

**THE KILL KINETICS OF AZADIRACHTA INDICA A. JUSS. (MELIACEAE)  
EXTRACTS ON STAPHYLOCOCCUS AUREUS, ESCHERICHIA COLI,  
PSEUDOMONAS AERUGINOSA AND CANDIDA ALBICANS.**

P.O. Okemo,<sup>a\*</sup> W.E. Mwatha,<sup>a</sup> S.C. Chhabra<sup>b</sup> and W. Fabry<sup>c</sup>

<sup>a</sup>Department of Botany

<sup>b</sup>Department of Chemistry

Kenyatta University, P.O. Box 43844, Nairobi, Kenya.

<sup>c</sup>Institut für Medizinische Mikrobiologie

Der Universität-GH Essen, Hufelandstrasse 55, D-45147 Essen, GERMANY.

**ABSTRACT:** Crude extracts of the neem plant *Azadirachta indica* A. Juss (meliaceae) which were previously determined to have strong antibacterial activity were investigated for their rate and extent of bacterial killing (kill kinetics). Various extract dilutions related to the minimum inhibitory concentrations (MICs) of type culture strains of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* were used. Inoculated strains were serially diluted and plated at time intervals of 0, 2, 4, 6, 8 and 24h. Results obtained show that *A. indica* extracts killed all the bacterial cells of *S. aureus* at a concentrations of 1 mg/ml in 8h. A higher concentration of 2mg/ml had the same effect after 6h. The extracts killed a whole population of *C. albicans* at a concentration of 8 mg/ml in 24h. Both *E. coli* and *P. aeruginosa* which had previously shown an MIC of 8 mg/ml/ were not killed by the extracts even after 24h. at any of the concentrations tested (2, 4, or 8 mg/ml). It is concluded that the killing ability of *A. indica* extracts is time and concentration dependent and cell wall related.

**Keywords:** Plant extracts, minimum inhibitory concentrations (MICs), *Azadirachta indica*, minimum bactericidal concentrations (MBCs), kill kinetics, Mueller Hinton Broth, artificial drying, but not for sundrying. The exponential model only agreed accurately with drying of one layer.

## INTRODUCTION

Antimicrobial agents have been used for over 40 years (Zhanel et al., 1991). Dosing regimens for agents were designed from research done on penicillin G (Eagle et al., 1950). These dosing regimens are supposed to maintain antimicrobial serum concentrations above the minimum inhibitory concentration (MIC) (Kunin, 1981). This kind of microbial antibiotic sensitivity research done on penicillin G worked well in practice with other antibiotics for a long time.

Presently the emergence of immuno-compromised cases and new strains of disease causing bacteria require that antibiotics are closely monitored (Kirby and Craig, 1981, Kunin, 1981). For example, a system that shows the rate and extent of bacterial killing (kill kinetics) provides more accurate description of antimicrobial activity than does

the MIC (Zhanel et al., 1991; Craig and Vogelmann, 1987, Vogelmann and Craig, 1986). Kill kinetics have been used to demonstrate better killing synergism in methicillin-susceptible *Staphylococcus aureus* (MSSA) (Tuazon et al., 1978). It has also given better sensitivity trends to physicians than disk diffusion methods (Westh et al., 1992).

*Candida albicans* (a fungus). *A. indica* had shown antimicrobial activity over a wide range of organisms (Fabry et al., 1998). *A. indica* (neem) originated from Sri Lanka, India and Burma (Schmutterer and Ascher, 1986). It is now grown in Bangladesh, Cambodia, Nepal and in many other countries around the world (Ruskin, 1992). In Sri Lanka the seed paste is used as a shampoo for removing lice from the head (Schmutterer and Ascher, 1886). Seed oil is used against worm infection in man and livestock, and also

against chronic forms of skin diseases, stomach ulcer and rheumatism. Neem oil is used to manufacture soap, the use of which safeguards the skin from microbial infection. It has a natural insecticide, azadirachtin (Schmutterer et al., 1980). Other insecticides (limonoids) isolated from the neem include salannin, meliontrirole and nimbin. It was introduced to Africa earlier this century (Ruskin, 1992). In Kenya it grows along the coast and is traditionally used to treat several diseases. The Kiswahili name for the plant is 'Mwarubaini' which means a cure for forty diseases.

## MATERIALS AND METHODS

### Plant Materials

*A. indica* (stembark) was collected by P. Okemo and S. Mathenge from Kwale District along the coastal region of Kenya in September 1994. The taxonomic identification of the plant was established by Mr. Simon Mathenge and a voucher specimen has been deposited in the herbarium, Botany Department, University of Nairobi. The stembark was shade dried and crushed into powder using a crushing machine (Christy and Norris Ltd., Chelmsford, England, Model 8 Lab Mill). The powdered sample was hermetically sealed in polythene bags until the time of extraction.

### Extraction

Dried and powdered plant material (50 g) was Soxhlet extracted with methanol for about 10 h. or until the extract was clear (Chhabra et al., 1982). The solvent was removed under reduced pressure below 50°C to give a crude extract. The crude extract was further dried in a vacuum dessicator over anhydrous copper sulphate to give a dry solid of the extract for bioassay.

## BIOASSAY

### Dilutions

The MICs and minimum bactericidal concentrations (MBCs) for each organism by *A. indica* stembark extracts were reported earlier (Fabry et al., 1998). These were used as a guide for the series of concentrations that were tested. 320 mg of the extract powder was dissolved in 1 ml of dimethyl formamide (DMF). This was made to 10 ml using sterile Mueller Hinton Broth (MHB)-stock solution. 2.5 ml of the stock solution was added to 7.5 ml of MHB to form a final concentration of 8 mg/ml. Doubling dilutions of this concentration were made in MHB containing 2.5% DMF to form concentrations of 4, 2, 1 and 0.5 mg/ml. *S. aureus*, *E. coli* and *P.aeruginosa* were tested on extract concentrations of 2, 4, and 8 mg/ml., while *C. albicans* was tested on 4 and 8 mg/ml.

### Inoculums

Bacteria were grown on blood agar (BA) and *C. albicans* on Mueller Hinton agar (MHA). They were both incubated at 35°C for 24h. Log phase cultures were obtained by suspending about 10<sup>6</sup> to 10<sup>7</sup> cells/ml in MHB and then incubating at 35°C for 24h. 20µl of the 24h culture of each organism were added to each of the 10 ml of the corresponding concentrations of the extract in MHB, and incubated further at 30°C. Initial control counts of organism were obtained by serial dilution and spread plating of 0.1 ml of this inoculum on MHA just before incubation. Subsequent 0.1 ml of each extract concentration were serially diluted and spread planted at intervals of 2, 4, 6, 8 and 24h. All incubated MHA plates were allowed to stand for 30 min at 4°C before spreading. They were incubated at 35°C for 24h. Plate counts were made after 24h of incubation and only plates containing between 30-300 counts for each series of dilutions were counted. The procedure was repeated five times for each organism and the means (X) of the readings computed and recorded.

### Electron micrographs

Mueller Hinton broth solutions containing *A. indica* extracts of various concentrations were withdrawn at intervals of 0, 2, 4, 6, 8 and 24h. The concentration and the time of withdrawal of the samples depended on the trend of counts on duplicated spread plates. Withdrawn samples were fixed with 10% gluteraldehyde and then submitted for electron microscopy.

## RESULTS

The means (X) of readings obtained at each concentration plated for each organism are presented graphically in Figures 1-4. As presented in Fig.1, *S.aureus* was consistently killed by extract concentrations of 0.5 mg/ml. The initial inoculum contained 1.06x10<sup>7</sup> cfu/ml. of the organism. After 24h. the population had been reduced to 2.3x10<sup>2</sup>cfu/ml. A higher extract concentration of 1 mg/ml completely wiped out all viable bacteria after 8h. Subsequent stronger concentrations of the extracts (4 mg/ml and 8 mg/ml) completely killed all viable *S.aureus* cells in 6h.

*E. coli* (Figure 2), did not respond to concentrations of 2 mg/ml and 4 mg/ml. In each case, the counts increased from 2.03x10<sup>4</sup>cfu/ml in the initial inoculum to 2.95x10<sup>8</sup>cfu/ml and 9.2x10<sup>7</sup>cfu/ml respectively in 24h. In the highest concentration tested, (8 mg/ml) the initial inoculum was substantially reduced to 1.1x10<sup>2</sup>cfu/ml in 24h.

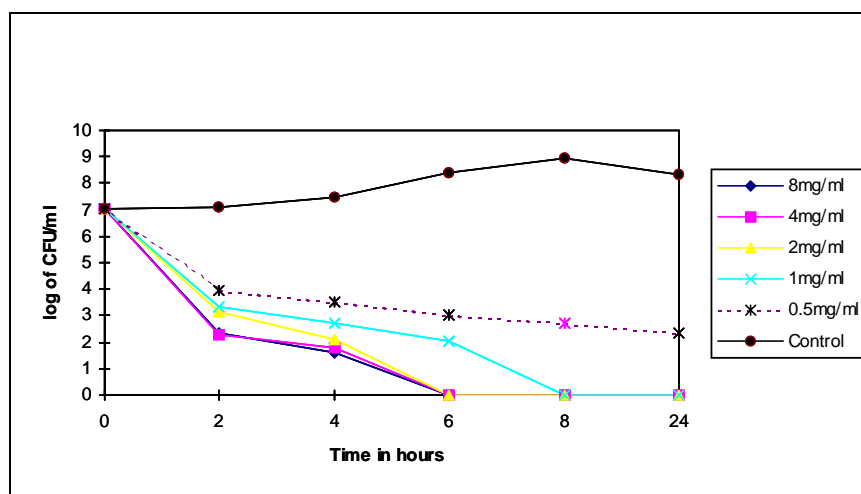


Fig. 1: Activity of varying concentrations of *A. indica* extracts against *S. aureus*

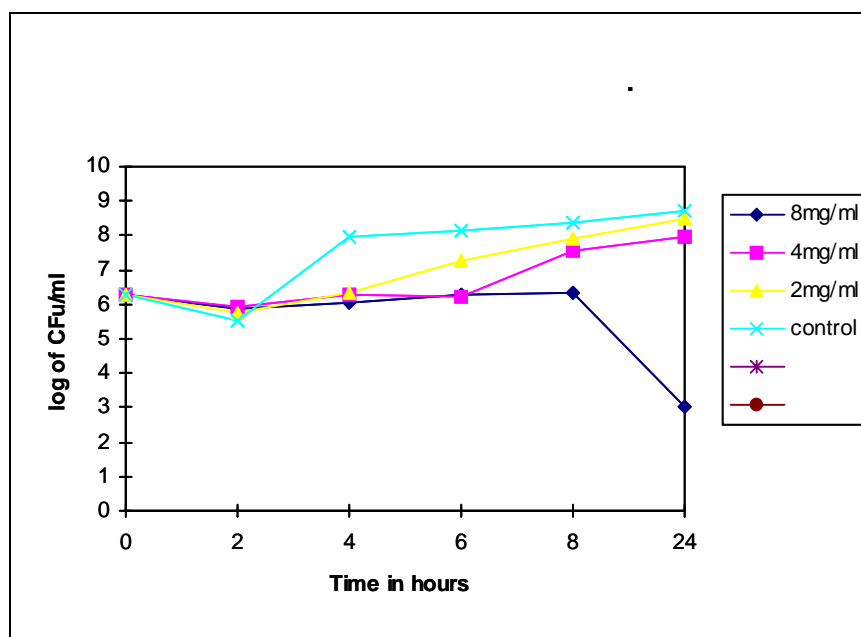


Fig. 2: Activity of *A. indica* extracts against varying concentrations against *E. coli*

*P. aeruginosa* (Figure 3) showed similar trends as *E. coli* in their response to extract concentrations. Concentrations of 2 mg/ml and 4 mg/ml did not significantly reduce the population of the initial inoculum. The response of the organism seems more bacteriostatic than bactericidal. A concentration of 8 mg/ml however has some little effects in the first 6h. The population is nonetheless noticeably reduced after 8h to  $3.4 \times 10^2$  cfu/ml and then increased to  $6.2 \times 10^3$  cfu/ml after 24h.

*C. albicans* (Figure 4) seems to show a fungistatic response for an extract concentration of 4mg/ml throughout the incubation period. An initial inoculum of  $1.4 \times 10^4$  cfu/ml is reduced to  $6.8 \times 10^3$  cfu/ml in 8h but increases to  $3.3 \times 10^4$  cfu/ml in 24h. A concentration of 8mg/ml initially reduces the population slightly but after 8h the population is drastically reduced and completely killed after 24h.

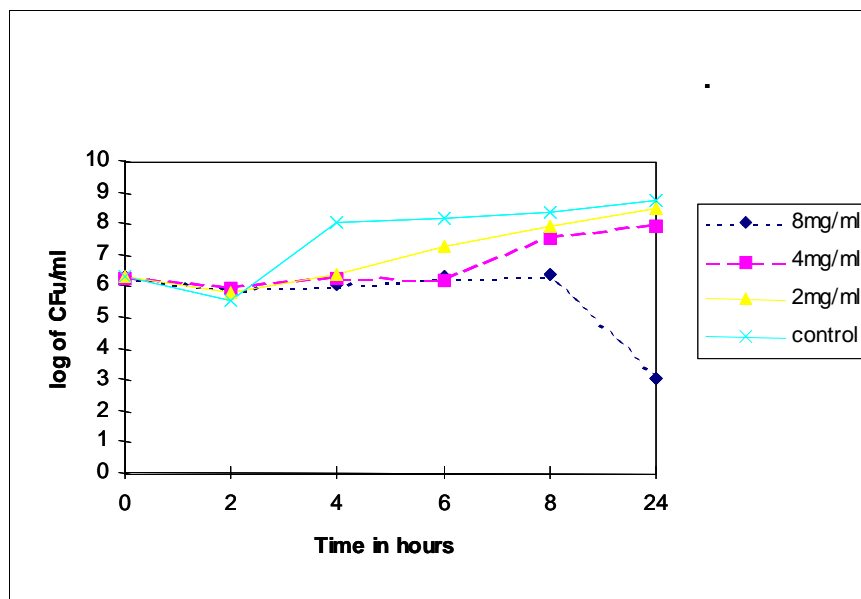


Fig. 3: Activity of varying concentrations A. indica extracts against Pseudomonas aeruginosa

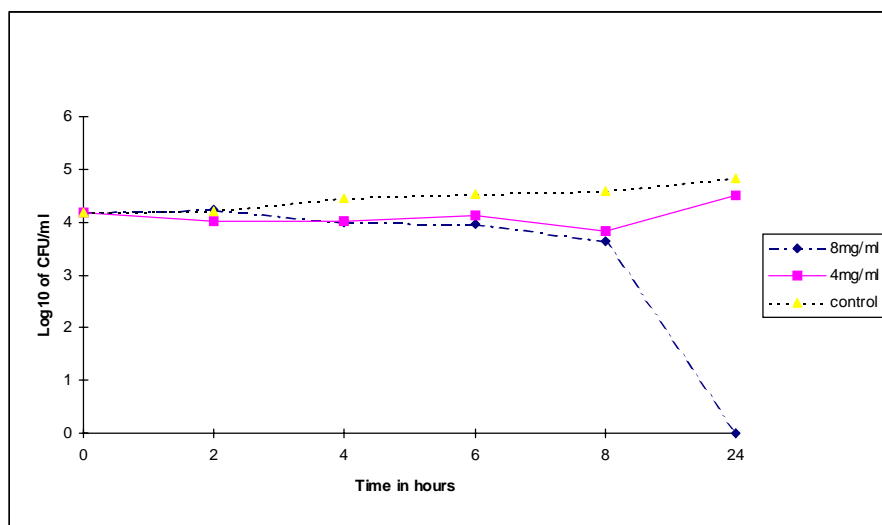


Fig. 4: Activity of varying concentrations of A. indica extract against Candida albican

### DISCUSSION

These results show that at sub-inhibitory concentrations ( $1/4 \times$  and  $1/2 \times$  MIC); bacterial growth for *P.aeruginosa* is decreased for 4h and remains static for the rest of the time (Figure 3); Inhibitory concentration ( $1 \times$  MIC) has very effective killing for 8h then resistant strains start to develop and even increase in population. Zhanel et al., (1991) observed similar post antibiotic effect (PAE) on the activity

of aminoglycosides against *P.aeruginosa*. *P.aeruginosa* is very adaptable and has been incriminated in several infections for its tolerance to antibiotics (Morse et al., 1986). If MIC determination by disk diffusion methods had been used to assess antimicrobial activity, it would have been difficult to notice the PAE in *P.aeruginosa* to *A.indica* as an antimicrobial agent.

*E. coli* (Figure 2) was tested at  $1/4$  x and  $1/2$  x MIC and found to decline in population for 2h then rapidly increase almost at the same rate as the control. The mechanisms by which microorganisms generally survive the action of antimicrobial agents are poorly understood and remain debatable (Woolfrey et al., 1990). This is true for the case of *E. coli* also, because the resistance attributed to *Pseudomonas* could be capsule related, but that of *E. coli* could probably be due to genetic factors or due to cell membrane permeability. A higher dosage of *A. indica* (1 x MIC) has the initial effect to decline in growth in the first 2h. This is followed by a paradoxical growth effect and a final continued slow population decrease. Simard and Bergeron (1982), found *Haemophilus influenzae* strains slowly killed by cefoperazone (MBC and MIC >32µg/ml) and mentioned that this could be due to the production of cell wall defective forms. When the *E. coli* used in this investigation was subjected to electron microscopy it indeed showed cell wall defective forms (Okemo, 1996).

*S. aureus* (figure 1) and *C. albicans* (figure 4) have both showed that inhibitory concentrations (1 x MIC) completely wiped out the organisms in 6 and 24h respectively. It is also noted that for *S. aureus* the killing was both dosage and time dependent and this is a more rational basis for determining optimal dosage for antimicrobial treatment regimens (Chalkley and Koornhof, 1985). The fact that both organisms, *S. aureus* and *C. albicans* which are gram positive are wiped out in less than 24h, unlike the gram negative rods, *P. aeruginosa* and *E. coli* is interesting. Perhaps the mode of action of *A. indica* extracts is indeed strongly cell wall related. When timed electron microscopy of *P. aeruginosa* were taken, "L" forms of the organism were noticed to have developed (Okemo, 1996).

The *Pseudomonas* (figure 3) and *Escherichia* (figure 2) which are not completely killed are likely to have reverted to "L" forms of the organisms (Woolfrey et al., 1990). This phenomenon is observed even after 24h of exposure and takes place in several organisms considered to be resistant to antibiotics. Such organisms usually revert to parental forms when the cell wall inhibiting agent is removed. It is proposed in this study that since *Pseudomonas* are increasingly resistant to commonly used antibiotics, a combination of the antibiotics for which *Pseudomonas* resistance has been observed should be applied to the organism together with extracts of *A. indica* to determine if the combination can kill the organisms. *A. indica* has shown that it affects all the cell walls of the organisms tested in this study. Other studies have shown that some antibiotics affect organisms in different ways than the cell wall (Morse et al., 1986). For example ;intercalating drugs such as Proflavin are inhibitors of nucleic acid synthesis. Proflavin may not have strong cell wall penetrating mechanisms on

its own, but may be useful when combined with *A. indica* extracts.

Most studies have isolated the majority of compounds found in *A. indica* (Ascher, 1981; Schumutterer and Zebitz, 1983). Of all the compounds isolated from the plant, azadirachtin is the most toxic (Ascher, 1981). Other less toxic compounds isolated include, melianol, salannin, nimbin and vepaol (Schumutterer and Zebitz, 1983). If *A. indica* extracts are used as chemotherapeutic agents in proper doses as this study proposes, they are therefore unlikely to be harmful. Already, neem products are consumed by humans and livestock along the coast of East Africa (Okemo et al, 1998) and in Asia (Okemo et al., 1999). Toxicity studies done by Arnason et al. (1989) showed that neem plant concoctions can be used in fair quantities without apparent hazardous consequences. Besides, the seed kernel cake has been successfully used as protein supplements for livestock (Anandan et al., 1996; Nagalakshmi et al., 1996; Verma et al., 1996). This study therefore can still safely propose the use of neem compounds as chemotherapeutic agents to control human and fungal pathogens if used within predetermined concentrations.

#### ACKNOWLEDGEMENTS

The authors are grateful to Mr. Simon Mathenge, Botany Department, University of Nairobi for assisting in the collection and identification of plant materials. The Deutscher Akademischer Austauschdienst, who financed a 5-month stay for Dr. P. Okemo at the Institute of Medical Microbiology, University of Essen, Germany and to Dr. Ernst N. Schmid in whose Laboratory electron Micrographs were taken.

#### REFERENCES

- Anandan, S., Sastry, V.R.B., Musalia, L.M. and Agrawal, D.k. (1996). Growth-rate and nutrient efficiency of growing goats fed urea ammoniated neem (*Azadirachta indica*) seed kernel meal as Protein-supplement. *Small Ruminant Research*, 22:205-212.
- Arnason, J.T., Morand, P., Pinogene, R.T. (1989). Insecticides of plant origin: *American chemical society symposium, script 387*, Washington D.C. pp.110.
- Ascher, K.R.S. (1981). Some physical properties and biological effects of a dried Methanolic neem seed kernel extract. In: *Proceedings of 1<sup>st</sup> International Neem Conference*. Rottach Eern. Pp.63-74.

- Chalkley, L.J.; Koornhof, H.J. (1985). Antimicrobial activity of ciprofloxacin against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* determined by the kill curve method: Antibiotics comparison and synergistic interactions. *Antimicrobial Agents and Chemotherapy* 28:331-342.
- Chhabra, S.C.; Shao, J.F. and Mshiu, E.N. (1982). Antifungal activity among traditionally used herbs in Tanzania. *Dar-es-Salaam Medical Journal*, 9:68-73.
- Craig, W.A.; Vogelman, B. (1987). The Postantibiotic effect. *Annals of Internal Medicine*. 106: 900-902.
- Eagle, H.; Fleishman, R., Musselman, A.D. (1950). Effects of Schedule of administration on the therapeutic efficacy of penicillin. *American Journal of Medicine*, 9:280-299.
- Fabry, W.; Okemo, P.O. and Ansorg, R. (1998). Antibacterial activity of East African Medicinal Plants. *Journal of Ethnopharmacology*, 60:79-84.
- Kirby, W., Craig, W.A. (1981). Theory and application of pulse dosing: A summary of The symposium. *Review of Infectious Disease*, 3: 28-37.
- Kunin, C.M. (1981). Dosage schedules of antimicrobial agents: A historical review. *Review of Infectious Disease*, 3: 4-11.
- Morse, S.A.; Johnson, S.R.; Biddle, J.W., and Roberts, C.M. (1986). High-level of tetracycline resistance in *Neisseria gonorrhoeae* is a result of acquisition of streptococcal tet M. determinant. *Journal of Antimicrobial Agent and Chemotherapy*, 30(5): 664-670.
- Nagalakshmi, D.; Sastry, V.R.B.; Agrawal, D.K.; Katiyar, R.C.; and Verma, S.V.S. (1996). Performance of broiler chicks fed on alkali-treated neem, (*Azadirachta indica*) kernel cake as a protein supplement. *British Poultry Science*, 37: 809-818.
- Okemo, P. O. (1996). Antimicrobial efficacy of selected medicinal plants used by Kenyan herbal doctors. Ph. D. Thesis pp158-196.
- Okemo, P.O.; Mwatha, E.W.; and Chhabra, S.C. (1998). Antibacterial activity of plant extracts. A comparison of agar dilution and microtitre broth dilution methods. *Discoveries and Innovations*, 10(1/2), 111-115.
- Okemo, P.O.; Mwatha, E.W. and Ngigi, S.K. (1999). Activity of some medicinal plant Pathogens. *East Africa Journal of Science* 1(2): 1-7.
- Ruskin, F.R. (1992). Neem-a tree for solving global problems. Report of an ad hoc panel of the Board on Science and Technology for International Development. *National Research Council, National Academic Press*, Washington, D.C. pp.22-39.
- Schumutterer, H.; Ascher, K.R.S. and Rembold, H. (1980). Nature pesticides from the neem tree (*Azadirachta indica* A. Juss) Eschborn: *German Agency for Technical Cooperation*.
- Schumutterer, H. and Zebitz, C.P.W. (1983). Effects of methanolic extracts from seeds of a single neem tree of African and Asian origin, on *Epilachna varivestis* and *Aedes aegypti*. *Proceedings of 2<sup>nd</sup> International Neem Conference*. Ranisohholzhausen, pp.83-90.
- Schumutterer, H.; Asche, K.R.S. (1986). Natural pesticides from the neem tree (*Azadirachta indica* A. Juss) and other tropical plants. *Proceedings of the Third International Neem Tree Conference (10-15 July 1986)*, Nairobi.
- Simard, P. and Bergeron, M.g. (1982). Inoculum side effect on the MIC of Cefoperazone, Mexalactam, Cefotaxime, Cefoxitin and Cephalothin for 118 strains of *Haemophilus influenzae*, including 'tolerant' microorganisms. *Journal of Antimicrobial Agents and Chemotherapy*: 10: 397-402.
- Tuazon, C.V., Lin, M.Y.C., and Sheaghen, J.N. (1978). In vitro activity of rifampin alone and in combination with nafcillin and vancomycin against pathogenic strains of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 13: 759-761.
- Verma, A.K.; Sastry, R.B. and Agrawal, D.K. (1996). Chevon characteristics of goats fed diets with water-washed neem (*Azadirachta indica*) seed kernel cake. *Small Ruminant Research*. 19: 55-61.
- Vogelman, B. and Craig, W.A. (1986). Kinetics of antimicrobial activity. *Journal of Paediatrics*, 108: 835-840.
- Westh, H.; Fimodt, N., Crutschick, E. and Bangsberg, J. (1992). Killing curve activity of Ciprofloxacin is comparable to synergistic effect of  $\beta$ -lactam tobramycin combination against *Haemophilus* species endocarditis strains. *APMIS*. 100:856-860.
- Woolfrey, B.F. and Enright, M. (1990). Ampicillin killing curve patterns for Ampicillin-susceptible non-typeable *Haemophilus influenzae* strains by the agar dilution plate count method. *Antimicrobial Agents and Chemotherapy*, 39: 1074-1087.
- Zhanel, G.G., Karlowsky, J.A., Bohan, D.J., and Davidson, R.J. (1991). Antimicrobial activity of sub-inhibitory concentrations of aminoglycosides against *Pseudomonas aeruginosa* as determined by the postantibiotic effect. *Antimicrobial agents and Chemotherapy*, 37: 114-121.