Affinity Effects of Some Antibiotics on Xanthine Oxidase Enzyme Activities *In Vitro*

Bazı Antibiyotiklerin Ksantin Oksidaz Enzimi Üzerine In Vitro Afinite Etkileri

Research Article / Araştırma Makalesi

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ABSTRACT

Xanthine oxidase (XO) is the last enzyme of purine catabolism. XO was separately purified by ammonium sulphate precipitation and affinity chromatography. The effect of some antibiotics which is commonly used in clinical on purified xanthine oxidase was determined *in vitro*. The name of antibiotics was gentamycin sulfate, sodium ampicillin, cefazolin sodium, chlarithromycin, rifamycin SV, clindamycin phosphate and kanamycin sulfate. XO was determined using xanthine as a substrate and IC_{50} values of these antibiotics exhibiting inhibition effects were found from graphs of activity (%) by plotting concentration of the antibiotics. Macrolid group of antibiotics, the effects of gentamycin sulfate and kanamycin sulfate were determinated on xanthine oxidase. Gentamycin sulfate increased xanthine oxidase enzyme activity but kanamycin sulfate caused an inhibitory effect on xanthine oxidase enzyme activity. In addition, sodium ampicillin and rifamycin SV caused activation on enzyme activity. Cefazolin sodium, chlarithromycin and clindamicin phosphate indicated inhibitory effect on xanthine oxidase enzyme activity. Especially, cefazolin sodium is the most effective inhibitor in studied antibiotics with the value of 5.4×10^{-4} mg/mL. This value is close to the other values found for XO's classical inhibitors.

Key Words
Xanthine oxidase, clarithromycin, gentamycin sulphate, sodium ampicillin, cefazolin sodium, clindamycin phosphate, rifamycin SV, kanamycin sulphate, inhibition.

ÖZET

Ksantin oksidaz (KO), pürin katabolizmasının en son enzimidir. KO, amonyum sülfat çöktürmesi ve afinite kromatografisi ile saflaştırıldıktan sonra tıpta yaygın olarak kullanılan antibiyotiklerin etkileri incelendi. Etkisi araştırılan antibiyotikler; klaritromisin, gentamisin sülfat, sodyum ampisilin, sodyum sefazolin, klindamisin fosfat, rifamisin SV, kanamisin sülfattır. Antibiyotiklerin inhibisyon etkileri IC_{50} değerleri ile verilirken, ksantin oksidazın enzim aktivitesi, substrat olarak ksantin bileşiğini kullanılarak ölçüldü. İnhibisyon konsantrasyonuna karşı % enzim aktivitesi grafiği çizildi ve grafikten yararlanılarak IC_{50} değerleri hesaplandı. Ksantin oksidaz enzimi üzerine makrolid grubu antibiyotiklerinden gentamisin sülfat ve kanamisin sülfatın etkisi incelendi, gentamisin sülfat ksantin oksidazın enzim aktivitesini artırırken kanamisin sülfat enzim aktivitesini azaltmıştır. Ayrıca sodyum ampisilin ve rifamisin SV de enzim aktivitesini artırmıştır. Sefazolin sodyum, klaritromisin ve klindamisin fosfat enzim aktivitesini inhibe etmişlerdir. Özellikle, sefazolin sodyum, 5.4×10^{-4} mg/mL IC_{50} değeri ile çalışılan antibiyotikler arasında en etkili inhibitördür. Sefazolin sodyum için bulunan IC_{50} değeri, ksantin oksidazın bilinen diğer inhibitörleri ile kıyaslandığında oldukça yakın bir değer olduğu görülmektedir.

Anahtar Kelimeler
Ksantin oksidaz, klaritromisin, gentamisin sülfat, sodyum ampisilin, sodyum sefazolin, klindamisin fosfat, rifamisin SV, kanamisin sülfat, inhibisyon.

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INTRODUCTION

Xanthine oxidoreductase (XOR) is a member of the molybdenum hydroxylase family of proteins [1]. The enzyme is a 300 KDa dimer composed of identical subunit consists of 1333-1358 amino acids, depending on the species, and contains binding sites for molybdopterin, iron and flavin cofactors [1,2]. In mammals, XOR can exist in two intraconvertible enzymatic forms: a dehydrogenase (XD; EC 1.1.3.204) which utilizes NAD^+ as an electron acceptor and an oxidase (XO; EC 1.2.3.22) which utilizes O_2 as an electron acceptor [2]. Both enzymatic forms are identical in size, subunit composition, and cofactor requirements and are capable of oxidizing a wide range of substrates [1]. In the organisms studied to date XD appears to be the primary gene product and the predominant form of the enzyme in mammalian tissues [3,4].

One of the primary biological functions of XOR in mammals is purine degradation where the enzyme catalyzes the rate-limiting step in the oxidation of xanthine and hypoxanthine to uric acid [5]. High levels of the XO form have been associated with tissue injury and certain diseases [6,7] and are believed to contribute to oxidative damage of cells through the generation of cytotoxic oxygen metabolites (H_2O_2; O_2^- and OH^-) [6]. The XD form, on the other hand, may be an important component in the defense against oxygen radical damage through its role in the synthesis of uric acid, a potent antioxidant [8].

Xanthine oxidase, a cellular redox enzyme, is highly expressed in mammary epithelial cells. During lactation these cells synthesise milk-fat globules that are packaged in a membrane of which xanthine oxidase is the predominant protein, thus milk is a rich source of the enzyme. Xanthine oxidase can generate weakly microbicidal superoxide and hydrogen peroxide. These may give antimicrobial protection to the neonatal stomach [9].

Most of the drugs affect the enzyme systems as an activator or inhibitor [10-12]. Many drugs exhibit the same effects both *in vivo* and *in vitro*, but some of them may not show the same effects on enzymes [13]. Many antibiotics are being used in therapies. There are few reports related to changes in enzyme activities [12].

Gentamycin sulfate is aminoglycoside antibiotic, which is widely used for the treatment of bactericide effects of *Pseudomonas aeruginosa*, *Klebsiella-Enterobacter-Serratia*, *Citrobacter*, *Staphylococcus*, *Shigella* and *Salmonella* species. Gentamycin sulfate is an inhibitor for NADPH oxidase and glucose 6-phosphate dehydrogenase [14,15].

Cefazolin sodium is a cephalosporin antibiotic, which is effective on *Streptococcus pneumoniae*, *Echerichia coli*, *Proteus mirabilis*, *Klebsiella* species, *Enterobacter aerogenes* and *Haemophilus influenzae*. The antibiotic cefazolin sodium causes significant decrease in level of liver glucose 6-phosphate dehydrogenase and human carbonic anhydrase I and II [15,16].

Clarithromycin is a macrolide antibiotic that is widely used for the treatment of a myriad of infections such as those caused by *Hemophilus influenzae*, *Mycobacterium avium*, and *Helicobacter pylori*. Clarithromycin is oxidatively metabolized to 14-(*R*)-hydroxyclarithromycin or N-demethylated to N-desmethylclarithromycin and members of the CYP3A subfamily mediate these reactions [17]. Like erythromycin, clarithromycin is a potent mechanism based inhibitor of CYP3A [18].

Clindamycin, a semi-synthetic derivative of lincomycin, is an antibiotic that is highly effective against Gram-positive and Gram-negative anaerobic pathogens, as well as Gram-positive aerobes. Its phosphate ester, clindamycin-2-phosphate, is produced by chemical modification of clindamycin. Although the ester is not biologically active, it is rapidly hydrolyzed to the active clindamycin *in vivo* [19-21].

Rifamycin sodium is a semi-synthetic macrocyclic antibiotic derived from natural rifamycin B that is produced by *Amycolatopsis rifamycinica sp*. Because of its broad spectrum of activity against Gram-positive and Gram-negative bacteria it is used for wound cleaning before closure [22-25]. Rifampicin and rifamycin SV are the most important antibiotics of the group with excellent therapeutic action in the treatment of several infectious

diseases, such as tuberculosis. Drug-monitoring in patients during antituberculosis therapy is important, especially in AIDS patients, owing to a global increase in the prevalence of drug-resistant tuberculosis [26–32].

Many antibiotics are used to deal with some tissues disorders but there are few studies of their effects on enzyme activities. The effects of some widely used antibiotics on serum and liver paraoxonase have been investigated. The other study was investigated *in vitro* the effects of chloramphenicol and clarithromycin on serum hPON1 and on liver hPON1 in HepG2 cell line [33].

Some studies were to determine the effect of some antibiotics, sodium ampicillin, ciprofloxacin, rifamycin SV and clindamiycin phosphate, on purified human serum paraoxonase *in vitro*, and mouse serum and liver paraoxonase *in vivo* [34]. Other studies were on the effects of streptomycin sulphate, gentamicin sulphate, ampicillin, netilmicin, cefotaxime sodium, cefodizime sodium, teicoplanine, and thiamphenicol on the activity of glutathione reductase (GR) from bovine erythrocyte [35].

A few reports have indicated that some increases and decreases were found in human liver enzyme activity levels such as aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase [36–39]. In addition, inhibitory effects of some antibiotics, such as sodium ceftizoxime, sodium ampicillin, sodium cefuroxime, sodium cefazolin, sod-ium cefoperazone, streptomycin sulfate, gentamicin sulfate, and netilmicin sulfate on glucose 6-phosphate dehydrogenase from human erythrocytes have been investigated [40].

Since the effects of many of the known antibiotics have not been analysed on XO enzyme yet. In the present study, the *in vitro* effect of sodium ampicillin, clarithromycin, gentamycin sulphate, cefazolin sodium, clindamycin phosphate, rifamycin SV, kanamycin sulphate on XO purified from milk were investigated. Using the IC_{50} values obtained (causing 50% inhibition of enzyme activity), some undesirable side-effects can be diminished on XO enzyme activity and body metabolism in therapy [41].

MATERIAL AND METHODS

Materials

Sepharose 4B, L-tyrosine, benzamidine , protein assay reagents and chemicals for electrophoresis were obtained from Sigma Chem. Co. All other chemicals used were of analytical grade and obtained from either Sigma or Merck.

Enzyme Purification

Fresh bovine milk, without added preservative, was cooled down to 4ºC, overnight. EDTA and toluene were then added to give final concentrations of 2 mM and 3% (v/v), respectively. The milk was churned with a blender at top speed for 30 min at room temperature. This sample was brought to 38% saturation by addition of solid ammonium sulphate [42]. The suspension was centrifuged at 15000 rpm for 30 min and the precipitate formed was discarded. The supernatant was brought to 50% saturation with solid ammonium sulphate. The precipitate formed was collected by centrifugation at 15000 rpm for 60 min and dissolved 0.1 M Tris-HCl, pH= 7.6.

The pooled precipitate obtained from bovine milk by using ammonium sulfate precipitation was subjected to affinity chromatography. The sample prior to that it was loaded onto the affinity column containing benzamidine.

Affinity column equilibrated in 0.1 M glycine, 0.1 M NaCl, pH=9. The sample was applied to the affinity gel was washed with 0.1 M glycine, pH=9.0. XO was then eluted with 25 mM benzamidine in 0.1 M glycine, 0.1 M NaCl, pH=9. Fractions of 1.5 mL were collected and their absorbance measured at 280 nm.

Activity measurements

Xanthine oxidase activity was determined at 37°C by the modified method of Massey et al [43]. The conversion of xanthine uric acid was followed by monitoring the change in absorbance at 295 nm, using CARY 1E, UV-Visible Spectrophotometer-VARIAN spectrometer (ε_{292}=9.5 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM Tris-HCl, pH 7.6, and 0.15 mM xanthine, at 37°C. The assay was initiated by the addition of the enzyme. One unit of enzyme activity was defined as the amount of

enzyme that converts one μmol of xanthine to uric acid per min under defined conditions [42].

In vitro inhibition kinetic studies

For the inhibition studies of some antibiotics different concentration of medical drugs were added to the enzyme activity. Xanthine oxidase enzyme activity with medical drugs was assayed by following the oxidation of xanthine. Activity % values of xanthine oxidase for six different concentrations of each medical drug were determined by regression analysis using the Microsoft Office 2000 Excel. Xanthine oxidase activity without a medical drug was accepted as 100% activity. The inhibitor concentration causing up to 50% inhibition (IC_{50} values) on enzyme were determined from the graphs.

Total protein determination

The absorbance at 280 nm was used to monitor the protein in the column effluents. Quantitative protein determination was achieved by absorbance measurements at 595 nm according to Bradford [44] with bovine serum albumin as a standard.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed after purification of the enzyme. It was carried out in 10% and 3% acrylamide-bisacrylamide concentration for the running and stacking gel, respectively, containing 0.1% SDS according to Laemmli [45]. Sample was applied to the electrophoresis medium. Gel was stained overnight in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained by frequently changing the same solvent, without dye. The electrophoretic pattern was photographed with the system of produce as an image of the gel.

RESULTS AND DISCUSSION

Xanthine oxidase, a cellular redox enzyme, is highly expressed in mammary epithelial cells [46] but XO was extracted from fresh bovine milk without added preservative using toluene and EDTA in this study. Toluene together with the gradual increase in temperature (from 4 to 45°C) during churning caused an efficient extraction of the enzyme from lipid micelles. Filtration of the churned milk through filtration paper could be fractionated directly with ammonium sulphate. The entire enzyme was successfully collected in a narrow range of ammonium sulphate concentration (38-50% saturation). At this step, a 35-fold purification was achieved [42]. The precipitate form was collected and dissolved 0.1 M Tris-HCl, pH= 7.6. The dissolved sample prior to that it was loaded onto affinity column containing benzamidine. The affinity gel was equilibrated in 0.1 M glycine, 0.1 M NaCl, pH= 9. The sample was applied to the affinity gel was washed with 0.1 M glycine, pH= 9. XO was then eluted with 25 mM benzamidine in 0.1 M glycine, 0.1 M NaCl, pH= 9. Fractions of 1.5 mL were collected and their absorbance measured at 280 nm.

The purification of enzyme was controlled with SDS-polyacrylamide gel electrophoresis (Figure 1). In Figure 1, the results of graphics were listed in terms of mg/mL of the test chemical causing a 50 % reduction in enzyme activity, and the values

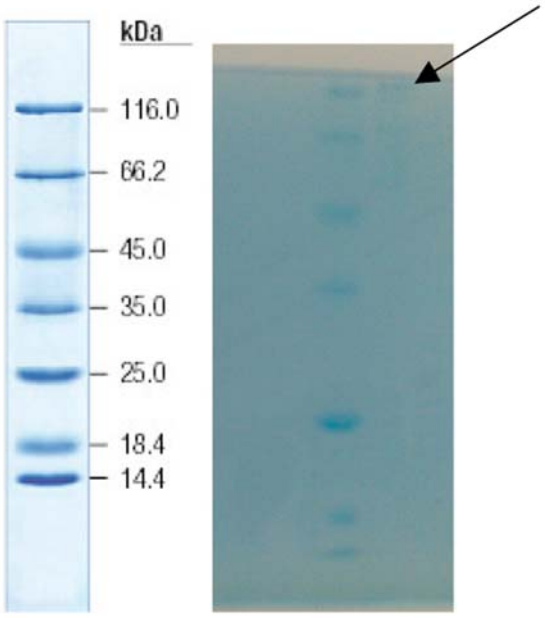

Figure 1. SDS-PAGE of xanthine oxidase. The poled fractions from affinity chromatography was analyzed by SDS-PAGE (%12 and %3) and revealed by Coomassie Blue staining. Experimental conditions were as described in the method. Lane 1 contained 5 μL of various molecular mass standards: 3-galactosidase, (116.0), bovine serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase, (35.0), Restriction endonuclease (25.0), 3-lactoglobulin (18.4), lysozyme (14.4).

of the activation increasing in stock concentration were established in the graphics.

Many chemicals at relatively low doses affect metabolism by altering normal enzyme activity, particularly through inhibition of a specific enzyme [47]. Therefore, we investigated the effect of widely used antibiotics, namely clarithromycin, gentamycin sulphate, sodium ampicillin, cefazolin sodium, clindamycin phosphate, rifamycin SV, kanamycin sulphate on the xanthine oxidase enzyme activity *in vitro* inhibition studies.

Gentamycin belongs to aminoglycoside class of antibiotics while cefazolin sodium is a class of Cephalosporin antibiotics. These antibiotics appear to prevent bacteria from making their cell walls, causing the cells die. These drugs are used to treat many sensitive Gram-negative and some Gram-positive bacteria.

Gentamicin, an aminoglycoside antibiotic, has been used effectively against Gram-negative infections since it was introduced in the 1970s [48,49]. Its chemical stability and rapid bactericidal action have made it a first-line drug in a variety of clinical situations [48,50]. However, clinical studies [51–53] report that gentamicin causes a dose-limiting nephrotoxicity that accounts for 20% of all cases of acute renal failure. Although a change from multiple daily doses to a single daily dose reduces the risk of nephrotoxicity, the incidence of gentamicin induced acute renal failure remains high [54–56]. Oxidative stress, caused by the overproduction of reactive oxygen species (ROS), is a central pathway responsible for gentamicin-associated nephrotoxicity [49,50,57,58]. Accumulated aminoglycosides in proximal tubular epithelial cells lead to the structural disturbance of cell membranes and to cell death because of ROS involvement [59]. Reactive oxygen species cause cellular injury and necrosis via several mechanisms: peroxidation of membrane lipids, protein denaturation, and DNA damage [60]. Hydroxyl radical, one of the most toxic ROS, is generated primarily from NO that interacts with superoxide anion [61]. In addition, the activation of renal iNOS and of xanthine oxidase are important for producing NO and superoxide anion, respectively [62,63].

Clarithromycin is a macrolide antibiotic used to treat pharyngitis, tonsillitis, acute maxillary sinusitis, acute bacterial exacerbation of chronic bronchitis, pneumonia (especially atypical pneumonias associated with *Chlemydia pneumoniae* or TWAR), skin and skin structure infections. In addition, it is sometimes used to treat LEgionellosis and lyme disease [33]. Ampicillin is used to treat a wide variety of bacterial infections. It is a penicillin-type antibiotic. It works by stopping the growth of bacteria [34].Clindamycin (CLDM) is a semisynthetic derivative of lincomycin, and was introduced in the 1960s as an antibiotic. Its antibacterial mechanism involves the inhibition of cell growth by blocking peptide bond formation via direct binding to functionally important sites on ribosomes [64,65]. Clindamycin is a lincosamide antibiotic. It is usually used to treat infections with anaerobic bacteria but can also be used to treat some protozoal diseases, such as malaria. It is a common topical treatment for acne and can be useful against some methicillin-resistant Staphylococcus aureus (MRSA) infections [33]. Rifamycin SV is the most important antibiotics of the group with excellent therapeutic action in the treatment of several infectious diseases, such as tuberculosis.

Macrolides are a long-used class of antibiotics which still play an important role in the chemotherapy of infectious diseases. They have been shown to affect several pathways of the inflammatory process, such as the migration of neutrophils, the oxidative burst in phagocytes, and the production of proinflammatory cytokines. Although the precise mechanisms of these effects are not clear, it has been suggested that the interaction between macrolides and leukocytes may be important [66]. Some studies have suggested that the antioxidant properties, shared by several macrolides, may play a role in the anti-inflammatory activity of these agents [66,67].

Xanthine oxidase (XO) is the last enzyme of purine catabolism. It catalyzes conversion of xanthine and hypoxanthine to uric acid and the production of superoxide radical anion, which is potentially toxic to cellular structures [68]. Free radicals (FR) and lipid peroxides have been implicated in the pathogenesis of a wide variety of diseases ranging from infectious, inflammatory and autoimmune disea-

ses to atherosclerosis and cancer [69-75]. FRs are known to play an important role in the intracellular killing of microorganisms by leukocytes. The challenge of polymorphnuclear cells with many activating agents, including immune complement, evokes a potent response that produces toxic oxygen species, such as O^{-2}, and hydrogen peroxide [69]. Increased FRs may cause cell and tissue damage [76-78]. A major source of radicals in biological systems is molecular oxygen (O^2). XO is an important source of O^{-2} in cells and tissues. This enzyme catalyses the conversion of hypoxanthine and xanthine to uric acid and the rate-limiting step in purine nucleotide catabolism [79].

They was found that XO activities were significantly lower in erythromycin, azithromycin, roxithromycin, and clarithromycin- treated groups, while XO activities in experimental group were significantly higher than in the studied control group [69]In other study, treatment of cultured renal epithelial cells (LLC-PK_1) cells with cephaloridine (CLD) induced time- and concentration-dependent inhibition of Cytochrome c oxidase activity in the mitochondria [79]. Another study, in rats, a daily supplement of sesame oil significantly protected against renal injury induced by a single daily dose of gentamicin. Sesame oil partially blocked gentamicin-induced renal oxidative stress and oxygen free radicals and NO generation, and partially inhibited renal xanthine oxidase activity and iNOS expression [80].

ROS have potential deleterious effects on the biological system, as they can damage proteins, lipids, and nucleic acids [81–83]. The other study showed that the macrolide antibiotics (erythromycin, azithromycin, roxithromycin, and clarithromycin) increase NOS activity, decrease XO activity and MDA level, which is an important indicator of oxidative stress [84].

Therefore, the effects of these widely used antibiotics (Figure 2) have initially been studied on the purified xanthine oxidase enzyme activity in vitro. Some antibiotics which we investigated their effects in our studies different levels effected the xanthine oxidase. For the antibiotics shown inhibition effect, the IC_{50} values of the chemicals caused inhibition were determined by means of activity percentage-[I] diagrams (Figure 3). Some antibiotics indicated activatory effect on the enzyme activity. The results showed that cefazolin sodium, clarithromycin, clindamycin phosphate and kanamycin sulphate inhibited the xanthine oxidase. The values of IC_{50} were 5.40 x 10^{-4} mg/mL, 1.68 mg/mL, 1.39 mg/mL and 1.49 mg/mL, respectively. The most effective antibiotic for XO was sefazolin sodium. On the other hand, the antibiotics showed activation increasing on XO were gentamycin sulphate, sodium ampicillin, rifamycin SV.

Antibiotics showing inhibitory effects on XO give rise their effect by bound to active center of enzymes or bound to the other part of enzymes. On the other hand, it can be said that the reason of IC_{50} values changing in respect to enzyme depend to the range, number and kind of enzyme amino acids.

Table 1. The values of IC_{50} in terms of concentration of the test chemical causing a 50% reduction of XO enzyme activity.

Inhibitors	IC_{50} (mg/mL)
Gentamycin sulphate	-
Cefazolin sodium	5.40 x 10^{-4}
Sodium ampicillin	-
Clarithromycin	1.68
Rifamycin SV	-
Clindamycin phosphate	1.39
Kanamycin sulphate	1.49

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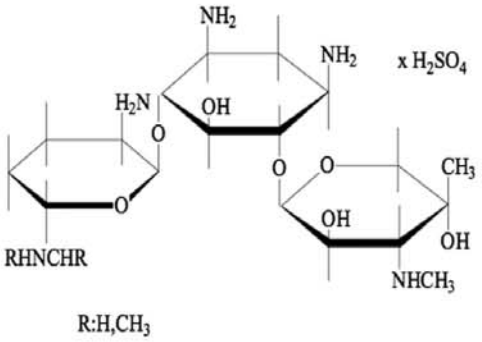
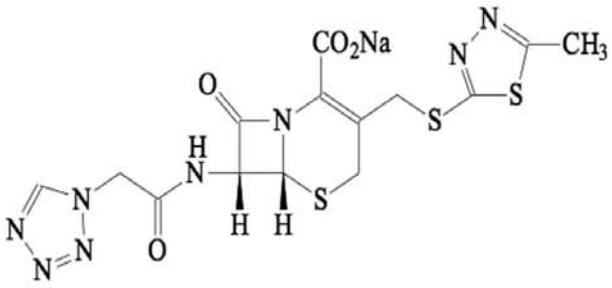
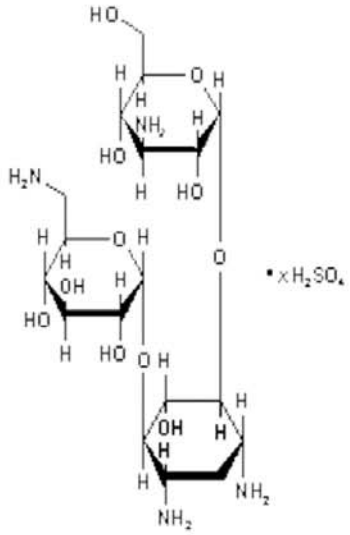


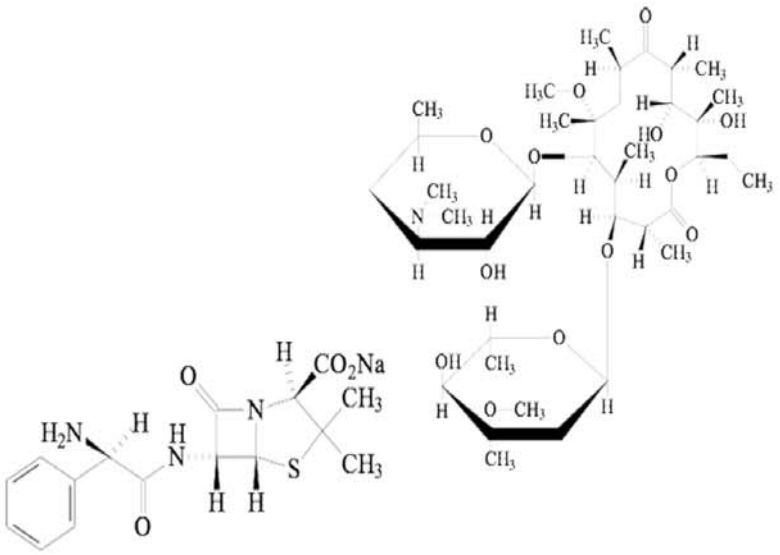
Gentamycin sulphate (A)



Cefazolin sodium (B)

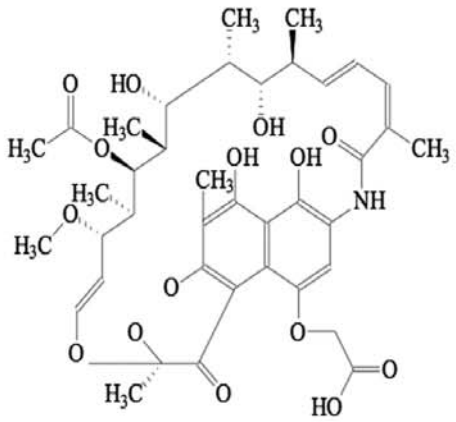


Kanamycin sulphate (C)

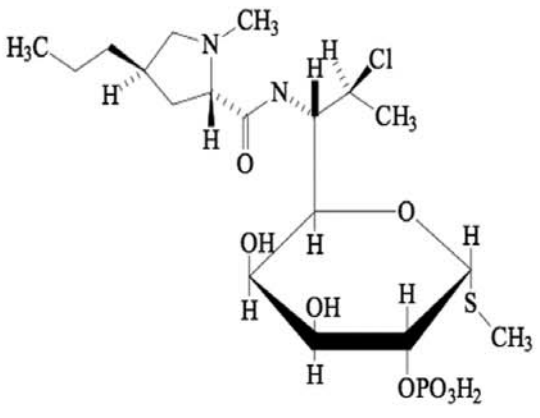


Sodium ampicillin (G)

Clarithromycin (D)



Rifamycin SV (E)



Clindamycin phosphate (F)

Figure 2. Structure of gentamycin sulphate (A), cefazolin sodium (B), kanamycin sulphate (C), clarithromycin (D), rifamycin SV (E), clindamycin phosphate (F), sodium ampicillin (G)**.**

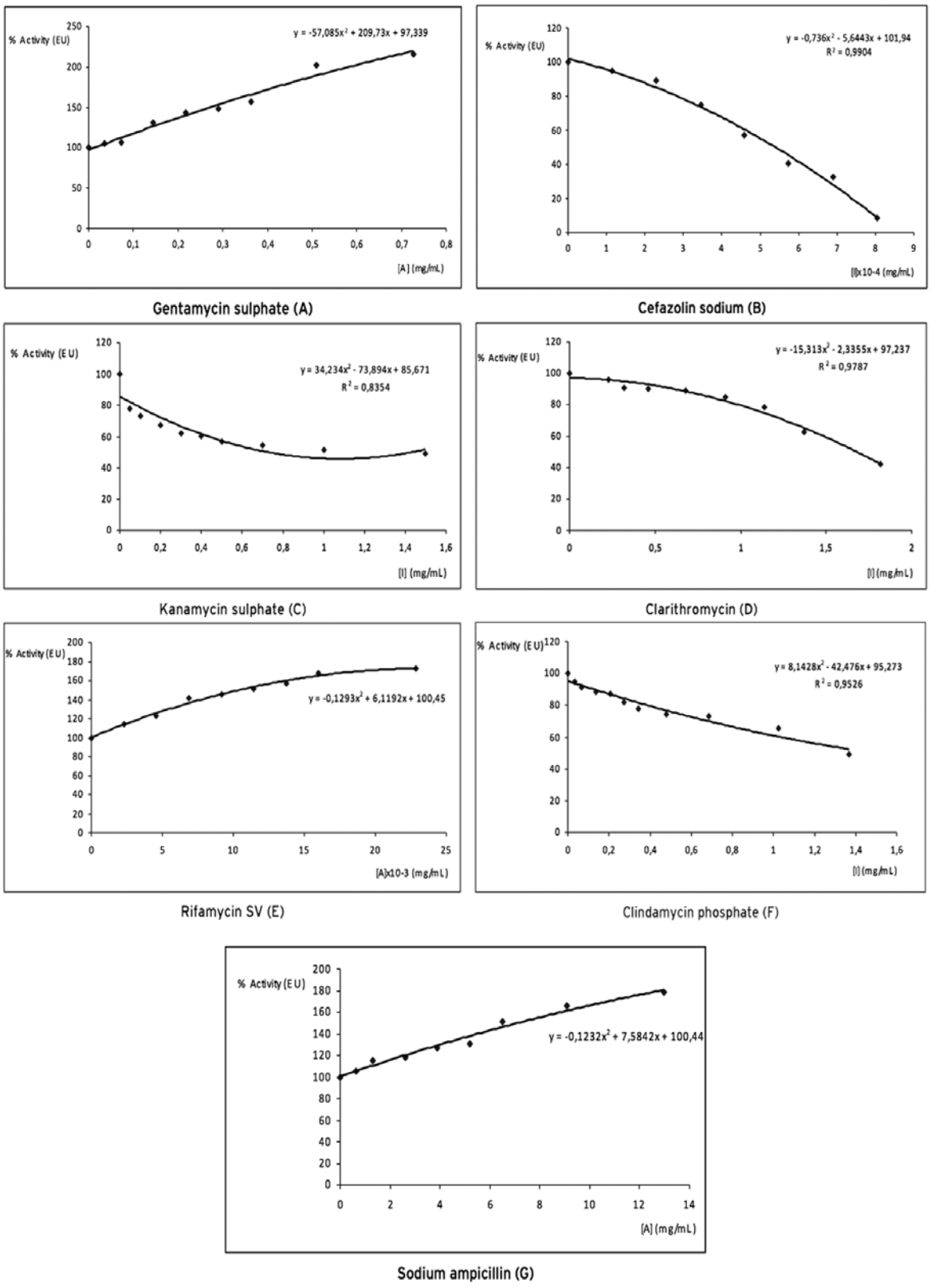


Figure 3. Effect of gentamycin sulphate (A), cefazolin sodium (B), kanamycin sulphate (C), clarithromycin (D), rifamycin SV (E), clindamycin phosphate (F), sodium ampicillin (G) on the enzyme activity of a purified xanthine oxidase from milk was assayed for enzyme activity in the presence of various concentrations of above antibiotics. IC_{50} values were determined from these graphs.

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