CIBTech Journal of Pharmaceutical Sciences ISSN: 2319–3891 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/cjps.htm
2014 Vol.3 (2) April-June, pp.07-14/Odumosu et al.
Research Article

ANTIMICROBIAL SCREENING AND KINETIC STUDY OF TWO NIGERIAN MEDICINAL PLANTS AGAINST ORAL PATHOGENS

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ABSTRACT
Oral diseases associated with microbial infections are of serious health concerns. There is a need for the use of medicinal plants as an alternative remedy against the growing antimicrobial resistant strains. Antimicrobial activities of methanolic extracts of dried stems of *Terminalia glaucescens* and *Anogeissus leiocarpus*were investigated against 7 clinical strains of *Streptococcus mutans*, and 3 *Candida* species (*C. albicans*, *C. tropicalis* and *C. krusei* ATCC 6825) by agar well diffusion assay. Zones of inhibition produced by different extracts against the test isolates were compared with standard antibiotic disc. The minimum inhibitory concentration (MIC) of the extracts ranged from 3.125 to 100mg/mL. Extracts of *T. glaucescens* displayed the highest activity against all the tested isolates and was able to kill *S. mutans* and *C. albicans* completely within 4h in the time kill studies.The phytochemical screening of the extract revealed the presence of taninns, saponins and flavonoids. *Terminalia glaucescens* and *Anogeissus leiocarpus* are promising alternative chemotherapeutic agent which can be use as a novel drug in replacement of convectional antibiotics.

INTRODUCTION
Oral diseases and associated infections in humans are one of the major public health concerns with high prevalence and impact in all regions of the world. Dental caries, also known as tooth cavities or tooth decay is the most prevalent among oral infection characterized by progressive destruction of the mineralized tissues of the tooth from the surface (Robinson *et al.,* 2000). Microorganisms that colonize the tooth surfaces plays a major role in dental caries by metabolizing and producing sufficient acids to demineralise the enamel covering of the tooth crown or the cementum covering the root (Liljemark and Bloomquist, 1996). Dental caries and other periodontal diseases as well as many disease of the mucous membrane such as tongue, and salivary glands are opportunistic infections (Loesche, 2007). Oral infections have profound systemic as well as local effects hence maintenance of oral health goes beyond the physiological needs of proper nutritional intake and protection of the oral tissues. It also includes protection against oral sources of systemic infection and encompasses a range of social and psychological attributes (Mandel, 2002). Streptococcus mutans is considered as one of the main etiological agents of human dental caries due to its efficient dental colonization and ability to dominate the major biofilm in the oral cavity as a result of its acidogenic and acidoduric nature which facilitate its role in development of root caries (Marsh, 2006, Marsh, 2009).

Terminalia glaucescens Planch. Ex Bth. and *Anogeissus leiocarpus* (DC) Guill and Perr are two important plants in the order *Myrtales* belonging to the family Combretaceae commonly used as chewing sticks among the South Westerners in Nigeria. Both plants are known especially as a source of secondary metabolites, such as alkaloids, phenols, saponins, steroids, glycosides, flavonoids, tannins and other aromatics and their derivatives. Some of these substances have antifungal, antibacterial, anti-cancer and hepatoprotective indications (Mann *et al.,* 2008). Ethnopharmacologically, the root and stem bark of *T. glaucescens* and *A. leiocarpus* has been reported to be used traditionally in the treatment of broad spectrum of infections, including diarrhoea, dysentery, tuberculosis, cough, asthma, stomach disorder, leprosy, schistosomiasis and treatment of infectious wound (Burkill, 1985; Ibrahim *et al.,* 1997). Antimicrobial activities of the aqueous and ethanol root extracts of both plants against Gram positive and

Gram negative bacteria has been previously reported (Ndukwe *et al*., 2005; Ogundiya *et al.,* 2007) Other pharmacological activities of the plant already investigated include antimalarial, leishmanicidal, trypanocidal, antihelminthiasis and antiscabies activities (Okpekon *et al.,* 2004).

Most of the available studies on plants used as medicinal purpose particularly chewing sticks, report their antimicrobial activities on Gram-negative bacteria and other human infections. Since these plants have gained a wide use as chewing sticks, there is a need for more information on their antimicrobial activities against pathogens mostly implicated in orodental infections such as *S. mutans* and *Candida* spp. Such studies will ameliorate the search for natural products that will enhance or alternate the current antibacterial and antifungal drugs in clinical use especially in the treatment of oro-dental infections, which are gradually suffering from resistant strains of bacteria.

The purpose of this study is to determine the antimicrobial activities and kinetics of killings of *T. glaucescens* and *A. leiocarpus* against clinical strains of *Streptococcus mutans* isolated from individuals with active dental caries, as well as some *Candida* spp in Ibadan, Oyo state Nigeria.

MATERIALS AND METHODS

***Plants Collection*:** *T. glaucescens* and *A. leiocarpus* stems were collected in Onigambari in Oyo state and authenticated at the Forest Research Institute of Nigeria (FRIN) Herbarium with specimen numbers FHI 108282 and FHI 108279 respectively. Both plants were air-dried at room temperature for 2 months, pulverized and stored at 4^{o}C before use in this study.

Preparation of Methanolic Extract: Five hundred grams (500g) of each pulverized plants samples was exhaustively extracted with (70%) methanol in a soxhlet extractor. Extracts were evaporated to dryness using a rotary evaporator at 40^{o}C and weighed. This was stored in a glass vials and kept at 4^{o}C before use.

Phytochemical Screening: A small portion of the dry powder was used for the preliminary phytochemical screening for secondary metabolites such as tannins, saponins, anthraquinones e.t.c as previously described (Harborne, 1998).

Bacterial Strains: *Candida krusei* ATCC 6825 is a standard strains while *Candida albicans* and *Candida tropicalis* are industrial cultures obtained from Federal Institute of Industrial Research (FIIRO) Lagos. *Streptococcus mutans* strains were isolated from dental plaque and saliva samples of human volunteers with active caries according to the protocol previously described (Takada and Hirasawa, 2005). A written informed consent was obtained from the selected participants. All *S. mutans* strains were grown on brain heart infusion broth (Difco Laboratories), as well as on Mueller Hinton agar (Oxoid England) with bacitracin (100 U/ml Glaxosmithkline), 5% defibrinated blood and 20% sucrose. All the strains were stored at 4^{o}C until required for further study.

Preliminary Antibiotic Sensitivity Test:

The antibiogram of the test isolates (*S. mutans*) against augument in (30µg), amoxicillin (25µg), erythromycin (30µg), cotrimoxazole (25µg), gentamicin (10µg), ofloxacin (5µg), cloxacillin (5µg) and tetracycline (30µg) was carried out by Kirby Bauer disk diffusion method (Bauer, 1966)

Determination of Antimicrobial Activity

Antimicrobial activities of both extracts were determined by agar well diffusion assay as described previously (Adeniyi *et al.,* 2010) with modifications. Two hundred microlitre of McFarland standard overnight broth cultures of the test organism were added to molten Mueller Hinton agar (Oxoid, England) at 40 – 45^{o}C. Agar plates were allowed to set and hardened before boring holes using a sterile cork borer of diameter 8mm. A 50µl of each extracts at varying concentration 200mgmL,$^{-1}$, 100 mgmL^{-1}, 50 mgmL^{-1}, 25 mgmL^{-1}, 12.5mgmL^{-1}, 6.25mgmL^{-1}, 3.125mgmL.$^{-1}$were carefully introduced into each corresponding well and then allowed to diffuse for 1 hr before incubation. The medium for *S. mutans* was supplemented with 5% defibrinated blood while fungal carpeted method on Sabouraud Dextrose agar (Oxoid, England) was used for the fungal assay as previously described (Adeniyi *et al.,* 2010). To ensure quality control, gentamicin and ketoconazole were used as positive controls for the antibacterial and antifungal assay respectively. Experiments were carried out in duplicate to ensure accuracy of results.

The minimum inhibitory concentration (MIC) of the extracts against the organism was determined by the agar dilution method as described previously by Okeke *et al.,* (1999) with modifications. One thousand microlitre of different dilutions of the extracts in the following concentrations 200mgmL^{-1},100 mgmL^{-1}, 50 mgmL^{-1}, 25 mgmL^{-1}, 12.5 mgmL^{-1},6.25 mgmL^{-1}, 3.125 mgmL^{-1} were each added to exactly 19mL of a sterile molten Mueller Hinton agar maintained at 45^{o}C, properly mixed for even distribution and allowed to set. Each plate was divided into six sections. Bacteria culture adjusted t 0.5 McFarland standard culture was used to inoculate each section of the solidified agar-drug mixture in duplicates. A medium without extract was similarly inoculated and served as quality control. The plates were examined for the presence of colonies after the incubation period. The least concentration that gave no visible colonies was taken as the minimum inhibitory concentration of the extract for the particular dilution of the organism while the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) was recorded as the lowest concentration preventing a growth after sub-culturing the organisms into another solid media without any extracts (recovery media) (Aibinu *et al.,* 2007).

Bactericidal Activity of the Extract: Kinetics of antimicrobial activities was carried out according to method described by Rotimi *et al.,* (1988) with modifications. Killing curves were determined for each bacterial and fungal strain in 5-ml glass tubes with samplings at 0, 30, 60, 120, 180 and 240 mins. Extracts were tested at 2 × MIC, 4 × MIC and 8 × MIC. The tubes were inoculated with 100µl of 24 h broth culture diluted to give a final concentration of about 10^6 cfu/ml. Ten microlitre (10 µl) of the test sample (extract-culture mixture) was withdrawn immediately, diluted out in normal saline and two drops of each dilution plated. Broths without any extracts were inoculated with the same test organisms for quality control. The media for *S. mutans* was supplemented with 5% defibrinated blood. The procedure was carried out in duplicate to ensure reproducibility. Viable counts were done at 24 h and after 48 h of incubation. A graph of percentage viable count against time in minutes was plotted on a semi-logarithm graph.

RESULTS

Phytochemical screening of the plants revealed the presence of tannins, saponins and flavonoids in varying proportions in both plants. Resistance pattern of the *S. mutans* isolates against selected antibiotics is shown (Table 1). Result of the antibiogram shows that the clinical isolates are resistant to most of the antibiotics tested. All the crude methanolic extracts of both plants demonstrated varying degrees of antimicrobial activities against both bacteria and fungi investigated with notable zones of inhibitions (Tables 2, 3). However, the results of the methanolic extracts of both plants are comparable with that of standard antibiotics. From the results obtained from the antimicrobial susceptibility studies of the plant extracts, extract of *T. glaucescens* was more active against the entire organisms in this study compared to the extracts of *A leiocarpus*. The results of MIC, MBC and MFC assay of both extracts against the bacteria and fungi varied from plant to plant extracts and are shown in Table 4. The MICs obtained from both extracts against the tested pathogens ranged from 3.125 to 100mg/mL.

Table1: Antibiotics susceptibility testing result for *S. mutans* isolated from individual

Organisms	Source	Resistance phenotypes
S. mutans ODM 01	Saliva	AUG, AMX, ERY, CXC
S. mutans ODM 02	Dental plaque	AUG, AMX, ERY, GEN, CXC
S. mutans ODM 03	Dental plaque	AUG, AMX, ERY, GEN, CXC, TET
S. mutans ODM 04	Dental plaque	AUG, AMX, ERY, GEN, CXC, TET
S. mutans ODM 05	Dental plaque	AUG, AMX, ERY, COT, GEN, CXC, TET
S. mutans ODM 06	Saliva	AUG, AMX, ERY, GEN, CXC
S. mutans ODM 07	Saliva	AUG, AMX, ERY, COT, CXC

Table 2: Antibacterial screening of the extracts showing varing concentrations

Extracts		*Streptococcus mutans* strains Diameter zone of inhibition (mm)							
	Concentration mg/mL	ODM 01	ODM 02	ODM 03	ODM 04	ODM 05	ODM 06	ODM 07	*S. aureus*
Anogeissus leiocarpus	200	13±0.00	13±0.50	13±0.00	13±0.00	13±0.00	8±0.00	13±0.00	14±0.50
	100	10±0.00	10±0.00	11±0.00	11±0.00	10±0.00	8±0.00	10±0.00	12±0.00
	50	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	10±0.50
	25	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00
	12.5	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00
	6.25	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00
	3.125	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00
Terminalia glaucescens	200	18±0.00	18±0.00	16±0.00	16±0.00	15±0.00	16±0.00	20±0.00	18±0.00
	100	17±0.00	15±0.50	14±0.00	14±0.00	13±0.00	14±0.00	18±0.00	17±0.50
	50	16±0.00	14±0.00	13±0.00	11±0.00	10±0.00	13±0.00	15±0.00	15±0.50
	25	15±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	11±0.00	14±0.00
	12.5	14±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	9±0.00	12±0.00
	6.25	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	10±0.50
	3.125	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00	8±0.00
Gentamicin	7.8*	20	17	22	20	20	22	23	25±0.00
Methanol 50%		—	—	—	—	—	—	—	—

**Gentamicin concentration is in µg/ml, Diameter of cork borer = 8mm. All experiment were performed in duplicates*

Table 3: Antifungal screening of the extracts

Extracts		Organisms Diameter zone of inhibition (mm)		
	Concentration mg/mL	*C. albicans*	*C. tropicalis*	*C. krusei*
Anogeissus leiocarpus	200	16±0.00	12±0.50	32±0.50
	100	14±0.50	10±0.00	30±0.50
	50	13±0.50	8±0.00	25±0.50
	25	12±0.00	8±0.00	20±0.00
	12.5	11±0.50	8±0.00	19±0.50
	6.25	10±0.00	8±0.00	17±0.00
	3.125	8±0.00	8±0.00	14±0.50
Terminalia glaucescens	200	18±0.00	20±0.00	33±0.00
	100	16±0.50	18±0.00	25±0.00
	50	14±0.50	14±0.50	22±0.00
	25	12±0.50	12±0.00	20±0.00
	12.5	10±0.00	10±0.50	18±0.00
	6.25	8±0.00	8±0.00	15±0.00
	3.125	8±0.00	8±0.00	13±0.00
Gentamicin	7.8*	28±0.00	30±0.00	35±0.00
Methanol 50%	—	—	—	—

**Gentamicin concentration is in µg/ml, Diameter of cork borer = 8mm,*
All experiment were performed in duplicates

Table 4: Minimum inhibitory, bactericidal and fungicidal concentrations of *T. glaucescens* and *A. leiocarpus*

Test Organisms	Concentrations (mg/ml)			
	T. glaucescens		*A. leiocarpus*	
	MIC	MBC	MIC	MBC
S. mutans	25.00	50.00	100.00	200.00
C. krusei	6.25	12.5	3.125	25.00
C. albicans	25.00	50.00	25.00	50.00
C. tropicalis	25.00	50.00	25.00	50.00

DISCUSSION

Effective oral care is paramount especially in this era of infectious diseases and alarming rate of resistance to chemotherapeutic agents. Hence an alternative method of combating such menace especially pathogenic organisms implicated in several oral infections through medicinal plant is essential. *T. glaucescens* and *A. leiocarpus* extracts assayed in this study have demonstrated good antimicrobial activities as evidenced in zones of inhibition on the selected microorganisms. From the result of the present study, the extracts of *T. glaucescens* displayed more antibacterial activities compared to extracts of *A. leiocarpus*, while both plants extracts displayed better antifungal activities at lower concentrations (≥62.5mg/ml) than antibacterial. This is suggestive of the presence of more antifungal principles than antibacterial in both plant extracts. There was also an observed decreased in *A. leiocarpus* extracts activities against the bacterial isolates with MIC of 100mg/mL, compared to its antifungal activity which was demonstrated against *C. krusei* ATCC 6825 with MIC of <3.125mg/mL. This may also suggest the presence of more antifungal principles than antibacterial in the extract. This current findings on antifungal activities of *A. leiocarpus* corresponds with a previous a study by Mann *et al.,* (2008b). The minimum inhibitory concentration (MIC≤25mg/mL) observed for *T. glaucescens* against *S. mutans* in this study is higher than a previously reported study from Oshomoh and Idu (2011) whose MIC was 6.25mg/ml against S. auricularis and S. mutans. Although the MIC obtained against fungal isolates in this study corresponds with their results, the observed higher antibacterial MIC in our study could be due to the choice of solvent for the extraction in this study. The ability of plant extract to efficiently kill or inhibit the growth of a bacterial or fungal isolate indicates the presence of active antimicrobial principles in the extract. This suggests that the extracts of both *T. glaucescens* and *A. leiocarpus* contains active phytochemical compounds with appreciable antimicrobial activities. The phytochemical screening of the two plants extracts revealed the presence of the following secondary metabolites; tannins, saponins and flavonoids. These secondary metabolites have been previously reported to possess antibacterial and antifungal activities (Man *et al.,* 2008). The fact that these plant species are being used locally and as a means of oral hygiene (Rotimi *et al.,* 1988) suggests that they are active against microbes although the level of active principles extracted in this study is low or a different technique may be is required for a better antibacterial activities. The presence of tannins in both plants especially *T. glaucescens* could explain the reason for its broad spectrum of activity. Burapadaja and Bunchoo (1995) attributed the inhibition of cell wall formations in fungi leading to cell death to the presence of tannins in *Terminalia citrine* extracts. Similar findings were observed in this study as the fungi isolates investigated displayed a high sensitivity to the extracts compared to the bacterial isolates. *Candida krusei* in particular was found to be inhibited at larger zones of inhibitions compared to other fungi. The reason for this is unclear. The susceptibility of *S. mutans* to *T. glaucescens* in this study suggests that the plant can effectively reduce plaque and thus prevent caries among its users since *S mutans* is known to dominate the major biofilms in the oral cavity due to its acidogenic and aciduric nature (Marsh, 2006). The bactericidal activity of the *T. glaucescens* corresponds well with its MIC, killing both *S.mutans* and *C. albicans* within 4hrs of contact time. Destruction of these oral floras in the kinetic study suggests the plant active metabolites are capable of inhibiting the growth of microorganism.

Conclusion

Terminalia glaucescens and *A. leiocarpu*s stems extracts has demonstrated good antibacterial and antifungal properties against the selected oral pathogens. The presence of secondary metabolites in the plants could be responsible for the antimicrobial activities. Therefore the use of both plants as chewing sticks for maintaining oral health should be encouraged. Our findings in this study suggests the use of these plants as an alternative means of oral chemotherapeutic agent. The reported activities as well as traditional and local uses of these plants against several infectious diseases showed that they are promising chemotherapeutic agent which could be use as a novel drug in replacement of convectional antibiotics which have suffered resistance by some microorganisms.

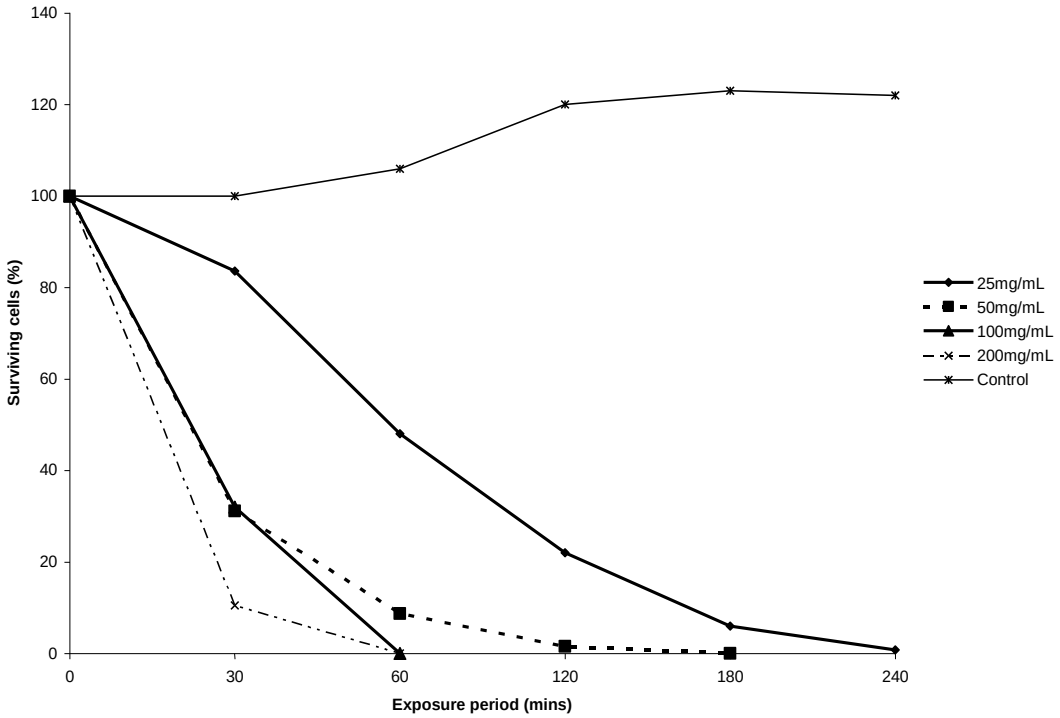

Figure 1: Kinetics of bactericidal activities of *Terminalia glaucescens* on *Candida albicans*

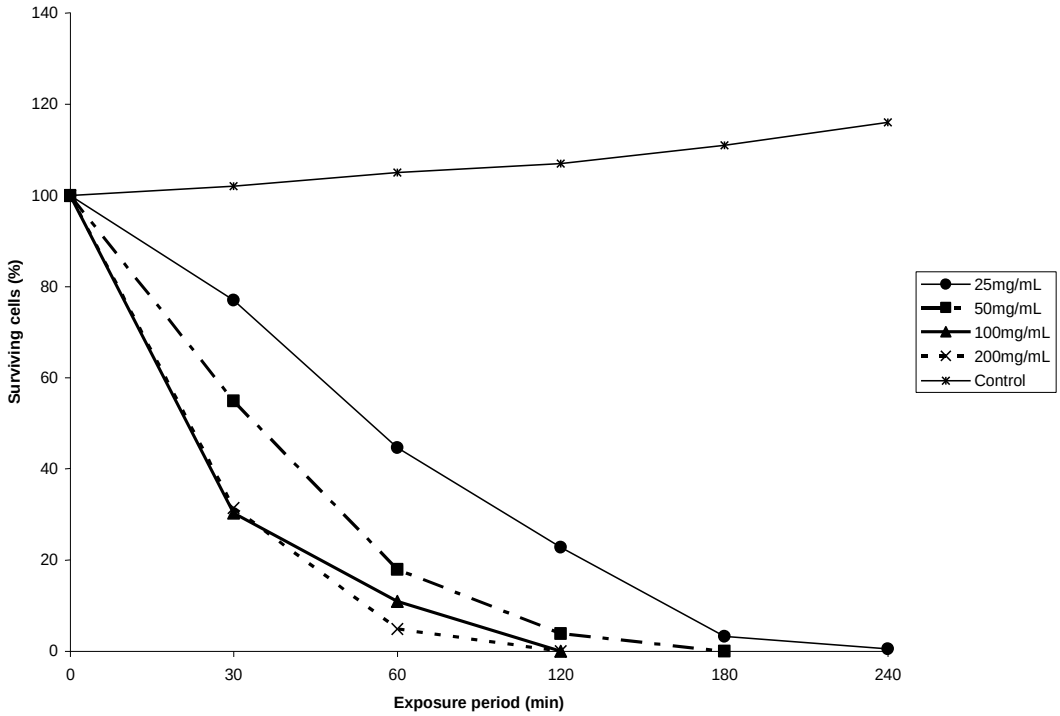

Figure 2: Kinetics of bactericidal activities of *Terminalia glaucescens* on *Streptococcus mutans*

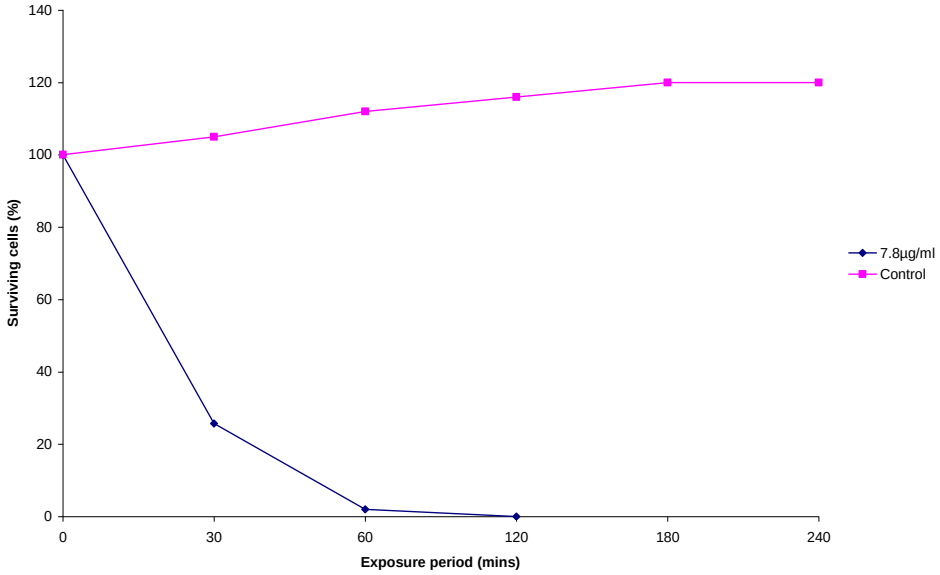

Figure 3: Kinetics of bactericidal activities of Gentamicin on *S. mutans*

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