill of the extract and a graph of the percentage survival against exposure time was plotted on a semi-logarithm graph.

3. Results and Discussion

There is no doubt that herbotherapy raises the hope of a more natural alternative to the fast-failing chemotherapy. For this reason, scientists have been combing the earth for plants with the right combination of phytochemicals that could be developed for the treatment of various diseases (WHO Factsheet 134, 2006). This study examined the activity and bactericidal kinetics of the extracts of *Allium ascalonicum* Linn (whole plant) against *Helicobacter pylori* strains. The percentage yield per unit mass of the plant sample was observed to be higher (17.7%) in the methanol fraction than the *n*-hexane fraction (1.98%). The phytochemical screening revealed the presence of alkaloids, saponins, cardiac glycosides, flavonoids, and essential oil but tannins, anthraquinones and terpernoids were not detected (Table 1). Most of the clinical strains of the *H. pylori* screened and the standard strain were susceptible to the methanol extract, while the *n*-hexane fraction did not display any inhibitory potential (Table 2). and therefore not presented in the tables. The absence of inhibitory potential of the hexane extract could be attributed to its minimal polarity. Most phenolic substances would dissolve readily in polar solvents like methanol. It is also known that polarity of solvents affect the quantity and composition of secondary metabolites of an extract (Parekh *et al*, 2001). The minimum inhibitory concentrations of the susceptible strains ranged between 100 mg/mL to 200 mg/mL, while the kinetic studies showed that the methanol extract killed the susceptible organisms within 6 to 24 hours of contact time. The results of some of the kinetic studies are shown graphically (Figs. 1, 2 and 3). The significant inhibitory potential displayed by the methanol extract of *Allium ascalonicum* against susceptible strains of *Helicobacter pylori* may be due to the presence of secondary metabolites such as alkaloids, saponins, flavonoids, cardiac glycosides and essential oils. Though the mechanisms of antimicrobial actions of the secondary metabolites are not fully understood, many investigations have been conducted. Single compounds may not be responsible for bioactivity but rather a combination of compounds interacting in an additive or synergistic manner (Javed *et al*., 2011).

Flavonoids mechanism of action might be through cytoplasmic membrane, deoxyribonucleic acid (DNA) gyrase inhibition, and β-hydroxyacyl-acyl carrier protein dehydratase activities (Cushnie and Lamb, 2005; Zhang *et al*., 2008). The cell wall morphology can be changed by isoflavone genistein through formation of filamentous cells and inhibiting the synthesis of DNA and ribonucleic acid (RNA) of *Vibrio harvey,* according to Ulanowska *et al*., (2006). Terpenes which are abundantly present in essential oil, promotes membrane disruption; and also, essential oil being lipophilic in nature, makes it permeable to cellular membrane (Bakkali *et al*., 2008). Coumarins cause cell respiration reduction, and tannins bind to polysaccharides or enzymes, promoting inactivation and effect on microorganisms' membrane (Ya *et al*, 1988; Cowan, 1999). The result of the bactericidal kinetics of this extract, which showed an *in vitro* killing time of 6 hours (96-98% kill) to 24 hours (100% kill), is an indication of the potency of this plant, especially when compared with the drug-positive control which also recorded the same killing time. The mechanism of bactericidal activity of the extract was not studied however; the bactericidal activities may be attributable to the effects of the plants components on the cell wall, cytoplasmic membrane and deoxyribonucleic acid (DNA) of the test organism. Any interruption during the synthesis of the cell wall; disruption of the cytoplasmic membrane and inhibition of DNA synthesis will lead to loss of viability of the cell thereby leading to death of the organism This work therefore agrees with the report of Adeniyi and Anyiam (2004) and others on the anti-*Helicobacter pylori* properties of *Allium ascalonicum*.

Table 1: Phytochemical Screening of *Allium ascalonicum* Linn. (whole plant)

Phytochemical Compound	**Observation**
Alkaloids	++
Anthraquinones	-
Cardiac glycosides	++
Carbohydrates	-
Flavonoids	++
Saponins	+
Tannins	-
Terpenoid	-
Essential oil	++

Note: ++= present; - = Not detected; + = present in low concentration;

Table 2: Susceptibility of *Helicobacter pylori* strains to Methanol Extracts of *Allium ascalonicum* (whole plant). Diameter (mm) of zone of inhibition ± SEM

***Helicobacter pylori* Strains**	***Allium ascalonicum* methanol Extract (mg/mL)**					**Ofloxacin**	**DMSO**	**Methanol**
	200	**100**	**50**	**25**	**12.5**	**80µg/Ml**	**20%**	**50%**
H. pylori ATCC 43504	19±0.00	12±0.00	-	-	-	20±0.00	-	-
BA3	20±0.00	19±0.00	12±0.00	-	-	20±0.50	-	-
BA4	-	-	-	-	-	20±0.00	-	-
BA5	-	-	-	-	-	20±0.00	-	-
BA6	19±0.50	18±0.00	-	-	-	20±0.50	-	-
BA7	15±0.00	12±0.00	-	-	-	22±0.00	-	-
BA8	-	-	-	-	-	20±0.00	-	-
BA9	19±0.40	18±0.20	11±0.00	-	-	22±0.00	-	-
BA10	18±0.00	14±0.00	-	-	-	21±0.50	-	-
BA11	-	-	-	-	-	22±0.00	-	-
BA12	-	-	-	-	-	23±0.00	-	-
BA13	13±0.50	11±0.00	-	-	-	22±0.00	-	-
BA15	12±0.00	10±0.50	-	-	-	21±0.50	-	-
BA16	-	-	-	-	-	22±0.00	-	-
BA18	-	-	-	-	-	21±0.50	-	-
BA19	-	-	-	-	-	21±0.00	-	-
BA21	16±0.30	13±0.10	-	-	-	20±0.50	-	-
BA22	-	-	-	-	-	21±0.50	-	-
BA24	-	-	-	-	-	22±0.00	-	-
BA25	-	-	-	-	-	21±0.50	-	-
BA26	-	-	-	-	-	21±0.00	-	-
BA27	15±0.00	12±0.50	-	-	-	18±0.50	-	-
BA28	14±0.20	11±0.20	-	-	-	20±0.50	-	-
BA29	13±0.50	10±0.00	-	-	-	21±0.50	-	-
BA30	14±0.10	12±0.20	-	-	-	20±0.50	-	-
BA32	12±0.20	10±0.20	-	-	-	20±0.00	-	-
BA33	-	-	-	-	-	20±0.00	-	-
BA34	16±0.00	13±0.00	-	-	-	20±0.50	-	-
BA36	12±0.30	10±0.20	-	-	-	20±0.00	-	-
BA37	11±0.10	-	-	-	-	20±0.50	-	-
BA38	-	-	-	-	-	20±0.50	-	-
BA39	12±0.50	10±0.50	-	-	-	18±0.50	-	-
BA40	13±0.00	11±0.00	-	-	-	20±0.00	-	-
BA42	12±0.00	10±0.00	-	-	-	20±0.00	-	-
BA43	12±0.50	10±0.50	-	-	-	21±0.00	-	-
BA44	14±0.00	11±0.00	-	-	-	21±0.00	-	-
BA46	14±0.00	12±0.00	-	-	-	21±0.50	-	-
BA47	13±0.50	11±0.00	-	-	-	20±0.50	-	-
BA48	12±0.00	10±0.50	-	-	-	20±0.50	-	-
BA49	12±0.00	10±0.00	-	-	-	21±0.50	-	-
BA50	12±0.00	10±0.00	-	-	-	19±0.50	-	-
BA52	14±0.00	11±0.50	-	-	-	22±0.00	-	-

Note: Diameter of cork borer = 8mm; Result is average of duplicate experiments;- = no zone of inhibition

Table 3: Minimum inhibitory concentration (MIC) of *Allium ascalonicum* on selected susceptible *Helicobacter pylori* strains

***H. pylori* strains**	**Methanol Extract of *A. ascalonicum* (mg/mL)**	**Ofloxacin (µg/mL)**
H. pylori ATCC 43504	200	80
BA3	100	80
BA9	100	80

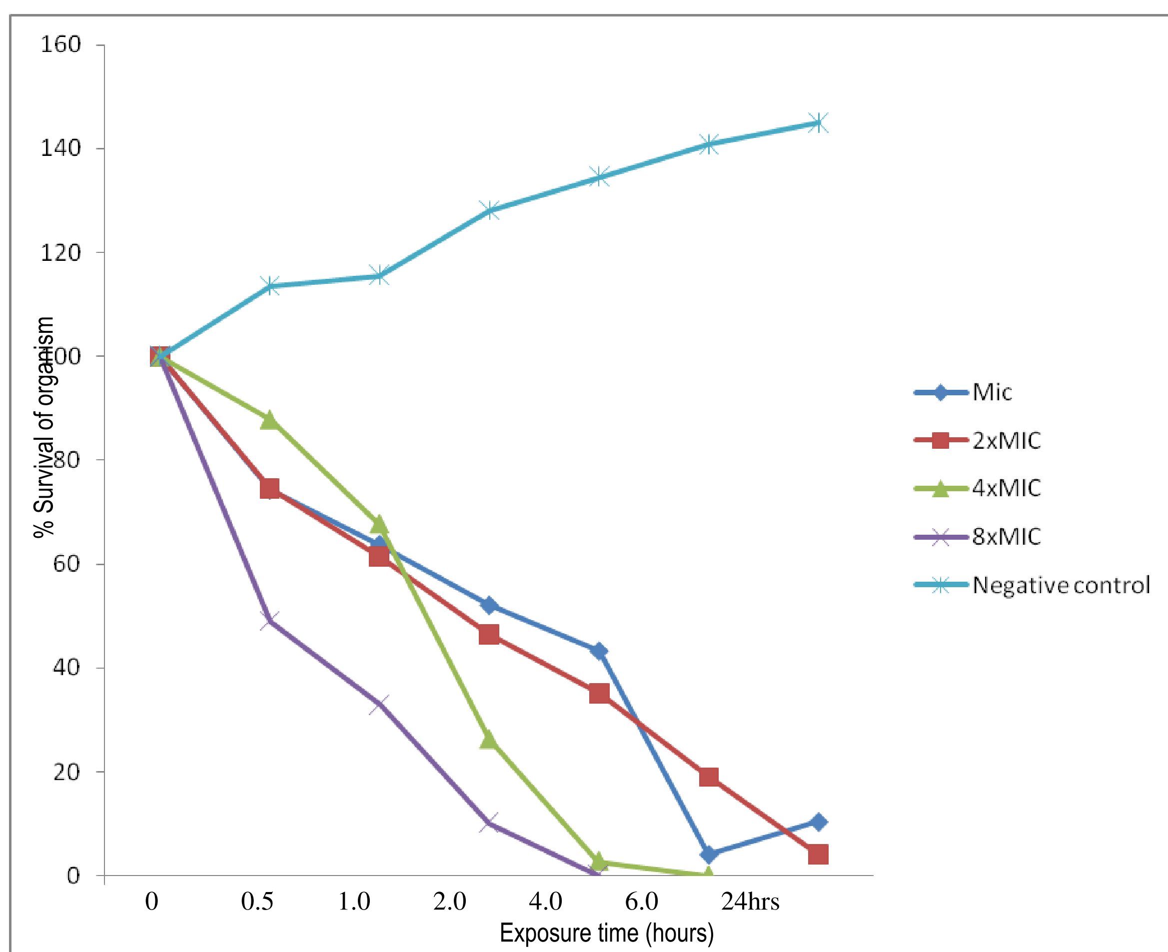

Fig. 1: Kinetics of bactericidal activities of methanol extract of *Allium ascalonicum* on BA9, showing the rate of kill of the organism by the extract.

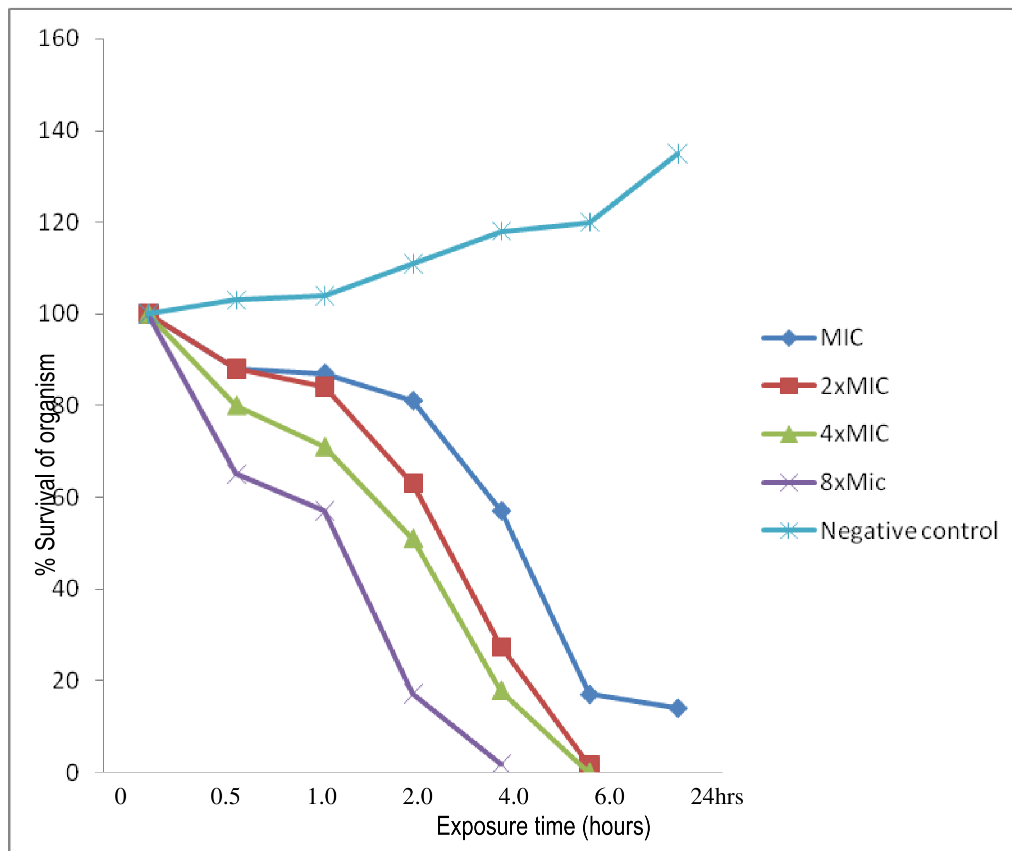

Fig. 2: Kinetics of bactericidal activities of methanol extract of *Allium ascalonicum* on BA3, showing the rate of kill of the organism by the extract.

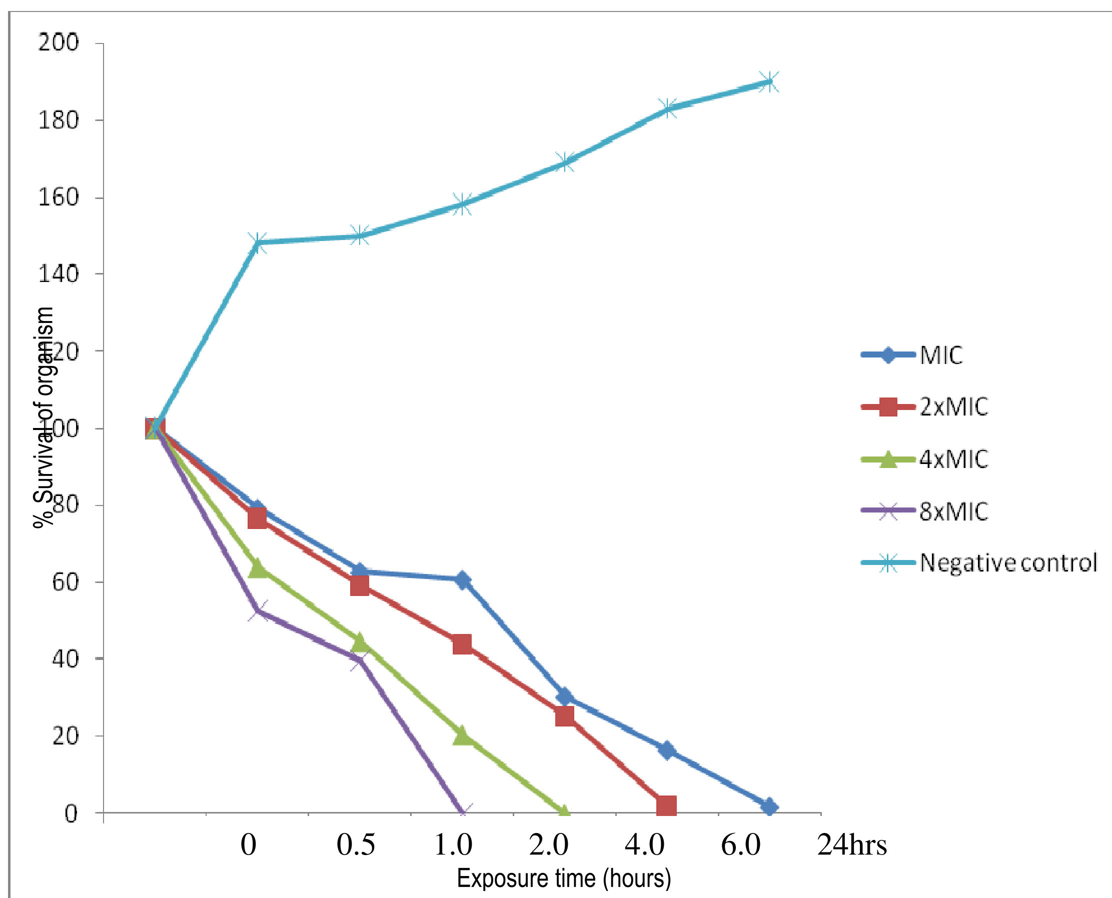

Fig. 3: Kinetics of bactericidal activities of methanol extract of *Allium ascalonicum* on *H. pylori* ATCC 43504, showing the rate of kill of the organism by the extract.

4. Conclusion

This study examined the activity and bactericidal kinetics of the extracts of *Allium ascalonicum* Linn (whole plant) against *Helicobacter pylori* strains. The significant inhibitory potentials displayed by the methanol extract of *Allium ascalonicum* against susceptible strains of *Helicobacter pylori* establish the scientific rationale for the use of this plant in folk medicine. Further research will be carried out to isolate the bioactive constituents in this plant species for the production of more active and less toxic drugs which can be used in the treatment of *Helicobacter pylori*-related gastroduodenal disorders.

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