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Evaluation of Antibacterial Activity and Phytoconstituents of the Aqueous Leaves Extract of *Alchornea Cordifolia*

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Abstract: *Alchornea cordifolia* Schum and Thonn. (Euphorbiaceae) is a plant widely used in traditional medicines of Ghana for the treatment of skin and other infections. This study evaluated the antibacterial properties and phytoconstituents of the aqueous leaves extract of *A. cordifolia* with particular reference to methicillin-resistant *Staphylococcus aureus* (MRSA). Minimum inhibitory and bactericidal concentrations (MIC and MBC) of the aqueous leaves extract of the plant were evaluated against 32 clinical isolates of MRSA and controls by the microdilution technique in Iso-sensitest broth. Growth and time-kill curves were also carried out using spectroscopy at 490 nm and viable cell counts method. Mean diameter of zones of inhibition ranged 18-30 mm of the aqueous extract of the plant were found against MRSA. MIC and MBC values ranged 1.6-3.1 mg/ml and 6.5-12.5 mg/ml of the aqueous leaves extract of *A. cordifolia* were found against the 32 clinical isolates of MRSA with most of the strains having MIC value of 3.1 mg/ml and MBC value of 12.5 mg/ml. Growth and time-kill curves indicate bacteriostatic activity of the plant extract on MRSA. Phytochemical analysis of the extract showed low concentrations of alkaloids and saponins but very high concentrations of tannins present in the leaves of the plant. From the results of the study, aqueous leaves extract of *A. cordifolia* may contain antibacterial compounds that justify its usage in traditional medicine.

Keywords: *Alchornea*, Aqueous, Extract, Staphylococci, Mrsa

INTRODUCTION

Alchornea cordifolia Schum and Thonn. (Euphorbiaceae) is a shrub that grows to about 8 m in height. It is found in secondary forests usually near water, moist or marshy places in Ghana. Leaves of the plant are not eaten but are freely used in Ghana and other West African countries traditional medicine for the treatment of different disease conditions including skin infections (Mshana *et al*., 2000).

Antimicrobial, anti-inflammatory, and antistress activities of the plant have been previously reported by different research groups (Ajali, 2000; Ebi, 2001; Banzouzi *et al*., 2002; Osadebe and Okoye, 2003; Ishola *et al*., 2008). It is also found to possess broad spectrum antimicrobial properties (Manga *et al*., 2004). Six antimicrobial compounds notably quercitin derivatives have been isolated from the methanol leaves extract of *Alchornea laxiflora* (Benth.) Pax and Hoffman, similar related species (Ogundipe *et al*., 2001). Minimum inhibitory and bactericidal concentrations (MICs and MBCs) values ranging from 3-125 µg/ml and 6-137 µg/ml of the compounds were found against *Candida albicans, Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus cereus.* Triterpenoids such as taraxerone, friedeline, epifriedelinol, taraxerol, seco-3,4-friedeline, and seco-3,4-taraxerone have also been previously isolated from *Alchornea latifolia* Sw. Alchiotillo (Setzer *et al*., 2000). Toxicity studies have shown that seco-3,4-friedeline and seco-3,4-taraxerone are cytotoxic *in vitro* assays in human cancer cells as well as potent inhibitor of topoisomerase II (Setzer *et al*., 2000).

S. aureus is a large problem worldwide because of the ability of the bacterium to develop resistance to

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antimicrobial agents. Methicillin-resistant *Staphylococcus aureus* (MRSA) is presently the most dangerous and prevalent form. The bacterium is important causal agent of most nosocomial infections worldwide (Guignard *et al*., 2005). It is therefore necessary that new treatment regimes be developed (Cutler and Wilson, 2004). Various natural products have been shown to have considerable activity on MRSA. For example, allicin from *Allum sativum* have been shown to have a potent activity against MRSA (Cutler and Wilson, 2004). The search for natural products from plant sources more especially those that are used in traditional medicine may be very important in the search for new drugs for the treatment of MRSA which has become important pathogen in hospitals and in the community throughout the world. However, different parts of *A. cordifolia* are used extensively in herbal medicine of Ghana and other West African countries to treat skin and other infections. Since there is the need for potent antibacterial agent(s) against MRSA and other microbial infections, this present study investigates the fundamental scientific bases of the anti-MRSA properties of the aqueous leaves extract of *A. cordifolia* based on a previous study on the antibacterial activities of plants used in the Akwapim-North district of Ghana for the treatment of skin diseases (Pesewu *et al*., 2008). Preliminary phytochemical screening for the detection of bioactive agents including, alkaloids, saponins, and tannins were also performed.

MATERIALS AND METHODS

Plant materials

Authenticated samples of *A. cordifolia* leaves were collected from the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong-Akwapim, Ghana. The leaves were cut into small pieces and air-dried in the shade at room temperature (25-28°C) for 1 week. The leaves were then dried at 45°C in a convention oven for 2 h to completely remove residual moisture, before milling into fine powder. The powder was sealed in air-tight bags to prepare it for storage and transport.

Plant extract

Aqueous extract was prepared from the leaves of the plant using modification of the method by Pesewu *et al*. (2008). Briefly, 25 g of dry milled powder of *A. cordifolia* leaves were placed in a beaker (250 ml), 100 ml distilled water (H_2O) was added and allowed to soak overnight. The next day the mixture was then vigorously stirred for 10 min and allowed to settle for 5 min. The supernatant liquid was passed through Whatman no.1 filter paper (Whatman International Limited, England) to remove solid plant material and the aqueous extract was freeze dried. The dry weight of the extract was expressed as a percentage of the dry weight of the original powdered plant material.

Evaluation of antibacterial activity

Test bacteria and media used

Multi-resistant strains of MRSA isolates (32) were obtained from the Royal London Hospital and Newham University (courtesy of Dr. P. Wilson) and maintained on nutrient agar slants and Oxacillin Resistance Screening Agar plates (ORSA: CM 1008, Oxoid Ltd, Basingstoke, Hampshire, UK). The following control bacteria were also obtained: methicillin-sensitive *Staphylococcus aureus*-National Collection of Typed Cultures (MSSA: NCTC 6571) and MRSA (biotypes 102 and 103). These are studied types of bacteria and are kept at the School of Health, Sport, and Bioscience (UELSHSB), University of East London, UK. Media used for the antibacterial evaluation of the plant extract were: Nutrient agar (NA), Iso-Sensitest broth (ISB), and Mueller-Hinton agar (MHA) obtained from Oxoid Ltd, Basingstoke, Hampshire, UK.

Agar-well diffusion antibacterial assay

The inhibitory effects of the aqueous leaf extract of *A. cordifolia* were determined by agar well diffusion assay based on BSAC procedures (Andrews, 2005) modified by Cutler and Wilson (2004). Briefly, wells 6 mm in diameter were punched in ISA and the plant extracts (100 µl of 50 mg ml^{-1} solutions) were added. Standard paper discs (Oxoid Ltd, Basingstoke, UK), of vancomycin (30 µg), were used as antibiotic controls. Plates were incubated for 24 h at 37°C and the diameter of the inhibition zones around the wells and control discs were used to assess antibacterial activity. All the tests were done in triplicates and standard deviations determined with their respective means.

Determination of MIC and MBC

MIC of the aqueous leaves extract of *A. cordifolia* against the 32 clinical isolates of MRSA and controls was determined using Clinical Laboratory Standard Institute (CLSI, 2007) methods in 96-well microtitre plates. Concentrations of the plant extract prepared by serial doubling dilutions were tested against the bacteria in the range 0.048-50 mg ml^{-1}. To determine the MBC, a set of NA plates were prepared and 10 µl of the cultures from all the wells and the controls were sub-cultured onto NA plates and incubated at 37°C for 48 h. The lowest concentration of the plant extract that does not show growth of the test bacteria on sub-culture was taken as the MBC.

Growth curve study

Single colonies each of MSSA and MRSA were inoculated into separate universal bottles containing 10 ml sterile ISB each, and incubated overnight at 37°C. Using ultra-violet (UV) spectrophotometer the

absorbance of the bacteria were measured (viability of each strain) and based on British Society for Antimicrobial Chemotherapy (BSAC) standard methods (Andrews, 2005) the necessary dilutions to obtain a final concentration of 10^6 colony forming units per millilitre (CFU)/ml were calculated and the bacterial suspensions prepared accordingly using serial doubling dilutions method.

Growth characteristics of MSSA and MRSA in the presence of aqueous leaves extract of *A. cordifolia* were studied in 96-well microtitre plate. Columns 1-12 of microtitre plate were marked and using an Eppendorf pipette, sterile ISB (100 µl) was distributed from the first well to the 96-wells in the microtitre tray. To wells A-H of column 1, 100 µl of different concentrations of the plant extract originally sterile filtered through 0.2 µm Millipore filters were added. Then using a multichannel micropipette with 8 sterile tips attached to each channels, the preparations in A-H of column 1 were mixed well and from these column 1, 100 µl of each was transferred to wells of column 2. The contents of column 2 were also thoroughly mixed and the above process was repeated up to wells A-H of column 11. No extracts were added to the wells in A-H of column 12 and serves as positive growth controls. Then (100 µl) of logarithmic phase cultures of the bacterial suspension (10^6 CFU/ml) prepared above were added to each well in rows A-F whilst 100 µl of sterile ISB were added to each of the wells in rows G and H that served as negative growth controls. To prevent dehydration the microtitre plate was covered with a sterile plastic cover. The plate was then incubated and scanned at 37°C overnight in an ELX-800 Absorbance Microplate reader that measures absorbance at 490 nm. Readings were taken hourly for 20 h. Growth curves of absorbance versus time of each suspension for MSSA, MRSA, and the controls were plotted. All tests were performed in duplicates.

Time-Kill study

Bactericidal activity of the aqueous leaves extract of *A. cordifolia* on MRSA was determined by the viable cell count method. Briefly, 5 ml each of sterile ISB were first inoculated with the bacterial cultures and incubated overnight at 37°C. After incubation, samples from the overnight bacterial suspensions were then inoculated into two fresh sterile 30 ml ISB each with two times the standard amount of bacteria according to BASC standard (Andrews, 2005) to yield 10^7 CFU/ml of bacteria culture. The broths were then incubated at 37°C for 2 h to obtain logarithmic phase growth cultures prior to use. Freeze dried aqueous leaves extract of *A. cordifolia* was also dissolved in sterile ISB and sterile filtered through 0.2 µm Millipore filters (Merck Millipore, Germany). The prepared extract was mixed with one of the 30 ml logarithmic phase bacterial suspensions prepared above whilst 30 ml of sterile ISB was added to the other and serves as a positive growth control. The preparations were poured into sterile petri dishes and incubated at 37°C with shaking. Aliquots (20 µl) of the plant extract and the control were obtained at different time intervals 0, 1, 2, 3, 4, 5, 6, 7, 8, and 24 h and serially diluted in 96-well microtitre trays containing 180 µl of sterile ISB in each well. Then 10 µl of the mixed suspensions were taken and seeded onto separate MHA plates. Numbers of surviving bacteria were determined by counting visible colonies after 24 h of incubation at 37°C and compared with that of the control in which the plant extract was replaced with sterile ISB. The experiment was repeated two more times and the results were expressed in viable cells as a percentage of the control.

Preliminary phytochemical analysis

Test for alkaloids

Alkaloids were tested in the aqueous leaves extract of *A. cordifolia* by modification of standard phytochemical methods (Otshundi *et al*., 2000). A sample of the freeze dried powdered plant extract was boiled in 10 ml alcoholic hydrochloric acid (HCl) in a boiling tube for 1 min. The preparation was allowed to cool, debris settled and the supernatant liquid was filtered into another tube. Part of this solution (1 ml) was taken and 3 drops of Dragendorff's reagent (Bismuth potassium iodide solution) were added. Presence of orange precipitates was taken as indicative of the presence of alkaloids in the plant extract.

An extract showing the presence of precipitates was further analysed by the addition of dilute ammonia (NH_4) to the rest of the extract until it was alkaline to litmus paper. Then 5 ml of chloroform were added to the extract, and shaken gently in a separating funnel. The layers were allowed to separate, chloroform layer ruined into another tube and extracted with 10 ml acetic acid and the chloroform discarded. The extract was divided into 3 portions. To one, 3 drops of Dragendorf's reagent were added. Three drops of Mayer's reagent (Potassium mercuric iodide solution) were also added to the second portion whilst the third untreated portion serves as a control. Presence of alkaloids was determined by the development of turbidity when compared to the untreated control.

Test for saponins

The presence of saponins in the plant extract was determined by the modification of the honey comb froth test and confirmed by the haemolysis test (Mojab *et al*., 2003). A sample of the plant extract was dissolved in 20 ml of distilled H_2O and divided into two portions; 10 ml of each suspension was then

shaken vigorously to froth and allowed to stand for 30 min. A dense froth 3 cm high above the liquid surface which persisted for 30 min was taken as the presence of saponins in the test plant extract. A further test of saponins was performed by the haemolysis assay. Cup-wells (6 mm diameter) equidistant apart to each other were made on a blood agar (BA) plate. One of the wells was filled with 100 µl of the crude aqueous leaves extract of *A. cordifolia* and the 2nd, 3rd, and 4th wells were also filled with "Tea saponin" (positive control), phosphate buffer solution (negative control), and distilled H_2O (minimal haemolytic control). The plate was allowed to stand for 1 h and examined for clear zones of haemolysis on the BA plate surrounding the plant extract and the controls.

Confirmation of saponins in the plant extract was performed using haemolysis testing in 96-wells microtitre plate. Aliquots of heparinised sheep blood (Oxoid Ltd, Basingstoke, UK) were washed 3 more times with sterile physiological saline solution by centrifugation at 180 x g for 5 min. The red blood cell suspension was prepared finally by diluting the pellet in %85 NaCl solutions. Serial doubling dilutions of the stock solution of the plant extract were prepared in a 96-well microtitre plate using physiological saline from the 1st-12th well with the first test well containing the highest dilutions of the plant extract. After that 100 µl of the washed blood cell suspension was added into each well that respectively contained 100 µl of the plant extracts and the controls. The plate was incubated at 37°C for 4 h, centrifuged at 70 x g for 5 min, and 100 µl of the supernatant removed into a fresh 96-well microtitre plate. Free haemoglobin were measured spectrophotometrically at 412 and 620 nm. "Tea saponin", phosphate buffer solution, and distilled H_2O were also used as positive, negative, and minimal haemolytic controls. The experiment was performed in duplicates at each test concentration of the plant extract and controls.

Test for tannins

Tannins were detected in the aqueous leaves extract of the plant by the gelatin-salt block test according to the method described by Otshundi *et al.* (2000). Reference standard control was tannic acid powder (TD 125) obtained from Sigma-Aldrich, UK.

RESULTS

Antibacterial activity

All the 32 clinical MRSA isolates, and the control bacteria were susceptible to the aqueous leaf extract of *A. cordifolia* with inhibition zone diameters ranging from 18-30 mm. Using a modification of BSAC break points (Andrews, 2005), 30 (94%) of the isolates were identified as fully susceptible (21-30 mm) zones sizes, while only 2 (6%) were classified intermediate as having inhibition zone diameters between 11-20 mm. MIC and MBC values ranged 1.6-3.1 mg ml^{-1} 6.5-12.5 mg ml^{-1} of *A. cordifolia* against the clinical isolates were found in this study with most of the MRSA strains having MIC value of 3.1 mg ml^{-1} and MBC value of 12.5 mg ml^{-1}. Inhibition diameter of zone sizes ranged 31-34 mm and MIC and MBC values ranged 0.4-3.1 and 0.8-12.5 mg ml^{-1} respectively of the aqueous leaf extract of *A. cordifolia* were found against the reference control bacteria used in the study.

Growth curve study

The growth of MSSA and MRSA in the presences of aqueous leaf extract of *A. cordifolia* are presented in Figures 1 and 2. When cultures of the two strains of bacteria with cell density of 10^6 CFU ml^{-1} were exposed to 3 different concentrations of the plant extract and the number of cells determined by measuring the absorbance using a spectrophotometer. It was observed that concentrations ranging from 0.2-0.8 and 1.6-6.3 mg ml^{-1} of the aqueous leaves extract of *A. cordifolia* show bacteriostatic effects on MSSA and MRSA used in the study as there were no appreciable growth of the bacteria when compared to the growth controls in which the plant extract was replaced with sterile ISB (Figs.1 and 2).

Time-kill assay

Aqueous leaf extract of A. cordifolia showed considerable bactericidal activity against the test bacteria used in the present study. It was observed from the time-kill assays that, when 107 CFU ml-1 of the MRSA was exposed to 1.6, 3.1, and 6.3 mg ml-1 (0.5x, 1x and 2x MIC values) of plant extract, there was a reduction in growth in the first 8 h and a complete kill after 24 h (Fig. 3).

Phytochemical screening

Results of the phytochemical screening of the aqueous leaves extract of the plant are presented in Table 1. When the crude extract of the plant was subjected to phytochemical analysis, alkaloids, saponins, and tannins were found in the aqueous extract. There were high concentrations of tannins in the aqueous leaves extract of the plant. When the extract was centrifuged at 180 x g for 5 min and the supernatant removed and tested again for tannins, it was observed that there were still high tannins contents in the aqueous leaves extract of *A. cordifolia* (Table 1).

DISCUSSION

In vitro antibacterial screening of plants extracts or products permits the selection of phytomedicines with potentially useful properties to be used for further chemical and pharmacological studies. Extracts of the leaves of *A. cordifolia* are used to treat

ringworm and other skin diseases in Ghana and other West African countries (Mshana *et al*., 2000). The MIC values ranging from 0.4-3.1 mg/ml of the aqueous leaves extract of the plant were found against the 32 clinical isolates and the controls which highlights the potential of the plant as an antibacterial agent and provides possible scientific evidence that supports the traditional usage of *A*. *cordifolia* in the treatment of skin and other infections in Ghana (Pesewu *et al*., 2008).

Antibacterial activities of acetone, methanol, ethanol, and chloroform-soluble extracts of the stem bark and leaves of the plant have been previously investigated against *S. aureus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae*, and *Ps. aeruginosa* in Nigeria (Ajali, 2000). Chloroform soluble extracts were active against *S. aureus*, *Ps. aeruginosa*, and *E. coli* but demonstrated greater anti-staphylococcal and anti-pseudomonal activity in the fractions that were insoluble in chloroform (Ebi, 2001). It has also been reported that ethanol leaves extract of *A. cordifolia* are active against several types of microorganisms including *E. coli and S. aureus* (Okeke *et al*., 1999). MBC values ranged 0.8-12.5 mg/ml of the plant extract was also found against MSSA and MRSA in this study. Wild type strains of microorganisms often have unpredictable susceptibility to antimicrobial agents due to the fact that some of the organisms may have variable degree of drug resistance (Olila *et al*., 2001). These highlights the need for investigating plant extracts against clinical isolates of MRSA. It was established that all the isolates were susceptible to the plant extract and shows the potential of the plant as useful anti-MRSA agent and confirms anti-MSSA property previously reported (Ebi, 2001). In terms of toxicity, it has been previously reported that extracts of *A*. *cordifolia* are toxic to grazing animals if ingested (Adadepo *et al*., 2007).

Phytochemical analysis of the aqueous leaves extract of *A*. *cordifolia* shows the presence of alkaloids, saponins, and tannins. From the amount of precipitate formed and the intensity of colour change after the addition of the tannin reagents to the plant extract, it was deduced that there were high presence of tannin contents in the extract. Another observable feature of the aqueous leaves extract of *A*. *cordifolia* was that when it is left to stand for few minutes there was precipitation. It is possible that the precipitates can contribute to the total antibacterial activity of the plant. High existence of tannins is also a characteristic of these plant species (Cui and Tan, 2004).

CONCLUSION

From the study, aqueous leaves extract of *A*. *cordifolia* has shown a good antibacterial activity against 32 clinical MRSA isolates and the controls. The results shows that all the test bacteria used in the study were sensitive to the plant extract and support the traditional usage of extracts of the plant for the treatment of skin and other bacterial infections. It also highlights the plant as a potential anti-MRSA agent. Preliminary phytochemical analysis has shown the presence of low concentrations of alkaloids and saponins but very high concentrations of tannins in the leaves of the plant. Work is on-going to isolate and identify the bioactive agent(s) of the plant extract using the high performance liquid chromatography (HPLC), matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF), and other analytical techniques.

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Declaration of interest: None

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Table 1. Phytochemical screening of the aqueous leaves extract of *A. cordifolia*

Plant extract	Alkaloids	Saponins	Tannins
Crude	+	+	+++
Spun	+	+	+++

(+), low concentration detected; (++), moderate concentration detected, (+++), high concentration detected

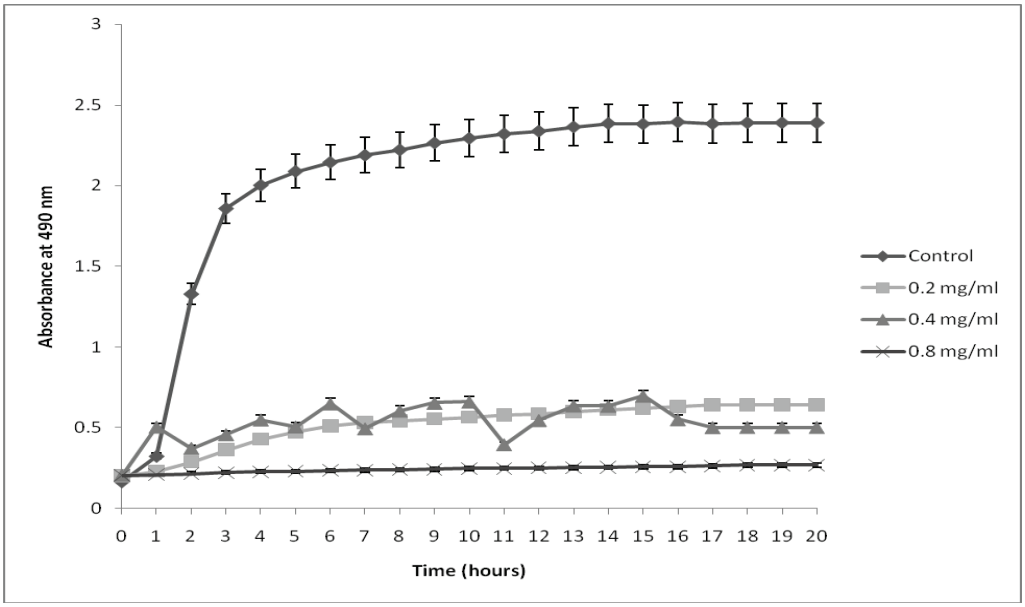

Fig. 1. Effects of different concentrations of aqueous leaves extract of *A. cordifolia* on the growth of MSSA NCTC 6571. Bacterial suspension of MSSA was incubated with (0.5XMIC, 1XMIC, and 2XMIC) or without (control) the plant extract in sterile ISB at 37°C with shaking.

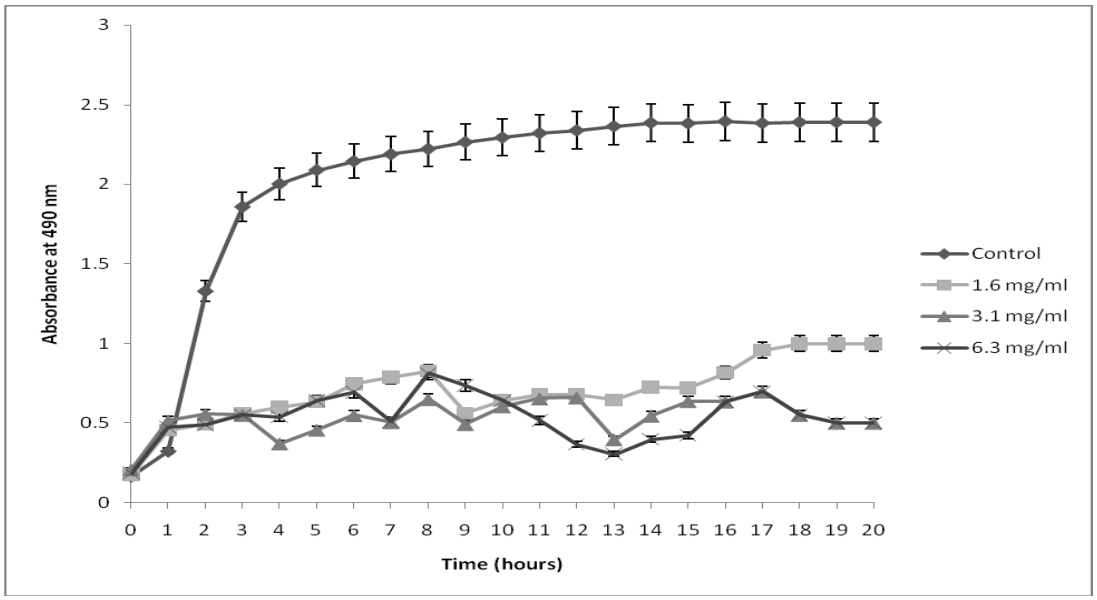

Fig. 2. Effects of different concentrations of aqueous leaves extract of *A. cordifolia* on the growth of MRSA UELSHSB 102. Bacterial suspension of MRSA was incubated with (0.5XMIC, 1XMIC, and 2XMIC) or without (control) the plant extract in sterile ISB at 37°C with shaking.

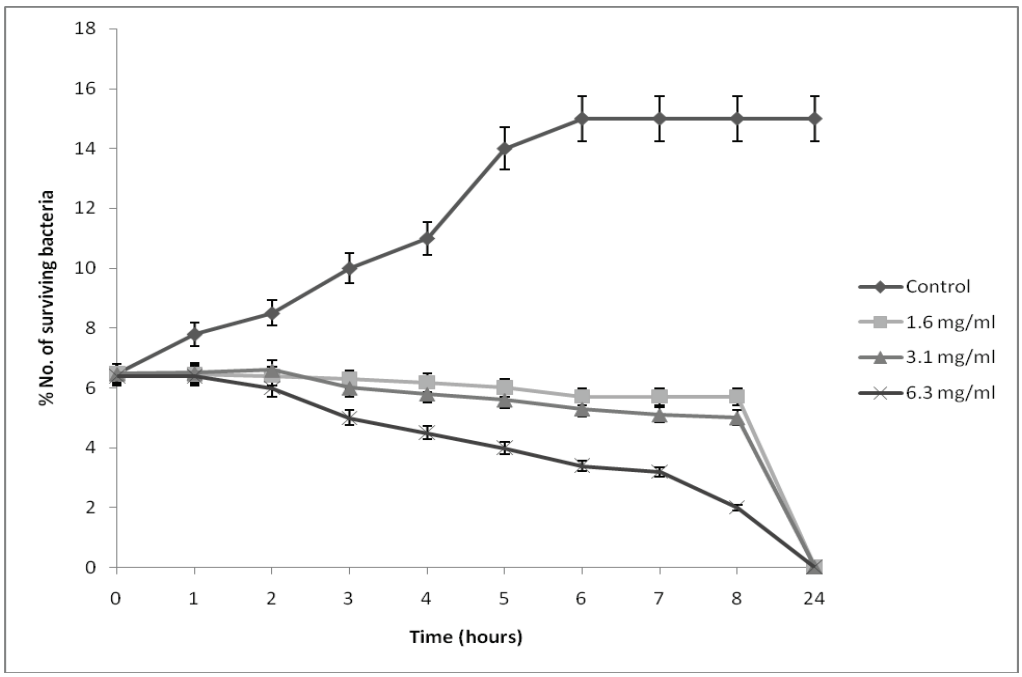

Fig. 3. Bactericidal effects of aqueous leaves extract of *A. cordifolia* against MRSA UELSHSB 102. Exponentially growing suspension of MRSA was inoculated into sterile ISB containing different concentrations of the plant extract (0.5XMIC, 1XMIC, and 2XMIC), incubated at 37°C with shaking, and sub-cultured onto sterile MHA at 1 h intervals.