# THE BIOCHEMICAL EVALUATION OF TYLOSIN AND ENROFLOXACIN ON SELECTED ENZYMES

**A MASTER'S THESIS**

**IN**

**CHEMICAL ENGINEERING AND APPLIED CHEMISTRY**

**ATILIM UNIVERSITY**

**BY**

**TAHER MEHEMED A. ALATRASH**

**MAY 2018**

# THE BIOCHEMICAL EVALUATION OF TYLOSIN AND ENROFLOXACIN ON SELECTED ENZYMES

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

OF

ATILIM UNIVERSITY

BY

TAHER MEHEMED A. ALATRASH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF

MASTER OF SCIENCE

IN

CHEMICAL ENGINEERING AND APPLIED CHEMISTRY DEPARTMENT

MAY 2018

Approval of the Graduate School of Natural and Applied Sciences, Atılım University.

_____

Prof. Dr. Ali Kara

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that we have read the thesis "THE BIOCHEMICAL EVALUATION OF TYLOSIN AND ENROFLOXACIN ON SELECTED ENZYMES" submitted by "TAHER MEHEMED A. ALATRASH" and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master.

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## ABSTRACT

### THE BIOCHEMICAL EVALUATION OF TYLOSIN AND ENROFLOXACIN ON SELECTED ENZYMES

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May 2018, 43 Pages

The antibiotics Enrofloxacin and Tylosin were used widely in the veterinary field due to its effectiveness against Gram positive and Gram negative bacteria. However, they can also be the reason of several negative effects such as the inhibition of antioxidant enzymes activities, for example Catalase (CAT), Superoxide Dismutase (SOD), Glutathione S Transferase (GST) and Glutathione Peroxidase (GPX) which may lead to an oxidative stress in the body. This study was conducted to evaluate the effects of two antibiotics on these enzymes. Enrofloxacin was dissolved in DMSO, whereas Tylosin was dissolved in water at a concentration 1mg/ml for both drugs. The stability measured by UV absorbance at 276 nm for Enrofloxacin and at 282 nm for Tylosin showed that both drugs were stable in dark environment at +4°C and at room temperature for three months. The enzyme assays with serial concentration of drugs resulted that Enrofloxacin decreased the activity of CAT and GST by 10% and 5% respectively, while increased GPX activity by 15%. There were no change in CAT and GST activities with Tylosin, hovewer, 10% increase of GPX activity was observed. The results showed that Enrofloxacin and Tylosin may not cause an oxidative stress in the body, and hence Enrofloxacin and Tylosin can be suggested to use safely among with other antibiotics that may have adverse effects on the antioxidant enzymes.



**Keywords:** Enrofloxacin Enr, Tylosin Tyl, Glutathione Peroxidase GPX, Superoxide Dismutase SOD, Glutathione S Transferase GST and Catalase CAT.

# ÖZ

## ENROFLOKSİN VE TYLOSİN'İN SEÇİLİ ENZİMLER ÜZERİNE BİYOKİMYASAL OLARAK DEĞERLENDİRİLMESİ

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Gram pozitif ve gram negative bakterilere karşı gösterdikleri etki nedeni ile Enrofloksin ve Tylosin antibiotikleri veterinerlikte yaygın olarak kullanılmaktadır. Bununla beraber özellikle Katalaz (KAT), Superoksit dismutaz (SOD), Glutatyon-S-Transferaz (GST) ve Glutatyon Peroksidaz (GPX) gibi bazı antioksidan enzimleri inhibe ederek vücutta negative etkilere sebep olabilmektedirler. Bu çalışmada söz konusu antibiyotiklerin adı geçen enzimler üzerine etkisi değerlendirilmiştir. Enroflaxin DMSO da çözünürken Tylosin suda çözünmüştür. Her iki antibiyotik de 1mg/ml stok derişimde hazırlanmıştır. İlaçlara ait stabilite testleri absorbsiyon spekturumu ölçülerek test edilmiş, maksimum absorbsiyon Enroflaksin için 276 nm ve Tylosin için de 282 nm olarak ölçülmüştür. Üç ay süren testler sonucunda her iki ilacın da karanlık ortamda +4 C° ve oda sıcaklığında kararlı olduğu sonucuna varılmıştır. Seri seyreltmelerle hazırlanmış olan farklı derişimlerdeki ilaçlarla yürütülen enzim çalışmalarının sonucunda, Enroflaksin'in KAT ve GST aktivitelerini sırasıyla %10 ve % 15 oranında azaltırken GPX aktivitesini % 15 oranında artırdığı bulunmuştur. Tylosin'in KAT ve GST üzerine etkisi bulunmazken GPX aktivitesini % 10 oranında artırdığı gözlenmiştir.

Sonuç olarak Enrofloksin ve Tylosin'in oksidatif stress yaratmadığı gibi, her iki ilacın da kullanılmasının antioksidan enzimler üzerine bir etki yaratmadığı da gözlenmiştir.

**Anahtar Kelimeler:** Enrofloxacin, Tylosin, Glutatyon Peroksidaz GPX, Superoksit Dismutaz SOD, Glutatyon– S – Transferaz GST, Katalaz CAT

## AKNOWLAGEMENT

First, I thank my God who gives me health, strength and guides me through my life.

This work is dedicated to my parents, may God have mercy on them, who taught me the first lessons of my life and were the source of inspiration and gave me motivation to achieve my goals.

I would like to express my deep and sincere gratitude to my research supervisor, Dr. Belgin Isgor, who has supported me throughout my thesis with her patience and knowledge. I greatly benefited from her keen scientific insight, her knack for solving intractable practical difficulties.

My special thanks to Dr Yasemin Isgor my co-supervisor, who has taught me the methodology to carry out the research and to present the research works as good as possible.

I also dedicated this work to my wife Mrs. Huda Al-Wefati for providing me unfailing support and encouragement throughout my time of this work as well as my children Ahmed, Mohammed, Alaa and Maad. It is truly the source of my happiness.

I would like to thank my friends and research colleagues, and special thanks to Mr Junaid Aldossary for his support and cooperation throughout the time of this research.

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| | |
|---|---|
| 4-AP | Dichlorohydroxy Benzene Sulfonic Acid |
| CAT | Catalase |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| DHBS | 1-chloro-2,4-dinitrobenzene |
| DMSO | Dimethyl Sulfoxide |
| EDTA | Ethylene Diamine Tetra acetic acid Disodium Salt Dihydrate |
| Enr | Enrofloixacin |
| FQ | Fluoroquinolones |
| GPx | Glutathione Peroxidase |
| GR | Glutathione Reductase |
| GSH | Glutathione |
| GST | Glutathione S Transferase |
| $H_2O_2$ | Hydrogen Peroxide |
| HRP | Horse Reddish Peroxidase |
| Mc | Macrolides |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| NBT | Nitro Blue Tetrazolium Chloride |
| RNS | Reactive Nitrogen Species |
| ROS | Reactive Oxygen Species |
| SOD | Superoxide Dismutase |
| Tyl | Tylosin |
| XOD | Xanthine Oxidase |

# CHAPTER 1

## INTRODUCTION

Antibiotics have been used widely and successfully for many decades against diseases caused by bacteria, and also as a treatment for secondary infections caused by bacteria in viral and fungal diseases. Although antibiotics have many effects and some of those are adverse, those are mostly neglected when compared with their benefits.

Many studies have been conducted to detect the potential effects of antibiotics on the components of biological systems such as antioxidant enzymes. Among the other families of antibiotics, Fluoroquinolones (FQs) and Macrolides (MCs) are considered to be the most important families since they are widely used in both human and veterinary medicine due to their pharmacokinetic properties.

In this study, we will focus on studying the influence of two families of antibiotics, namely Fluoroquinolones and Macrolides, on the cytosolic antioxidant enzymes which includes Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX), and Glutathione-S-Transferase (GST).

### 1.1 Antibiotic Drugs.

Antibiotics and their metabolites can be transmitted to the human body via four ways (**Figure 1)**:

1. As a treatment against infectious diseases, caused by bacteria, and also as treatment for secondary infection caused by bacteria in viral and fungal diseases.
2. By consumed animal products with residual of antibiotics such as meat, milk and eggs. (Cinquina et al. 2003) & (Gorla, N. et al. 1997).
3. By consumed crops or livestock fed with forages, provided that those plants have grown in a bio-solids fertilized land (Sabourin, L. et al. 2012).
4. Using contaminated surface, ground, and drinking water with micro-contaminants as antibiotics (Fick, J et al. 2009).

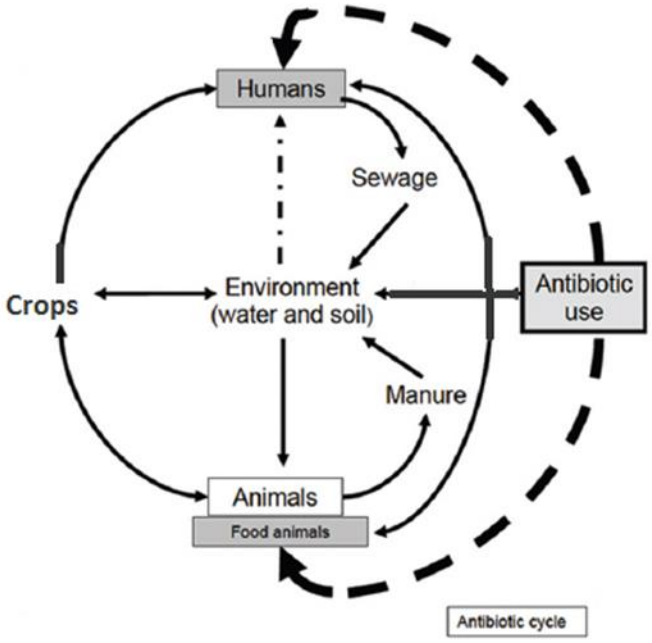



**Figure 1:** The cycle of the antibiotic transmission (Hodzic, E 2015).

### 1.1.1 Fluoroquinolones

Fluoroquinolon (FQ) family members are synthetic antibiotics. The first generation of this family is Nalidixic acid **(Figure 2)**, which discovered in 1962, and released in 1967 to be clinics as quinolones (non-fluorinated) for the treatment of urinary tract infections. The early forms of quinolones exhibited low efficiency due to their antibacterial effect was limited to Gram-negative bacteria with high binding capacity to plasma proteins. The modifications were done to improve the molecule's potency to effect Gram-positive bacteria withdecreased selection resistance (Wolfson et al. 1989). The outcome of advancements in developing the quinolone family is the addition of a flour atom on the 6$^{th}$ position of the ring structure **(Figure 3),** which converts the quinolone to fluoroquinolone and improves its antibacterial spectrum.

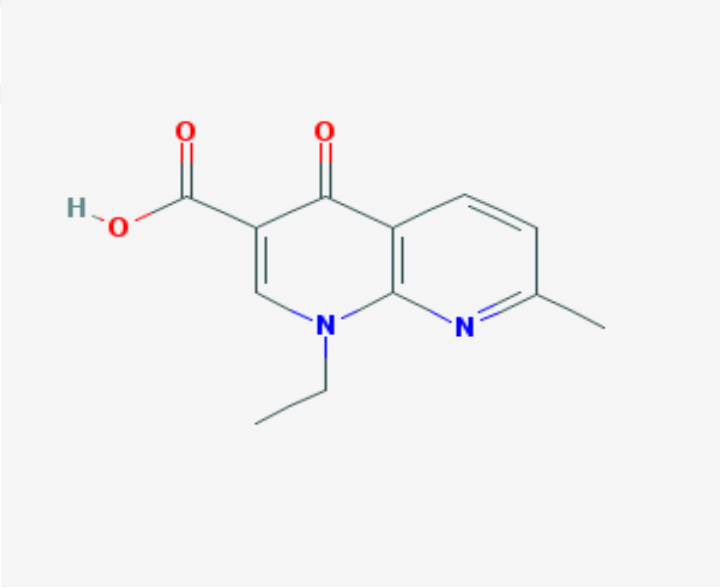



**Figure 2:** Chemical structure for Nalidixic acid.

With continuous development and improvement between 1962 and 2000 , four generations of fluoroquinolones were discovered and developed (Sharma, P. C et al. 2009) including Nalidixic acid, Enrofloxacin, Temafloxacin, Gatifloxacin **(Table 1)**.

**Table 1:** Classification of Fluoroquinolones (Sharma, P. C et al. 2009).

| **Generation** | **Drug** | **Characteristic features** |
|---|---|---|
| First | Naldixc acid, Oxolinc acid, Pipemic acid. | Active against some gram-negative bacteria. Highly protein-bound drugs. Short half-life. |
| Second | Norfloxacin, Enrofloxacin, Ciprofloxacin, Ofloxacin, Lomefloxacin. | Protein binding (50%) Longer half-life than previous agents. Improve activity against Gram- negative bacteria. |
| Third | Temafloxacin, Sparafloxacin, Grepafloxacin. | Active against Gram- negative bacteria. Also active against Gram positive bacteria. |
| Fourth | Clinafloxacin, Trovafloxacin, Moxifloxacin, Gatifloxacin. | Show extended activity against both strains of bacteria. Active against anaerobes and atypical bacteria. |

Enrofloxacin is one of Fluoroquinolones which is oxidized by liver microsomal enzymes of the cytochrome P450 family (Stratton, 1998).

#### 1.1.1.1 Enrofloxacin ($C_{19}H_{22}FN_3O_3$)

Enrofloxacin is a synthetic antibacterial drug with IUPAC name of 1-Cyclopropyl-6-fluoro-7-(4-ethyl-1-piperazinyl)-1,4-dihydro-4-oxo-3 quinoline carboxylic acid **(Figure 3)**.

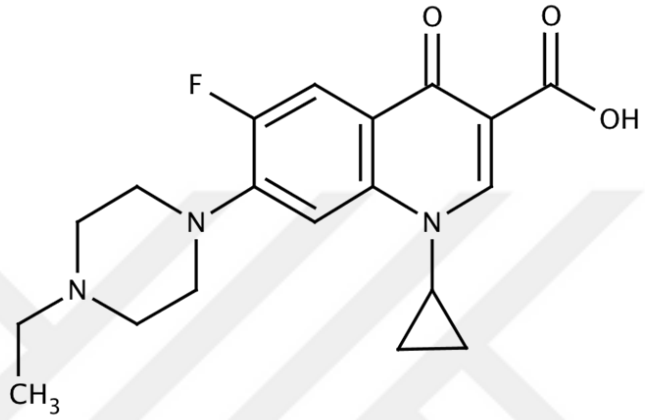



**Figure 3:** The chemical structure of Enrofloxacin.

It is the second generation of FQs, but the first member of this family to be approved as antibiotic in veterinary medicine. Its solubility in water at 25°C was found to be 146 µg/ml (Seedher, N., & Agarwal, P. 2009), and in dimethyl sulfoxide (DMSO) was 1 mg/ml (Kato, M. et al. 2007, TOKU-E EvoPure). However, its solubility in organic solvents decreases in the order of acetonitrile > acetic ether > 2-propanol > ethanol > water (Liu, M. Et al. 2014).

It is widely used in the veterinary medicine as a treatment for many diseases caused by Gram-negative and Gram-positive bacteria, such as Salmonella and E coli (Vancutsem, et al.1990). Enrofloxacin is one of Fluoroquinolones which is oxidized by liver microsomal enzymes of the cytochrome P450 family (Stratton, 1998).

The main target of the FQs is bacterial DNA gyrase enzyme (**Figure 4**), a member of the class type II topoisomerases, inhibition of which disrupts DNA replication and results in the death of bacteria (Brown, S. A.  1996).

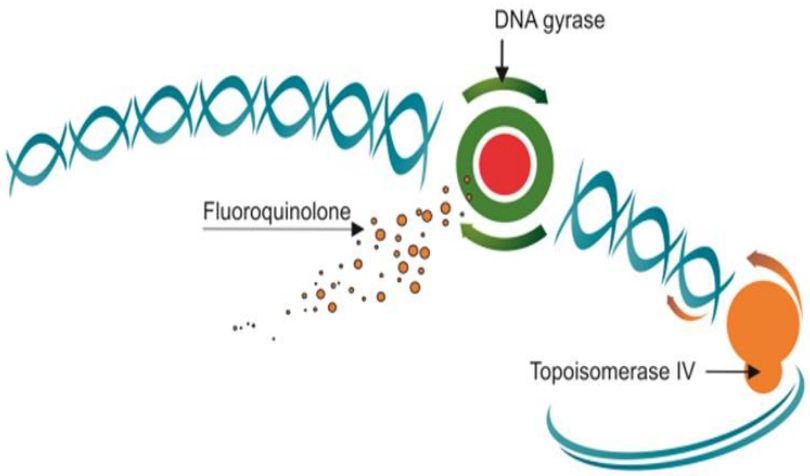



**Figure 4:** The mode of action of Fluoroquinolones (Hooper, D. C. 1999).

### 1.1.2 Macrolides

The most natural macrolides (MC) contain large lactone rings vary in size from 12 to 16 atoms and they are produced by actinomycete species (Zhanel, G. G.et al. 2001). MCs are considered to be one of the safest antibiotics available (Anca Mchiriac, Pascal Demoly 2016). It was shown that Macrolides affect a number of processes involved in inflammation, including the migration of neutrophils, the oxidative burst in phagocytes and production of various cytokines, although the detailed mechanisms are not clear yet. Therefore some MCs were considered as both antibacterial and anti-inflammatory (Zalewska-Kaszubska, J., & Górska, D. 2001).

Macrolides action by inhibiting bacterial protein biosynthesis by binding reversibly to 50S subunit of the bacterial ribosome and preventing translocation of peptidyl-tRNA (Dasgupta, A. 2012) as shown in **Figure 5.**

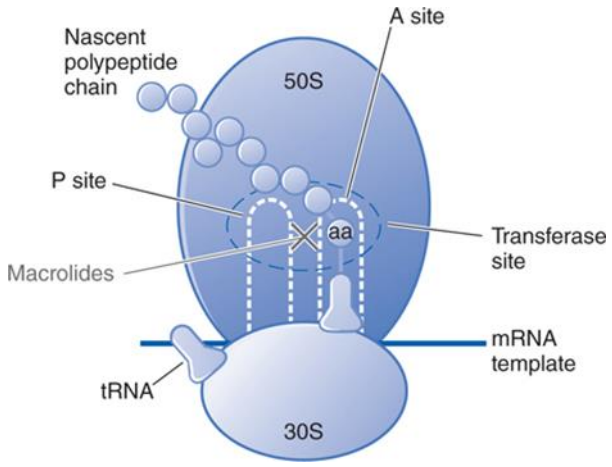



**Figure 5**: The Mode of Action of Macrolides (Mac Dougall, C., & Chambers, H. 2011).

### 1.1.2.1 Tylosin Tartrate ($C_{46}H_{77}NO_{17}.C_4H_6O_6$).

Tylosin is produced by Streptomyces *fradiae* and it contains a third sugar unit called mycinose attached at the $C_{23}$ hydroxy group in addition to mycaminose and mycarose at C 5. Studies suggested that mycinose increases the ability of the molecule to enter bacterial cells. Tylosin tartrate **(Figure 6)** is a medium-spectrum antibiotic used in veterinary medicine for the treatment of respiratory infectious diseases caused by most Gram-positive bacteria, Mycoplasmas, some Gram-negative bacteria and Chlamydia (McGuire, J. M., et al.1961).

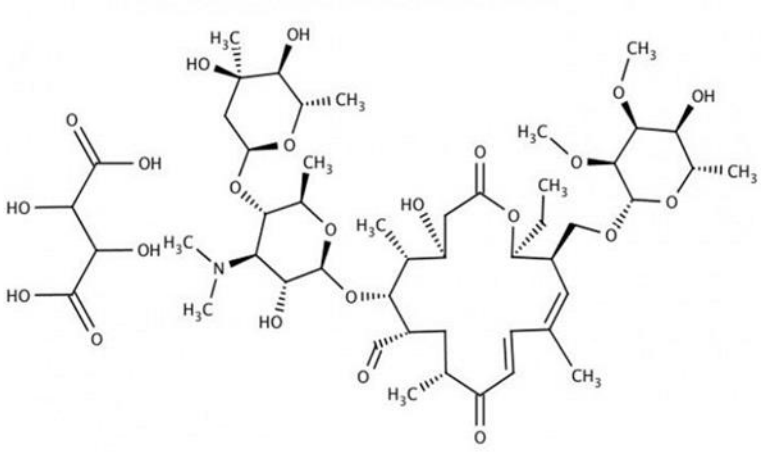


**Figure 6:** The chemical structure of Tylosin tartrate.

## 1.2 Free Radicals

Free Radicals are defined as any atom or molecules containing one or more unpaired electron that makes it unstable and highly reactive (Phaniendra et al. 2015). Free radicals can be generated from both endogenous sources in aerobic cells, such as mitochondria, peroxisomes, endoplasmic reticulum, phagocytic cells, as a product of normal cellular metabolism. In addition, they can be produced from exogenous xenobiotic metabolism, such as the metabolism of alcohol, heavy metals, pesticides, certain pharmaceutical products, some chemicals compounds including carbon tetrachloride, and also the radiations **(Figure 7)**. Moreover, it is produced in pathological disorders such as inflammations and traumas (Cheeseman, K. H et al.1993). Free radicals naturally found in living organisms are classified into two main types **(Table 2)**: A) reactive oxygen species (ROS) superoxide anion radical ($O_2^{\bullet-}$) which neutralized by Superoxide dismutase enzyme to generate hydrogen peroxide ($H_2O_2$), it may react to produce the hydroxyl radical ($HO^{\bullet}$). B) Reactive nitrogen species (RNS) are generated in higher organisms (Dröge, W. 2002) due to oxidation of the terminal guanido-nitrogen atoms of L-arginine and resulted in nitric oxide ($\bullet NO$) and nitrogen dioxide ($\bullet NO_2$), or peroxynitrite ($ONOO^-$).

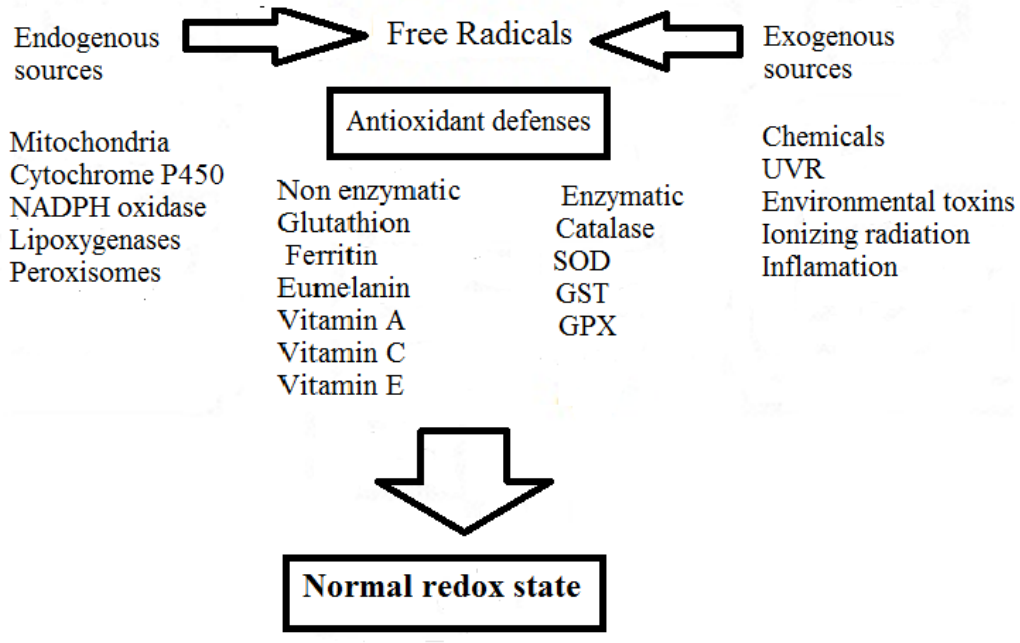



**Figure 7:** Free radical sources and defense system.

**Table 2:** Free radicals classification.

| Species | Name | Classification |
|---|---|---|
| $O_2^-$ | superoxide anion | ROS |
| $O_2$ | Singlet oxygen | ROS |
| NO | Nitric oxide | RNS |
| NO2 | Nitrogen dioxide | RNS |
| HO2 | hydroperoxyl | Free radical |
| HO | hydroxyl | Free radical |
| RO | alkoxyl | Free radical |
| ROO | peroxyl | Free radical |
| H2O2 | hydrogen peroxid | Non-free radical |
| HOCl | hypochlorous acid. | Non-free radical |

Under the normal physiological conditions, the mitochondria are the main organelle that generates ROS as a by-product in energy production. It is also produced due to the reduction of $NADP^+$ to generate NADPH by some oxidative enzymes in the cell such as xanthin and hypoxanthine **(Figure 8)**.

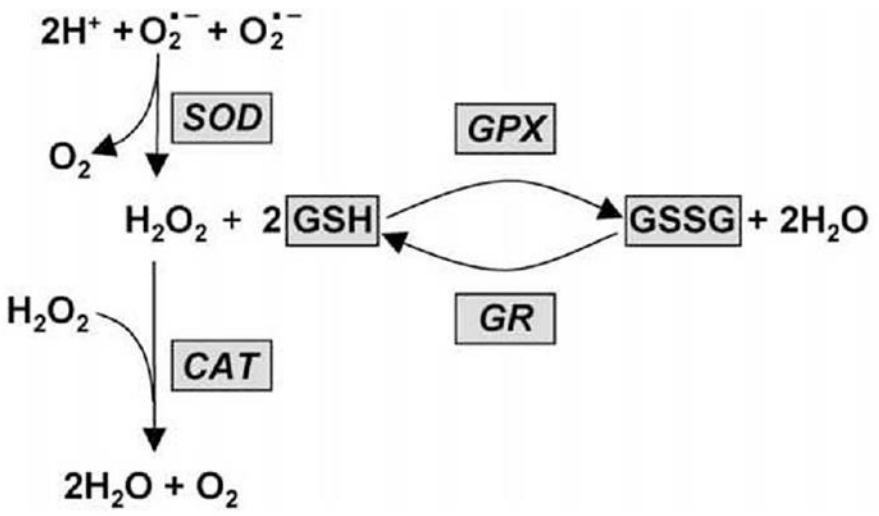



**Figure 8:** Enzymatic antioxidant defense system.

Reactive Oxygen Species (ROS) are also generated in the liver by a group of enzymes called P-450, which are iron-containing and responsible for detoxifying several of xenobiotics from drugs and even food sources. Macrophages and Neutrophils also produce ROS as a defense mechanism against foreign pathogens by NADPH oxidase enzymes to generate hydrogen peroxide which reacts to chloride ion producing hypochloride to destroy the pathogens (Bhattacharya, S. 2015).

### 1.2.1 Oxidative Stress

The imbalance between the free radical production and antioxidant defense system may occur due to the increase in the production of free radical or deficiency of antioxidant enzymes. Such imbalance may result in an increase of the cellular concentration of ROS/ RNS, which attack the subcellular molecular targets, and thereby cause oxidative stress. Free radical (such as $NO_2$, OH) attack the polyunsaturated fatty-acids or lipoprotein side-chain and attract hydrogen atom that leaves unpaired carbon and starting multiple chain reactions thateventually lead to the cell membrane breakdown. The most important effect of free radicals may occur on guanine of DNA which either results in a mutagenic lesion by oxidation, or halts DNA replication **(**Halliwell, B. 1994).

DNA constantly undergoes lesions caused by free radical which is repaired by its own mechanism (Breimer, L. H. 1991). High concentrations of ROS are determined in cancer cells compared to normal cells, in other words, the redox status of cancer cells differs from that of normal cells (Gorrini et al. 2013). Proteins are the target for free radical attacks that cause an alteration in amino acids and resulting in proteins dysfunction, loss of enzymes activity, and loss of cell receptors **(**Butterfield et al. 1998). Free radicals contribute significantly in many pathological conditions such as cancer, cardiovascular diseases (CVD), pulmonary disease, rheumatoid arthritis, nephropathy disease, ocular disease, fetus disease, and amyotrophic lateral sclerosis (ALS), Moreover they are found with neurological diseases including Alzheimer's disease, Parkinson's disease, multiple sclerosis (Pham-Huy et al. 2008).

### 1.3 Antioxidant defense system

The ratio of the concentration of the oxidized species to the concentration of the reduced species called redox state. Although free radicals can cause a serious damage to the cell may lead to apoptosis, they are necessary with certain concentrations for cell survival. They play a pivotal role as a part of the immune system against pathogenic microbes, as a response to infection phagocytes release free radicals to destroy foreign pathogens. ROS and RNS involve the physiological function of cell signaling system, for example, nitric oxide (NO) as a physiological signaling agent because it can so readily move through membranes and between cells (Pacher et al. 2007).

Antioxidant protection system is divided into two main categories:

1. Non-enzymatic antioxidants that are consisting of:

A) Endogenous antioxidants: NADPH, NADH, Glutathione and thiols (-SH), Ubiquinol (coenzyme Q), uric acid, bilirubin.

B) Dietary antioxidants: ascorbic acid (Vitamin C), tocopherols (Vitamin E), carotenoids, and poly phenols.

C) Metal binding proteins: ceruloplasmin (copper), metallothionein (copper), albumin (cupper), transferrin (iron), ferritin (iron), myoglobin (iron) (Jacob, R. A. 1995).

2. The enzymatic antioxidant system **(Figure 9)**: this system consists of Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX) and Glutathione-S-Transferase (GST).

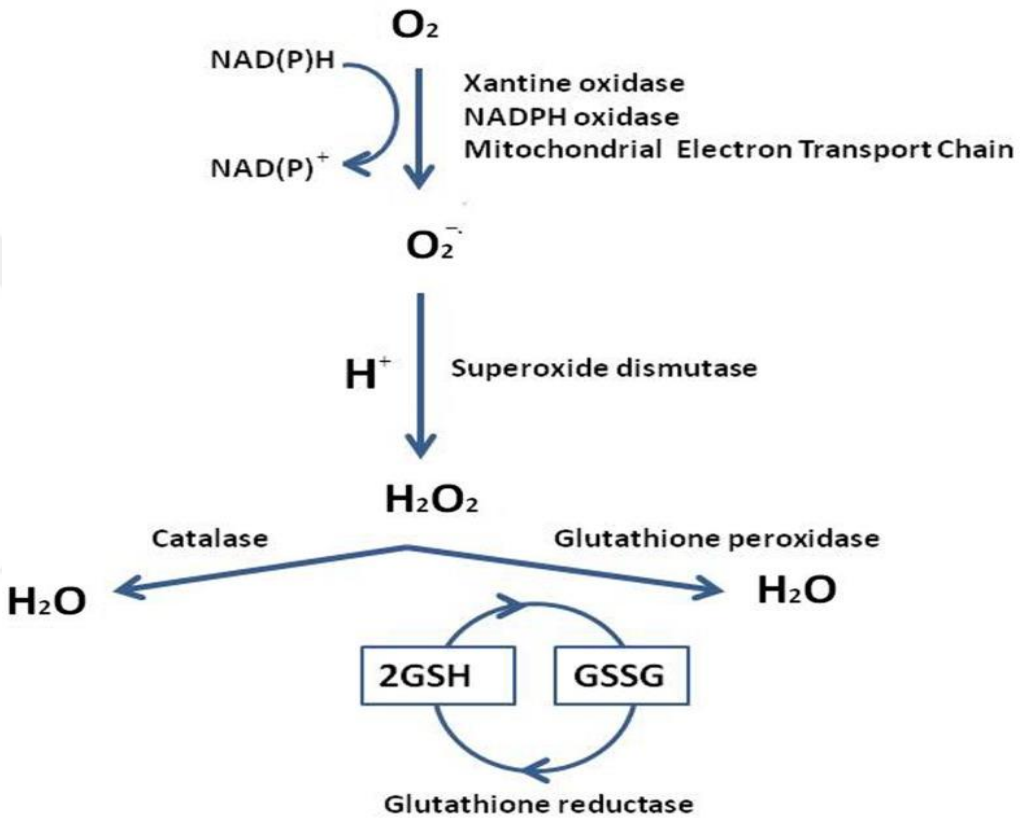



**Figure 9:** Enzymatic defense system (Sheehan et al. 2001).

### 1.3.1 Superoxide Dismutase (SOD)

Superoxide Dismutase (SOD) enzymes play a significant protective role against superoxide anion ($O_2$•−). They are classified into four types based on the presence of metal ion in their active sites. SODs contain manganese (MnSOD) found in the matrix of mitochondria and in prokaryotes, copper and zinc (Cu/Zn SOD) found in cytosol, and iron (FeSOD) which is found in prokaryotes with some exceptions (Duke et al & Salinet al. 1985). In addition, the novel type NiSOD which is purified from Streptomyces sp. is similar to specific activity per metal to that of bovine erythrocyte CuZnSOD (Hong-Duk, et al.1996).

SODs not only catalyze $O_2$•− to $H_2O_2$ which plays a role in cell signaling, but also play a critical role in inhibiting oxidative inactivation of nitric oxide, which thereby preventing peroxynitrite formation and endothelial and mitochondrial dysfunction (Fukai et al.2011). In the human body, there are three types of SOD classified based on subcellular location in the cell **(Figure 10)**:

1. SOD1 (Cu/ZnSOD) found in cytoplasm.
2. SOD2 (MnSOD) found in mitochondria of the liver cells but not in erythrocytes.
3. SOD3 (Cu/ZnSOD) extracellular. (Aldred, E. M. 2009).

SODs play an important role in mitochondrial function by inhibiting oxidation of bioavailable NO and thus preventing peroxynitrite ($ONOO^-$) generation or OH radical formation via inhibition the oxidation of Fe-S cluster-containing enzymes (Fukai et al. 2011).

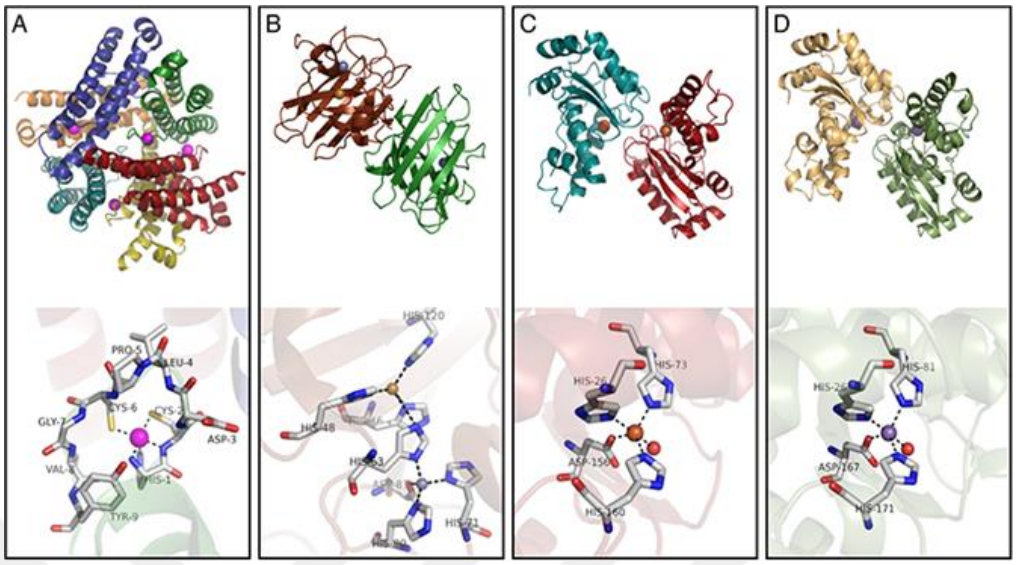



**Figure 10:** Types of SOD enzyme (Ma et al. 2017).

### 1.3.2 Catalase (CAT)

Catalase (CAT) is a heme tetrameric enzyme, its active heme site located deep in protein, its substrate is only hydrogen peroxide which dismutase into one dioxygen and two water molecules, **(Figure11)** (Domínguez et al. 2010).

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

**Figure 11:** Catalase enzyme function.

This reaction proceeds in two steps, in the first reaction the enzyme oxidized high-valent iron intermediate by capturing 2e forming $H_2O_2$ molecule to produce oxoferryl porphyrin (compound 1) which immediately reacts with another molecule of $H_2O_2$. The outcome of both reactions are two molecules of water and one molecule of oxygen (Alfonso-Prieto et al. 2009).

$$\text{Enz (Por-FeIII)} + H_2O_2 \text{Cpd I} \longrightarrow \text{(Por}^{\cdot+}\text{-FeIVdO)} + H_2O \quad (1)$$

$$\text{Cpd I (Por}^{\cdot+}\text{-FeIVdO)} + H_2O_2\text{Enz} \longrightarrow \text{(Por-FeIII)} + H_2O + O_2 \quad (2)$$

The Classifications of catalase enzyme can be divided into four main groups **(Figure 12):**

1. The heme-containing mono-functional catalase for which hydrogen peroxide is both electron donor and acceptor.
2. The heme-containing bifunctional catalase peroxidases (CPXs) with activity rate much higher than mono-functional.
3. The nonheme-containing catalase with its reaction center is manganese complex.
4. Miscellaneous group contain proteins with minor catalytic but no peroxidatic activities (Klotz, M. G., & Loewen, P. C. 2003).

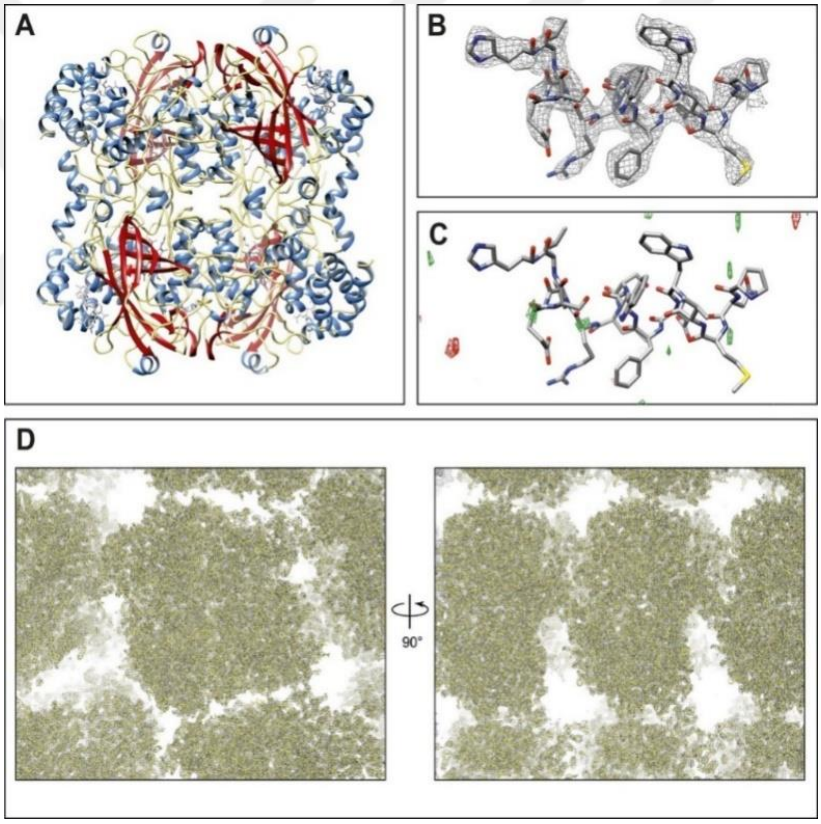



**Figure 12:** Catalase enzyme structure (Nannenga, B. L et al 2014).

### 1.3.3 Glutathione Peroxidase (GPX)

Glutathione peroxidase (GPX) (EC 1.11.1.9 and EC 1.11.1.12) is the general name for a family of multiple isozymes. Based on subcellular locations this family can be divided into six types: GPx1 found in the cytosol, nucleus and mitochondria, GPx2 accumulates in the cytosol and nucleus, GPx3 is a secreted protein and found in the cytosol, GPx4 is present in the nucleus, cytosol, mitochondria, and as bound to membranes, GPx5 and GPx6, identified in mammals. (Margis et al. 2008).

A cytosolic enzyme contains selenocysteine in its active site, it reduces $H_2O_2$ to $H_2O$ and also reduces lipid peroxides to lipid alcohols (Tabet, F., & Touyz, R. M. 2007). GPX catalyzes the reduction of hydroperoxides (ROOH) by glutathione (GSH) to produce water, alcohol and Glutathione disulphate (Minerva, B. 1985).

$$ROOH + 2\ GSH \longrightarrow ROH + H_2O + GSSH$$

$$GSSG + NADPH + H \longrightarrow 2\ GSH + NADP^+$$

### 1.3.4 Glutathione-S-Transferase (GST)

Glutathione-S-Transferase (GST) (EC 2.5.1.18) is a major group of detoxification enzymes catalyze the reaction of the sulfydryl group of tripeptide glutathione with the electrophilic sites of the xenobiotics. This conjugation leads to molecules less reactive and more soluble thus easily to eliminated from the cell (Woude et al. 2009). Not only exogenous xenobiotic conjugate with GST but also endogenous substance such as prostaglandins and leukotrienes. GST enzymes belong to the phase II enzymes, which involved to drug resistance in cancer chemotherapy (Hayes et al.1990). They are classified according to immunological, biochemical and partial sequence characterization into six types. Alpha class has presently 14 members, all of which are identified in vertebrate species, however, it may also present in fish **(Figure 13)**. Mu class, present in human which was identified as M1a, M1b, and M2. Pi class consist of only one member in every species. Sigma class comprises of the four S-crystallins from octopus and the two sequences from

squid lens, Theta class in yeast, bacteria, insects, also two cDNAs for theta class have been cloned from rat (İşgör, B. 200). Moreover, Microsomal GSTs which contain two enzymes cloned from human and rat (Buetler, T. M. & Eaton, D. L. 1992).

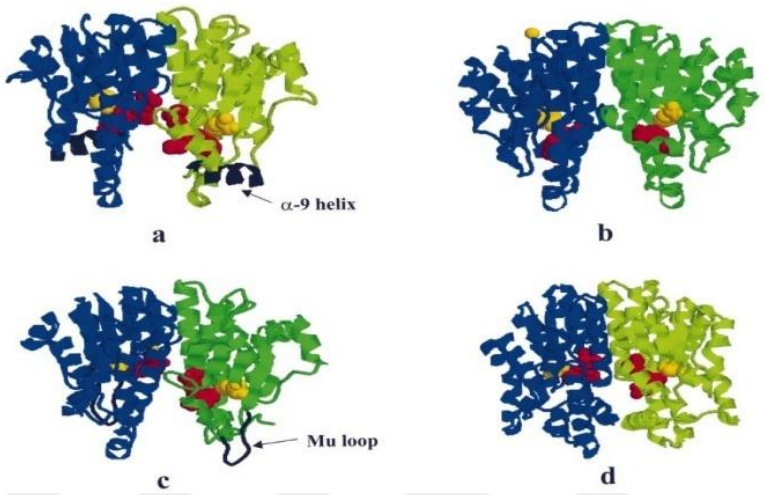



**Figure 13:** A. Human Alpha class 1GUH); (Sinning et.al.1993), B. human Pi class (1GSS); (Reinemer et al 1992), C. rat Mu class (6GST); (Xiao et al.1996), D. human Theta class (1LJR); (Rossjhon et al 1998). (Sheehan et al. 2001).

## 1.4 The Aim of the Study

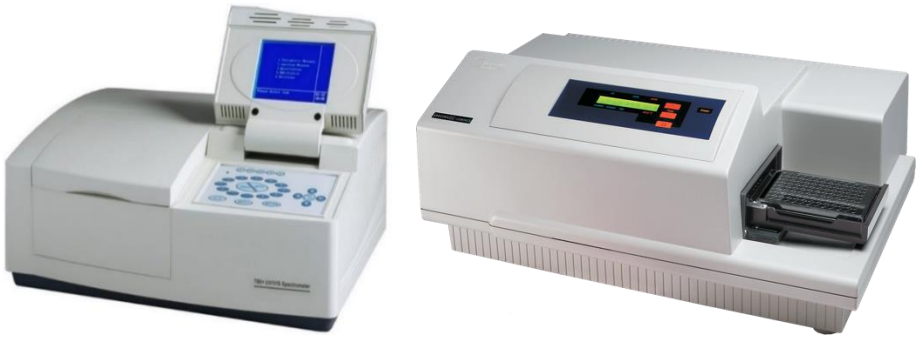
The aim of this study is to evaluate the effect of antibiotics Enrofloxacin and Tylosin on the intracellular antioxidant enzyme system such as Catalase (CAT), Superoxıde Dısmutase (SOD), Glutathione peroxidase (GPX), and Glutathione-S-Transferase (GST), and also to study if that effect may lead to increase the concentration of free radical and cause oxidative stress. Furthermore, we will be measuring the stability of these antibiotics in different environmental conditions, such as temperature.

# CHAPTER 2

## MATERIALS AND METHODS

### 2.1 Materials and Instruments:

Enrofloxacin lot #: LRAB3055, Tylosin tartrate lot #: LRAA3315 were purchased from Sigma Aldrich. Catalase, Superoxide dismutase, Dichlorohydroxy Benzene Sulfonic Acid (DHBS), Xanthine, Ethylene Diamine Tetra acetic acid Disodium Salt Dihydrate (EDTA), Reduced form of Glutathione, Glutathione Peroxidase and Glutathione Reductase (Sigma-Aldrich). Horse Reddish Peroxidase (HRP), 4-Amino Antipyrine (AP), tert- Butyl hydroperoxide, 70% Solution in water and Sodium Azide NaN3, Nicotinamide adenine dinucleotide phosphate reduced tetrasodium trihydrate (NADPH) (GERBU), (Across, Germany). Nitro Blue Tetrazolium Chloride (NBT) (Thermo), Xanthine Oxidase (Calbiochem), Bovine serum albumin (BSA), Sodium carbonate and bicarbonate salt (Merck), Hydrogen Peroxide ($H_2O_2$) (J.T. Baker), 1-Chloro-2,4- Dinitrobenzene (CDNB) (Fluka). Dimethyl Sulfoxide (DMSO) (Carloerba) Ethanol, Acetone, Mono potassium Phosphate and Dipotassium phosphate (Riedel de haen, Germany), Bovine liver cytosol was extracted in our laboratory from the bovine liver which brought from the slaughter house in Kazan-Ankara. Ultraviolet spectroscopic measurements were performed by double beam spectrometer T80 UV/VIS with band length 190-1100 nm **(Figure 14.A).** Enzymatic assays were carried out by a Multi-mode microplate reader Molecular Devices Spectramax M2 **(Figure 14.B).**



**A** **B**

**Figure 14:** (A) Double beam, T80 UV/VIS. (B) Molecular Device Spectramax M2.

## 2.2 Methods

### 2.2.1 Preparation of Standard Drug Samples:

**A.** Enrofloxacin was dissolved in dimethyl sulfoxide (DMSO) 1mg/1ml (Kato, et al. 2007). The stock solution was then divided into two samples each stored in different temperature, at +4 °C and +22 °C. We used these samples for the stability test where each sample is tested every two days for the period of three months.

**B.** The same procedure was followed to test the stability of Tylosin tartrate was dissolved in water at 1mg/ml then divided into two samples stored at +4 °C, 22 °C (room temperature) and tested every three days for the period of three months.

### 2.2.2 Determination of Drugs Stability

Since the maximum absorbance of Enrofloxacin at 276 nm in the concentration 35 ul/ml, and the range of wavelength is from 200 nm to 400 nm. Drug was dissolved in DMSO 1 mg/ ml and stored in the dark environment different temperature at +4 °C, and at room temperature to study its stability for three months. Tylosin tartrate was dissolved in water 1mg/ml, the stock solution was divided into two samples stored respectively at +4 °C, and at room temperature in order to measure the stability in different temperatures. The maximum absorbance of Tylosin was at 282 nm in the concentration 75 ul/ml and the range of wavelength is from 200 nm to 400 nm.

## 2.3 Enzyme Assays

### 2.3.1 Catalase Enzyme Assay

Serial dilutions of drugs (Enrofloxacin and Tylosin) solutions used in this assay were at 1:3 ratio of standard stock solution 1g/L **(Table 3).**

**Table 3:** Concentrations of Enrofloxacin and Tylosin used in CAT assay

| Dilutions | Stock Drugs Concentrations (g/L) | Final Drugs Concentrations in CAT Assay (mg/L) |
|---|---|---|
| 1 | 0.333 | 13.33 |
| 2 | 0.111 | 4.44 |
| 3 | 0.0370 | 1.48 |
| 4 | 0.0123 | 0.494 |
| 5 | 0.004115 | 0.165 |
| 6 | 0.00137 | 0.055 |

The indirect measurement of catalase activity is determined by measuring the remaining concentration of $H_2O_2$ from the catalase reaction inhibited by adding sodium azide (Aebi 1984). $H_2O_2$ remaining concentration is measured through the reaction of chromogen complex with enzyme horse reddish peroxidase (HRP) which produces red quinoneimine dye and change its color, measured at 520 nm. This assay was optimized to be feasible for microplate application (İşgör, İşcan et al. 2008). Each 5ml of chromogen contain three different compounds: (1) 1ml of 4 AP, 1.25 mM, (2) 1ml of DHBS, 10 mM; and (3) 3 ml of phosphate buffer at 150 mM, with pH 7.0.

The reaction mixture is prepared and incubated for 2 minutes, then 50 µL of sodium azide (15 mM) was added to stop CAT activity then incubated again for 5 minutes. After incubation period elapsed, 5 µL of each well was taken and added to the following column in microplate. Then 255 of chromogen containing HRP was added to each well and incubated for 45 minutes, this assay was measured at 520 nm **(Table 4)**. And based on the calibration curve for hydrogen peroxide concentration, ranging from 0.00192 mM to

0.12308 mM, we measured the $H_2O_2$ remaining concentration to determine catalase activity.

**Table 4:** The reagents used in CAT assay.

| Stock | Volume Added |
|---|---|
| Chromogen in total of 5 ml | |
| 1.25 mM 4 Amino Antipyrine | 1ml |
| 10 mM Dichloro Hydroxyl Benzene Sulfonic Acid | 1ml |
| 150 mM Phosphate Buffer | 3 ml |
| For each 5 ml chromogen, 5 µl HRP was added | |
| Reaction Mixture | |
| Drug | 4 µl |
| 100 U/ml Catalase | 20 µl |
| 10 mM Hydrogen Peroxide | 50 µl |
| 50 mM Phosphate Buffer | 26 µl |
| Incubation for 2 minutes | |
| 15 mM Sodium Azide | 50 µl |
| Incubation for 5 minutes | |
| Above mixture + Chromogen | 5 µl + 255 µl |
| Incubation for 40 minutes and read absorbance at 520 nm | |

***4 µL of phosphate buffer is used as drug control with Tylosin.**
***4 µL of DMSO is used as drug control with Enrofloxacin.**

### 2.3.2 Superoxide Dismutase (SOD) Enzyme Assay

This assay protocol (Işgor, B. S., 2013) is based on the measurement of blue colored Formazan which is produced due to the reduction of nitro blue tetrazolium (NBT) by superoxide anion **Figure 15**. The absorbance was read at 560 nm, the concentrations of drug was used as showed in **Table 5**, and the concentrations of assay mixtures are given in **(Table 6)**.

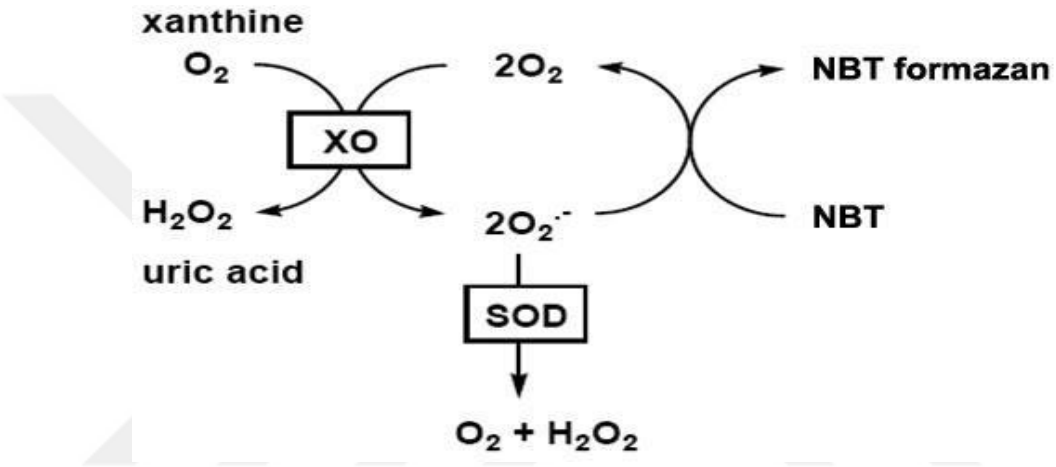



**Figure 15:** SOD enzyme reaction.

**Table 5:** Concentrations of Enrofloxacin and Tylosin used in SOD assay

| Dilutions | Stock Drugs Concentrations (g/L) | Final Drugs Concentrations in CAT Assay (mg/L) |
|---|---|---|
| 1 | 0.333 | 13.33 |
| 2 | 0.111 | 4.44 |
| 3 | 0.0370 | 1.48 |
| 4 | 0.0123 | 0.494 |
| 5 | 0.004115 | 0.165 |
| 6 | 0.00137 | 0.055 |

**Table 6**: The reaction mixture used in SOD Assay

| Reagents | Volumes added (µl) | Contents |
|---|---|---|
| Assay Buffer | 235 | 10 µl of 25mM NBT, 75 µl of 0.3 mM Xanthine, 150 µl of 200 mM Sodium Carbonate Buffer with 10 mM EDTA |
| Cytosol | 5 | Directly from stock |
| Drug | 5 | |
| Xanthine Oxidase | 5 | 2 U/ml |
| Incubation for 30 minutes and read absorbance at 560 nm | | |

### 2.3.3 Glutathione-S-Transferase (GST) Enzyme Assay

Glutathione –S–Transferase (GST) enzyme activity was measured by using the optimized and miniaturized protocol published in (Isgor and Isgor 2011) of Habig (Habit et al, 1974). The source of GST enzyme used is purified from bovine liver in our biochemistry laboratory. The assay mixture contains 50 mM of CDNB substrate, 200 mM of GSH, 100 mM of Phosphate buffer, and 10 µl drug of different concentrations with 10 µl of cytosol (the source of GST enzyme) all added to a total assay mixture of 250 µl. The absorbance was measured at 340 nm and the different concentrations of drugs used in this assay are shown in **Table 7**, where the reaction mixture and the procedure are shown in **Table 8.**

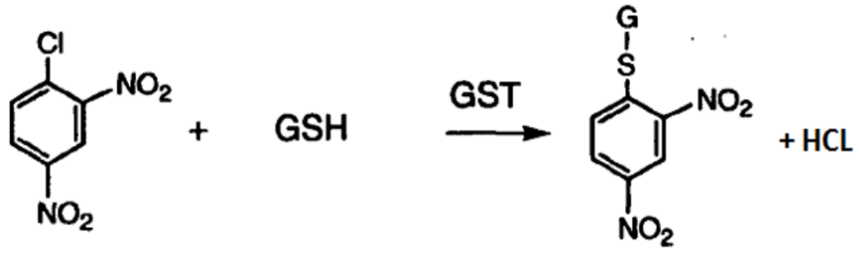



**Figure 16:** Conjugation GSH and CDNB (Hausheer, F. H 2007).

**Table 7:** Concentrations of Enrofloxacin and Tylosin used in GST assay

| Dilutions | Stock Drugs Concentrations (g/L) | Final Drugs Concentrations in CAT Assay (mg/L) |
|---|---|---|
| 1 | 0.333 | 13.33 |
| 2 | 0.111 | 4.44 |
| 3 | 0.0370 | 1.48 |
| 4 | 0.0123 | 0.494 |
| 5 | 0.004115 | 0.165 |
| 6 | 0.00137 | 0.055 |

**Table 8:** The reagent mixture used in GST assay

| Reagents | Volume added (µl) | Contents |
|---|---|---|
| Assay buffer | 200 | 184 µl Phosphate buffer 100 mM pH 6.5,<br>4 µl GSH 200 mM,<br>12 µl of CDNB 50 Mm |
| cytosol | 10 | Source of GST |
| Drug | 10 | Drug serial dilution |
| Phosphate buffer | 30 | 200 mm pH 6.5 |
| Read the absorbance at 340 nm | | |

### 2.3.4 Glutathione Peroxidase (GPX) Assay

$$2\ GSH + H_2O_2 \xrightarrow{GPX} GSSG + 2\ H_2O$$

$$GSSG + NADPH \xrightarrow{GR} NADP + 2\ GSH$$

The oxidation of NADPH to $NADP^+$ **(Figure 17)** is monitored spectrophotometrically by a decrease in absorbance at 340 nm (A340). Under conditions in which the GPx activity is rate limiting, the rate of decrease in the absorbance at 340 nm is directly proportional to the GPx activity in the sample. The reagents used in the assay shown in **Table 9**. The concentrations of drugs used in this assay are shown in **Table 10**.

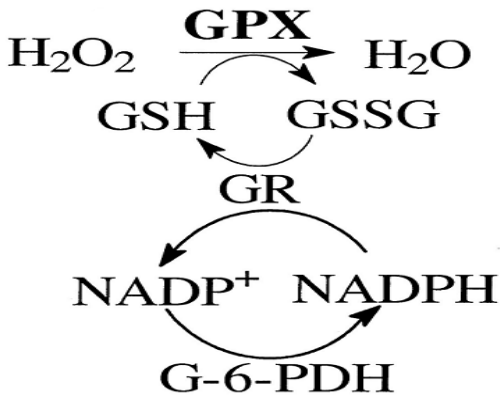



**Figure 17:** The chemical reaction of the oxidation (NADPH)

**Table 9:** The reagents mixture used in GPx assay.

| Reagents | Amount Per well (µL) | Details |
|---|---|---|
| GPx | 42µL | 0.25 U/mL |
| Drug | 8 µL | From drug serial dilutions |
| Assay mixture | 30 µL | 5 µL of NADPH 10Mm<br>20 µL of GSH 20mM 5 µL of GR 20 U/mL |
| Substrate buffer | 120 µL | 2 µL of t-BuOOH 30mM<br>118 µL of (Tris HCl,50mM pH 8 with EDTA 0.5 mM) |

**8µL of phosphate buffer instead of drug was used as control.**

**Table 10:** Concentrations of Enrofloxacin and Tylosin used in GPx assay.

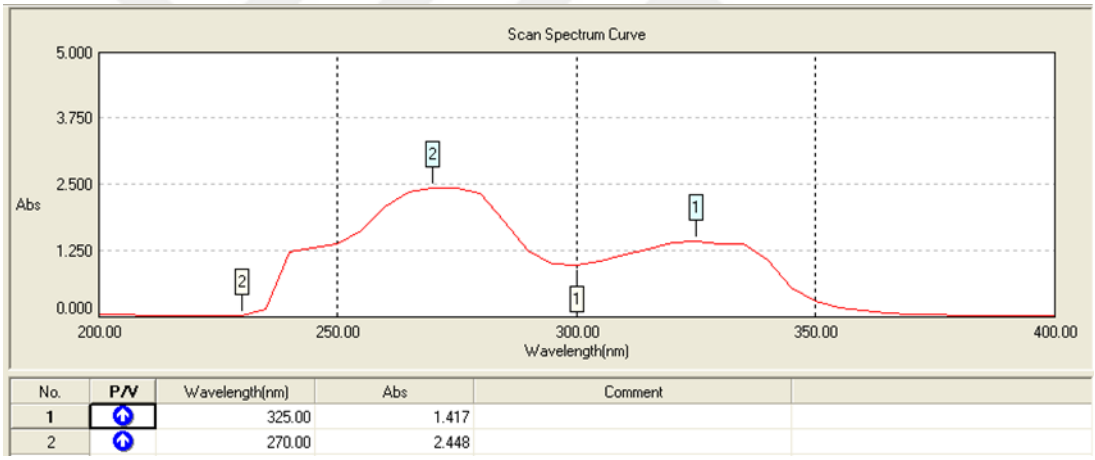
| Dilutions | Stock Drugs Concentrations (g/L) | Final Drugs Concentrations in GPx Assay (mg/L) |
|---|---|---|
| 1 | 0.333 | 13.33 |
| 2 | 0.111 | 4.44 |
| 3 | 0.0370 | 1.48 |
| 4 | 0.0123 | 0.494 |
| 5 | 0.004115 | 0.165 |
| 6 | 0.00137 | 0.055 |
| 7 | 0.000456 | 0.018 |

# CHAPTER 3

## RESULT AND DISCUSSION

### 3.1 Stability test results

Stability studies on Enrofloxacin showed that its half-life exceeded 120 days in a dark environment (Wu et al., 2005). Molar absorptivity (ε) of Enrofloxacin in several solvents at the wavelength of maximal absorption (λ max) is at 276 nm (Elizondo, M et al.1997), shown in **(Figure 18)**.




| No. | P/V | Wavelength(nm) | Abs | Comment |
|---|---|---|---|---|
| 1 | | 325.00 | 1.417 | |
| 2 | | 270.00 | 2.448 | |

**Figure 18:** Enrofloxacin absorbance (1mg/ml in dimethyl sulfoxide DMSO).

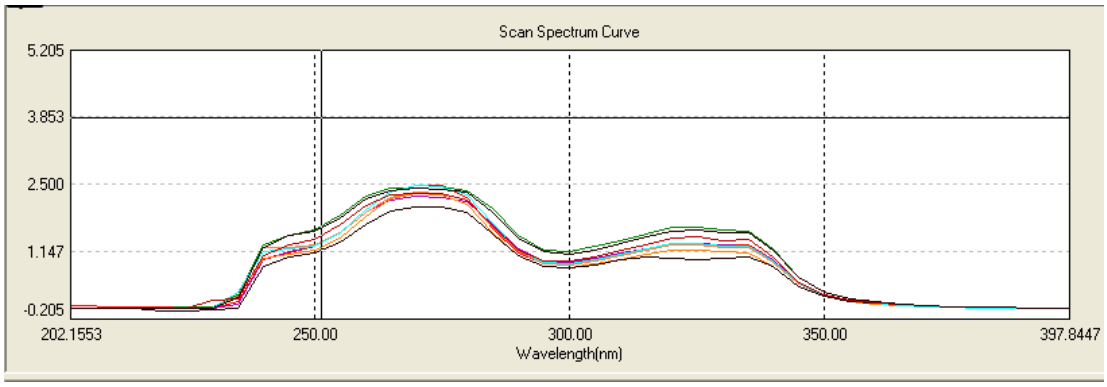
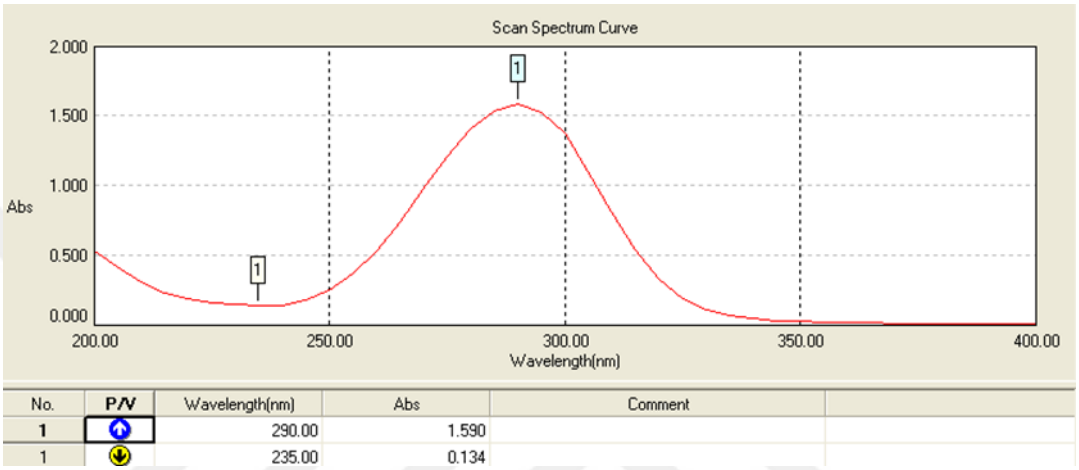



**Figure 19:** Combination of readings for three months for Enrofloxacin in DMSO.

**Figure 20** shows the maximum absorption (λ max) of Tylosin in water at wavelength of 282 nm (McGuire, et al., 1961). The stability study for Tylosin confirmed that it is stable for three months at +4 °C and also at room temperature in dark environment **(Figure 21)**.




| No. | P/V | Wavelength(nm) | Abs | Comment |
|---|---|---|---|---|
| 1 | ▲ | 290.00 | 1.590 | |
| 1 | ▼ | 235.00 | 0.134 | |

**Figure 20:** Tylosin Tartrate UV absorbance (1 mg/ ml $H_2O$).

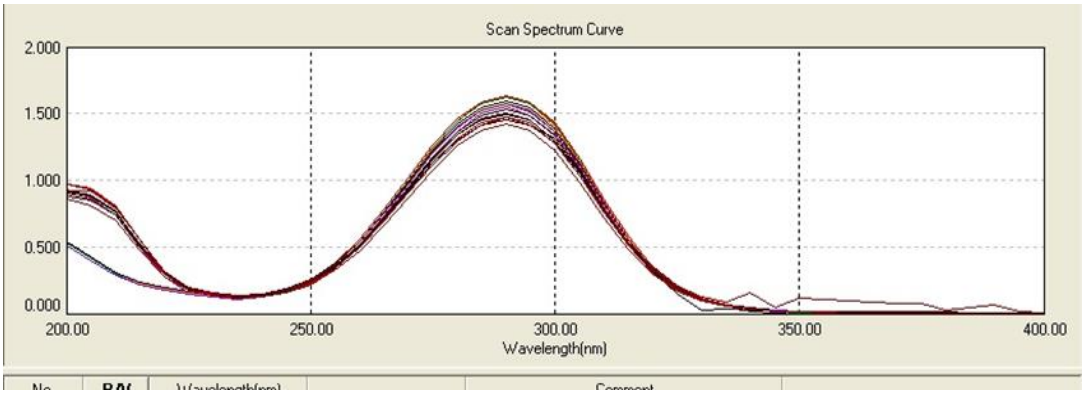



**Figure 21:** Combination of readings for three months for Tylosin tartrate in water.

### 3.2 Enzymes assay results

**Table 11:** Concentrations range of Enrofloxacin and Tylosin stock solutions used in enzyme assays.

| Dilutions | Stock Drugs Concentrations (g/L) |
|---|---|
| 1 | 1.000 |
| 2 | 0.333 |
| 3 | 0.111 |
| 4 | 0.0370 |
| 5 | 0.0123 |
| 6 | 0.004115 |
| 7 | 0.00137 |
| 8 | 0.000456 |

#### 3.2.1 The Effect of Drugs on CAT Enzyme Activity

The comparison of the different five concentrations of drug measured in our test with the control depicted in **Figure 22** shows that Enrfloxacin decreases the activity of CAT enzyme about 20%.

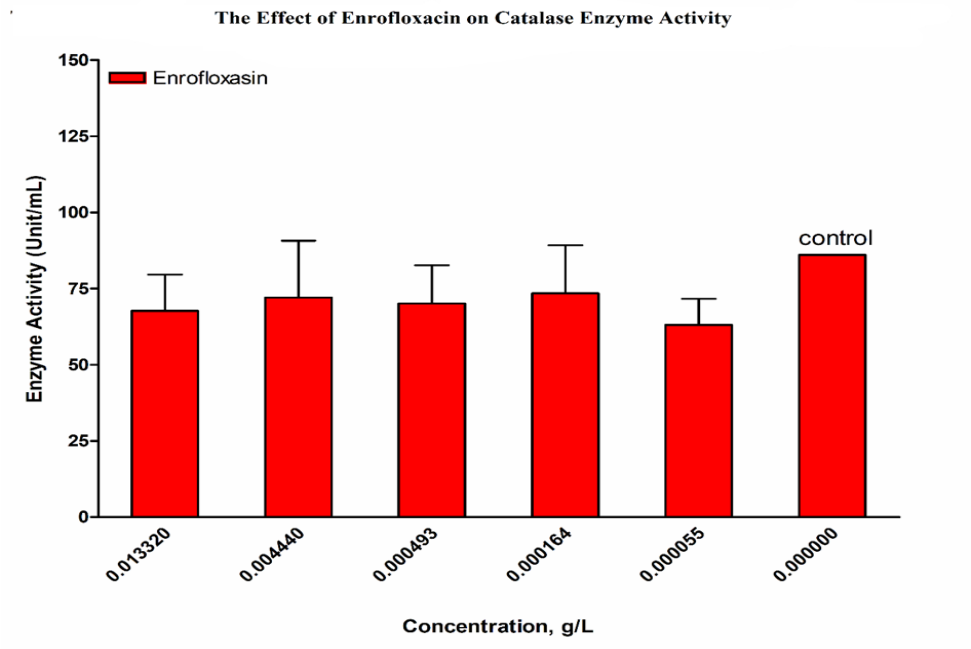



**Figure 22:** The effect of Enrofloxacin on Catalase enzyme activity.

However, our test shows no response (no effect) of Tylosin on CAT enzyme as we observed no decrease when comparing control with five different concentrations of drug shown in **Figure 23**.

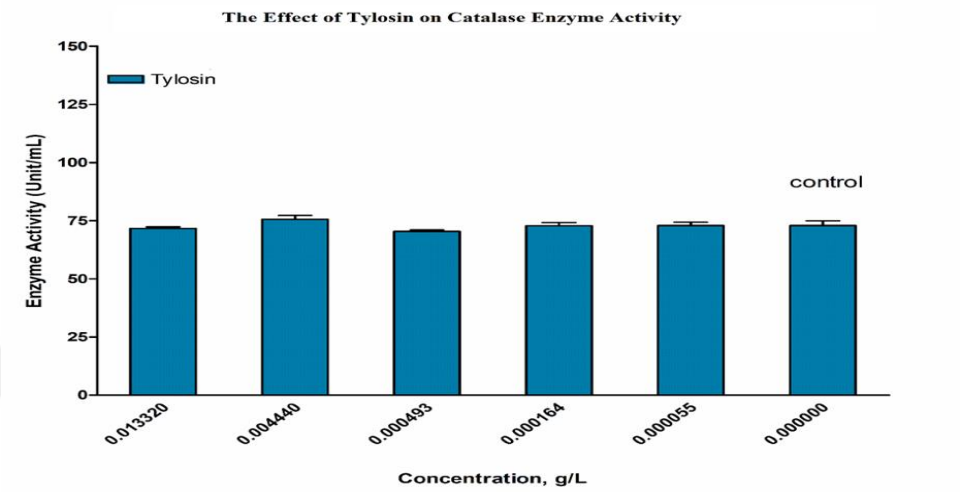



**Figure 23:** The effect of Tylosin on catalase enzyme activity.

### 3.2.2 The Effect of Drugs on GST Enzyme Activity

According to our test, Enrofloxacin showed less effect on GST enzyme. From the **Figure 24**, comparing the control with seven different drug measurements shows only 5% of decrease in enzyme activity.

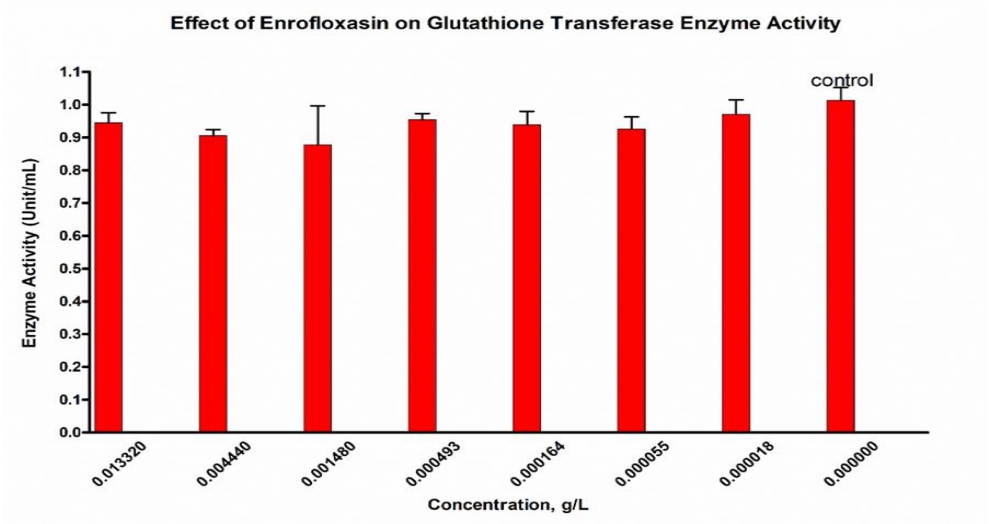



**Figure 24:** The effect of Enrofloxacin on Glutathione –S-Tranferase enzyme activity.

However, Tylosin showed no effect on Glutathione-S-Transferase enzyme activity during our test. **Figure 25** shows the control and seven different measured concentrations of the drug observed throughout the test.

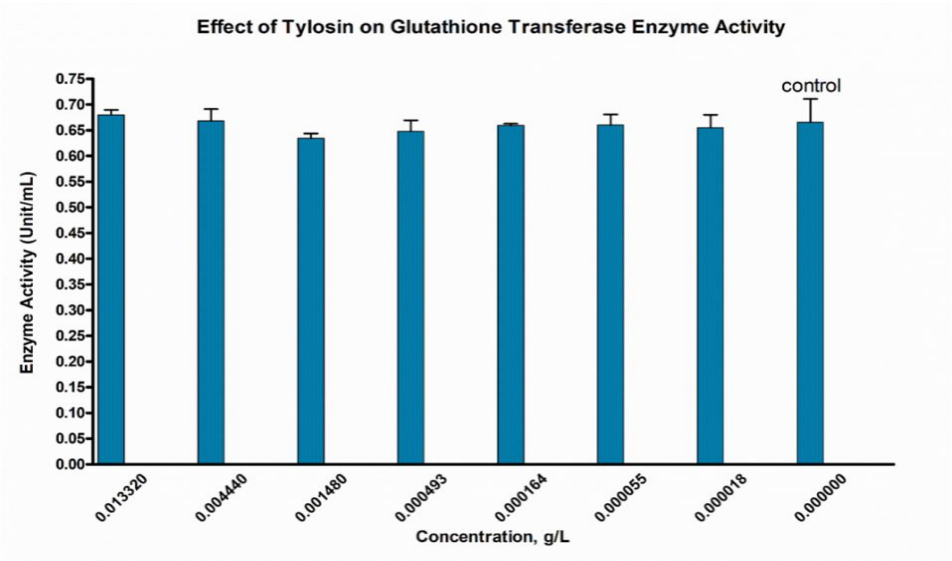



**Figure 25:** The Effect of Tylosin on Glutathione-S-Tranferase Enzyme Activity.

### 3.2.3 The Effect of Drugs on GPX Enzyme Activity

GPX enzyme activity was increased by 15% with Enrofloxacin concentration of 0.013320 g/L with respect to the control **(Figure 26).**

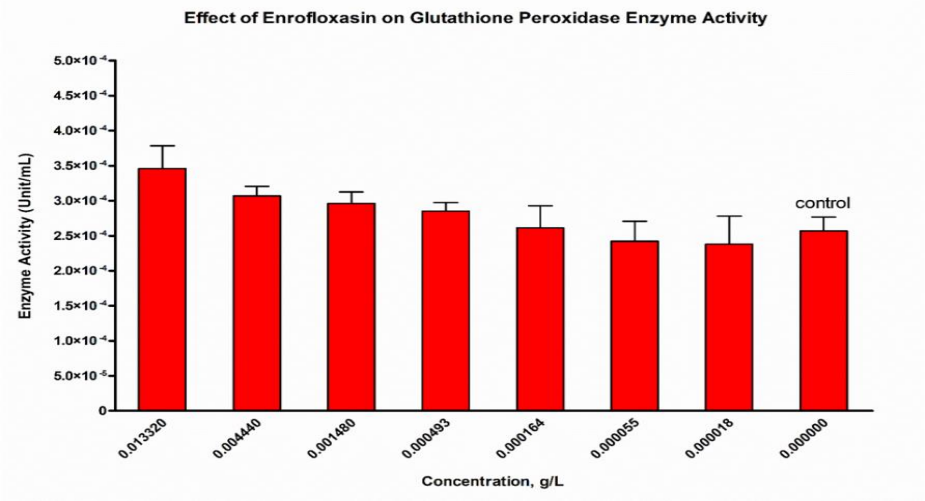



**Figure 26:** The Effect of Enrofloxacin on Glutathione Peroxidase enzyme activity.

However, we observed that the activity of GPX was slightly increased about 10% with concentration of Tylosin at 0.013320 g/L, shown in **Figure 27.**

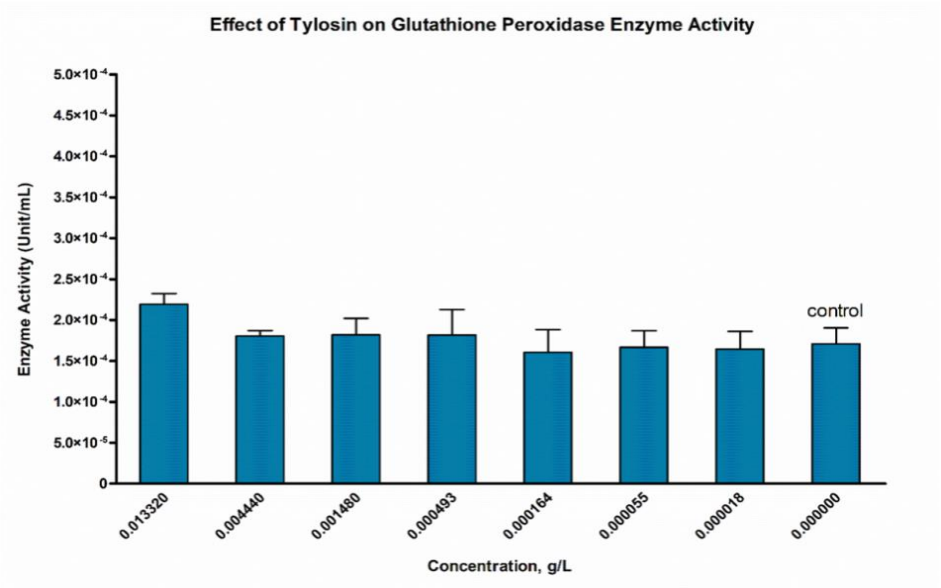



**Figure 27:** The effect of Tylosin on Glutathione Peroxidase enzyme activity.

### 3.2.4 The Effect of Drugs on SOD Enzyme Activity

Our study showed no effect of the selected drugs on SOD activity.

## DISCUSSION

In this study, we evaluated the effect of two antibiotic drugs Enrofloxacin and Tylosin on antioxidant enzymes. According to the beer-lambert law (mcnaught and mcnaught 1997), the absorbance of a molecule is directly proportional to its concentration. Therefore, the drug solubility and stability tests were observed at the wavelength where λ max is observed.

Since Enrofloxacin aqueous solubility in water at 25°C is 146 μg/ml according to (Seedher, N., & Agarwal, P. 2009), we dissolved Enrofloxacin in suitable solvent DMSO at 1 mg/ ml. However, Tylosin solubility in water is measured at 25 °C for 5 mg/ml and it is also soluble in lower alcohols, esters, ketones, chlorinated hydrocarbons, benzene, ether, acetone, and chloroform. The Enrofloxacin stability test for the period of three months, measured between 200 to 400 nm of wavelength with λ max at 276 nm shows that Enrofloxacin stability exceeds 120 days at +4 °C, and also when tested at room temperature in a dark environment (Lizondo, M et al.1997). Tylosin solutions stability are found to be stable with pH level from 4 to 9, with maximum stability at pH 7 (McGuire, et al., 1961), whereas UV detection wavelength for all Tylosin forms is 284.8 nm (Kolz, A. C., et al. 2005).

We examined the effect of Enrofloxacin and Tylosin drugs on four enzymes, including Catalase (CAT), Superoxide Dismutase (SOD), Glutathione S Transferase (GST), and Glutathione Peroxidase (GPX). The examination of Enrofloxacin effect in vitro on CAT enzyme showed 20% decrease of enzymatic activities. Similar result was found by (Gao, Y, et al. 2008) in vivo showed that the effect of Enrofloxacin on CAT activity was not significant in all treatment groups with different doses of the drug, and also the observed CAT activity changes only indicate short-term responses. Tylosin effect on antioxidant enzymes wasn't previously studied, however, a study conducted in vivo on similar macrolides antibiotics, namely Tilmicosin, showed that the decrease in CAT activity was observed in all experimental groups when compared to the control group (Eraslan, GOKHAN 2007). The observed decrease is statistically significant but only in the first

period of all experimental groups when compared to the control group. Our test in vitro of Tylosin effect on CAT enzyme shows no response (no effect) as no decrease or increase was observed when comparing control with five different concentrations of drug.

Enrofloxacin test on GST enzyme showed only 5% decrease of activity while Tylosin showed no effect according to the seven different measured concentrations of the drug. Enrofloxacin caused a decrease of GPX enzyme activity by 15% at concentration of 0.013320 g/L with respect to the control which considered to have no effect on the function of enzymatic antioxidant in the defense system (Elamaran, A., Hariharan, et al. 2015). However, we observed that the activity of GPX was slightly increased for about 10% with concentration of Tylosin at 0.013320 g/L. Last, our test in vitro showed no effect of both drugs on SOD Enzyme activity.

The study conducted on the effect of Ciprofloxacin as a Flouroquinolone on CAT and GPX activities showed an increase in CAT and GPX activities. At their maximum measurements values, the activities of these enzymes were increased by 115% of CAT activity and 127% of GPX with respect to the control (Nie, X. P., Liu et al. 2013). The present study shows that there is no significant effect of antibiotic Enr on CAT, GST and GPX with final concentrations used in the enzymes assays.

The studies conducted on healthy mice treated with flouroquinolones Enrofloxacin and Danofloxacin showed that Danofloxacin affectboth SOD and GPX activities, where ENR only affect GPX activity. It may be related to either DAN or ENR which may directly affect SOD and GPX activities in healthy mice or DAN and ENR may produce ROS, and so ENR and DAN may indirectly effect SOD and GPX activities (Author, E., & Tras, B. 2001).

The examination of Erythromycin as a macrolides antibiotic revealed that the activities of CAT, SOD and GPX were gradually decreased when concentration of Erythromycin increased, whereas the activity of GST did not respond to Erythromycin. (Nie, X. P., et al. 2013).

# CHAPTER 4

## CONCLUSION

Enzymes assays results showed that the effect of the drug Enr on Catalase enzyme activity with slight decreased of 20%, however it is not significant and may not cause a decrease in the function of catalase enzyme. Tylosin shows no effect on catalase enzyme activity for all concentrations of the drug with respect to the control. However, Glutathione-S-Transferase assay results showed that there is no effect for both drugs on the enzyme activity. In the assay of Glutathione Peroxidase enzyme, the results showed that both Enrfloxacin and Tylosin cause a little increase in the enzyme activity.

The results of this study show that Enrofloxacin as a fluoroquinolones antibiotic and Tylosin as a macrolides antibiotic have not significant effect on Catalase, Glutathione -S-Transferase and Glutathione peroxidase enzymes.

It is concluded that they don’t cause any activity on GST enzyme therefore, they don’t cause any resistance against other drugs which means it may advised to use safely with low concentrations as antibacterial drugs for patients who take chemotherapy drugs.

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