THE STUDY OF FLUORESCENCE QUENCHING OF NORFLOXACIN BY ASCORBIC ACID

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The Study of Fluorescence Quenching Norfloxacin by Ascorbic Acid

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Declaration

I hereby declare that this Master Thesis entitled **The Study of Fluorescence Quenching Norfloxacin by Ascorbic Acid** is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation

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_____ _____ _____

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We, the undersigned, members of the Board of Examiners of the thesis by B**edria Shukuarl** have read and evaluated the thesis entitled "**Study of the Fluorescence Quenching Norfloxacin by Ascorbic Acid**" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Laser spectroscopy.

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LIST OF ABBREVIATIONS

AA ----------Ascorbic Acid

DDW ----------Double distilled Water

FT-IR ---------- Fourier Transform Infra Red

HCL ----------Hydrocloric acid

NRF ----------Norfloxacin

UV ----------Ultraviolet Visible

ΔH---------- Change in enthalpy

ΔS ---------- Change in entropy

ΔG ---------- Change in energy

ABSTRACT

Currently, drug/drug or food/drug interactions are great field of study, due to rapid development of new types of drugs and increasing use of food supplements. The interactions of ascorbic acid (AA) with norfloxacin (NRF) were investigated by fluorescence quenching, UV/vis and Fourier transform infrared (FTIR) spectroscopic techniques. The results of the study indicated that the fluorescence quenching between ascorbic acid and norfloxacin could be rationalized in terms of static quenching or the formation of non-fluorescent AA-NRF complexes. From fluorescence quenching spectral analysis the binding constant (k_a), number of binding sites (n), and thermodynamic properties, were determined. The quenching constants (k_{sv}) is $2.27 \times 10^3 M^{-1}$ at 298K and binding sites n is 2. The thermodynamic parameters determined by using Van't Hoff equation. The determined thermodynamic parameters suggest that the binding process occurs spontaneously by involving hydrogen bond and van der Waals forces played the major role in the reaction of AA with NRF.

Keywords: *Ascorbic acid, binding constant, fluorescence quenching, norfloxacin,*

CHAPTER ONE

1. Introduction

1.1. Background of the Study

Currently, drug/drug or food/drug interactions are great field of study, due to rapid development of new types of drugs and increasing use of food supplements (Abraha et al., 2017). The interaction of antibiotics with bioactive components has attracted interest because they are found in various food sources and drugs. Bioactive components that are found in popular drinks and foods (such as, fruit, and vegetables) are the most useful compounds for human health. Moreover, they are the most widely consumed of all behaviorally active drugs in the world. This interaction may alter the pharmacokinetics or change the toxicity risk of the drugs. Understanding their binding in a biological system at the molecular level is useful.

Ascorbic acid (AA), also called vitamin C, is a kind of water-soluble antioxidant and plays a vital role in human life (Wang et al., 2021). It is required for the proper development and function of many parts of the body, including the bones, blood vessels, and skin (Llorent- et al., 2020). AA has various biomedical applications, including antioxidant, antibacterial, antiviral, food preservative, and drug adjuvant (Ganeshkumar et al., 2021). Ascorbic acid has also attracted considerable attention as an anticancer agent (Yuta et al., 2015). It plays a critical role in maintaining proper immune feature; inhibiting the conversion of irritants in smog, tobacco smoke, and certain ingredients into potentially carcinogenic substances; reducing the chance of developing high blood pressure and heart disease; supporting adjust levels of cholesterol; and preventing the development of scurvy. Vitamin C is a crucial co-enzyme in the oxidative stress pathways, able to ROS removal. It also hinders biofilm development, among others: Mycobacterium spp., Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, or Pseudomonas aeruginosa, by inhibiting the production of exopolysaccharides (Carneiro, 2020). It was revealed in previous studies ascorbic acid enhance the levels of interferon, antibody, hormones, ground substance(Shilpi et al., 2018). It is a chain breaking antioxidant and it give their effect by reacting with oxygen, hydroxyl, and superoxide radicals of the substances. It react with radicals of tocopheroxy to re-generate vitamin E (Shilpi et al., 2018). The usefulness of ascorbic acid together with antibiotics is significant high dose ascorbic acid has been shown to have synergistic effect when used with antibiotics. Moreover, there is a possibility of

potentiating the antibacterial activities of the antibiotic by antioxidants (Afzal, SAshraf, Buksh, Akhtar, & Rasheed, 2017)

Norfloxacin(NFX)[1-ethyl-6-fluoro-1,4-dihydro-4-oxo_7-(1-piperazinyl) quinoline-3-carboxylic acid] is a synthetic and potent fluoroquinolone antibacterial agent widely used in clinical treatment of many infections including prostate, skin, pulmonary, digestive, urinary tract infections (Muniz et al., 2014) soft tissue infections, and bone-joint infections (Abraha et al., 2017). The bacterial action of Norfloxacin results from inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, and recombination. The fluorine atom at the 6 position increases potency against gram-negative organisms, and the piperazine moiety at the 7 positions are responsible for anti-pseudomonal activity. The drugs contain several proton binding sites such as carboxyl, carbonyl, and amino groups (Woldegiorges et al., 2021) which help to interact with bioactive compounds.

In the presence of antioxidants such as glutathione and ascorbic acid, some research work involving the bactericidal effect of antibiotics has demonstrated that these antioxidants are protective against the action of antibiotics against E. coli, and the minimum inhibitory concentration of ciprofloxacin (MIC) significantly increased and streptomycin in the presence of antioxidants. Norfloxacin induced mutagenesis was found to be decreasing in Salmonella typhimurium in the presence of vitamins C and E (Paulraj et al., 2019). Carlsson et al. 2005 and Afzal et al. 2017 showed an increased antimicrobial activity of fluoroquinolones and aminoglycosides during AA addition (Kwiecińska-Piróg et al., 2019). Hence, as numerous ponder of the interaction between norfloxacin and ascorbic acid will offer assistance give fundamental data on the pharmacological activities, bio-transformation, bio-distribution of drugs. The authoritative marvels will moreover be valuable to clarify the relationship between the norfloxacin and ascorbic corrosive. Understanding their binding in a biological system at the molecular level is useful. The binding phenomena will also be useful to explain the relationship between the norfloxacin and ascorbic acid.

Various methods such as equilibrium dialysis, ion-selective electrodes, UV/vis, fluorescence and Fourier transform infrared (FTIR) spectroscopy have been used in binding studies (A. Belay, Kim, & Hwang, 2015). Fluorescence spectroscopy is becoming increasingly

important as a quantitative analytical technique because of its high sensitivity and good selectivity (Mi et al., 2013) and it is the most powerful techniques to investigate the interaction between biological molecules. Fluorescence measurements can give some information on the binding mechanism of small molecule biological molecules, including binding mode, binding constants, and binding sites (MM, Ghithan, Abu-Taha, Darwish, & Abu-Hadid, 2014). Among the several physical techniques fluorescence quenching is a useful technique in studying the interaction between two molecules, a fluorophore (M) and its quencher (Q) (Ferrie et al., 2017). Static quenching occurs when M and Q bind to create a dark, less fluorescent complex, [M–Q].

1.2. Statement of the Problems

The interaction of antibiotics with bioactive components has attracted interest because they are found in various food sources and drugs. Several research works dealing with bactericidal effect of antibiotics in the presence of antioxidants for instance glutathione and ascorbic acid have proved the protective role of these antioxidants against the actions of antibiotics on Escherichia coli with a significant increase in the minimum inhibitory concentrations (MICs) of ciprofloxacin and streptomycin in the presence of antioxidants. Norfloxacin induced mutagenesis was found to be decreasing in Salmonella typhimurium in the presence of vitamins C and E (Paulraj et al., 2019). Carlsson et al. (2005) and Afzal et al. (2017) showed an increased antimicrobial activity of fluoroquinolones and aminoglycosides during AA addition (Kwiecińska-Piróg et al., 2019). The study shows that interaction of norfloxacin with ascorbic acid increase the efficacy of antibiotics. However, detail of fluorescence spectral analysis such as binding constants, the binding sites, and thermodynamic parameters between norfloxacin and ascorbic acid are not investigated well.

1.3. Objective

1.3.1. General Objective

The general objective of this research is Study of the Fluorescence Quenching Norfloxacin by Ascorbic Acid

1.3.2. Specific Objectives of the Study

The specific objectives of this study are:

- Investigating of the quenching mechanism.

- Determine the binding constants and binding sites
- Calculate the thermodynamic parameters, binding force operating between the compounds and conformational changes from fluorescence quenching spectrum analysis.

1.4. Significance of the Study

Nowadays, commercial vitamins are available in additional to present in food and drink in the form of tablet. Among such vitamins ascorbic acid is one of important bioactive compound it has properties of antioxidant, antibacterial, antiviral, food preservative, and drug adjuvant, and so forth. This research will be focusing on the interaction of AA with NRF. It is important in treatment infection disease by fluoroquinolone and pharmaceutical industrial applications. Moreover, the final results of this study might be used as an input for other researchers and it will be intensively investigated with the field of pharmaceutical industrial.

CHAPTER TWO

2. Literature Review

In this chapter, the concepts of vitamins, the basic properties ascorbic acid and the antibacterial activities of ascorbic acid. The concepts of fluoroquinolone antibiotics, the basic properties norfloxacin, the bacterial activities of norfloxacin, the interaction ascorbic acid and norfloxacin. The concept of fluorescence, factors that reduce fluorescence intensity, fluorescence quenching mechanism, binding sites and binding constant, thermodynamic parameters and nature of the binding forces norfloxacin in the literature broadly.

2.1. Vitamins

Vitamins are organic compound (non-energy producing), which are essential for normal human metabolism that must be supplied in small quantities in the diet (Desai et al., 2019). The definition excludes the inorganic important trace minerals and important amino acids and fatty acids which can be required in plenty large quantities. Other materials needed for proper growth of microorganism or cells in tradition are known as growth factors. The exclusive chemical forms and precursors of vitamin may be known as its vitamers. The vitamins as a drug are essential in the prevention and treatment of deficiency disease. Some vitamins do have primary makes use of in pharmacological uses in pharmacological doses.

2.2. Ascorbic Acid

Ascorbic acid, (vitamin C) is one well known vitamin and first found in the Nineteen Twenties by a Hungarian biochemist, Albert Szent-Györgyi. We get vitamin C from the food particularly fruit and vegetables. Human bodies need vitamin C to make a substance collagen which is important for the health and repair of our skin, bones, teeth and cartilage (Desai et al., 2019). Ascorbic acid (AA) is a kind of water-soluble antioxidant and plays a vital role in human life (Wang et al., 2021). Figure 2.1 is the chemical structure of ascorbic acid. In nature, there are two isomeric molecules of vitamin C present in equal parts, the reduced form (D-ascorbic acid) and the chemically active and oxidized form (L-ascorbic acid), which are mutually interchangeable(Abdelraheem et al., 2022). Ascorbic acid cannot be synthesized by human, consequently dietary supplementation of about 75–1000 mg per day is needed. The common sources of vitamin C are citrus fruits and some other foods like tomatoes, broccoli, cauliflower, spinach, ladyfinger etc (Desai et al., 2019).. Ascorbic acid (after oral intake) is absorbed from the

small intestine and transported into cells through particular transporters: Sodium-depend Vitamin C Transporters (SVCTs)-1 or -2. After absorption, vitamin C is distributed through blood to the tissues, mainly the liver, brain, and adrenals. The biological functions of ascorbic acid stem from its ability to provide reducing equivalents for a variety of biochemical reactions.

Vitamin-C also has antiviral and antibacterial, by enhancing lympho-proliferation. It was revealed in previous studies Vitamin-C enhance the levels of interferon, antibody, hormones, ground substance(Shilpi et al., 2018). It is a chain breaking antioxidant and it give their effect by reacting with oxygen, hydroxyl, and superoxide radicals of the substances. It react with radicals of tocopheroxy to re-generate vitamin E (Shilpi et al., 2018). AA also plays an important role in maintaining proper immune function; inhibiting the conversion of irritants in smog, tobacco smoke, and certain foods into potentially carcinogenic substances; decreasing the risk of developing high blood pressure and heart disease; helping regulate cholesterol levels; and preventing the development of scurvy (Llorent- et al., 2020).

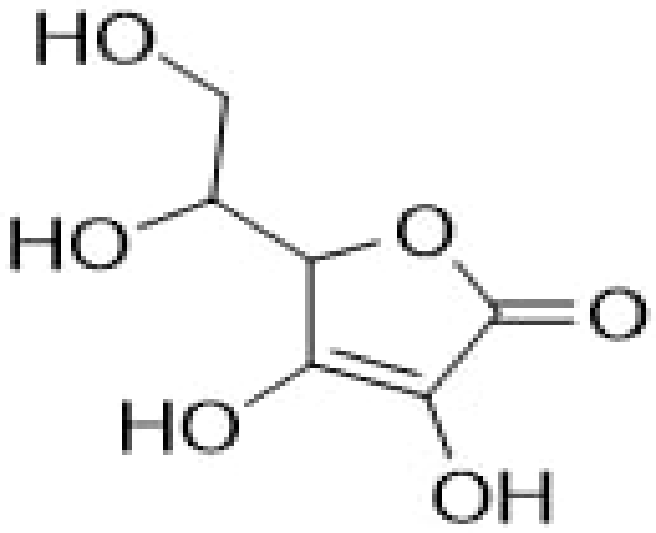

Figure 2.1. Molecular structure of ascorbic acid

2.3. Antibacterial Activities of Ascorbic Acid

Vitamin C is an important antioxidant, free radical scavenger, pro-oxidant, and an antibacterial molecule that can modify the antimicrobial activity of various antibiotics as well as significantly declines the adversative effects of reactive species (Mumtaz et al., 2021). Ascorbic acid has antioxidant properties, therefore any effects of its presence in the human body may be most prominent under conditions of the enhanced oxidative stress, i.e., during the process of

inflammation (Kwiecińska-Piróg et al., 2019). Viral, bacterial or fungal infections cause reactive oxygen species (ROS) release through phagocytes. It is useful in the challenge of contamination through deactivation of viruses or microorganism killing. ROS may additionally cause harm to the host cells, consequently the level of ROS released by phagocytes ought to be decreased without delay after infection. Vitamin C is a crucial co-enzyme in the oxidative stress pathways, able to ROS removal. It also hinders biofilm development, among others: Mycobacterium spp., Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, or Pseudomonas aeruginosa, by inhibiting the production of exopolysaccharides (Carneiro et al., 2020).

2.4. Fluoroquinolone Antibiotics

Fluoroquinolones are an important class of antibiotics used to treat human infections and prevent mortality in livestock (Ferrie et al., 2017). Fluoroquinolones are widely used because they are highly effective against bacteria and easily absorb into tissues and membranes. Extraocular bacterial infections require an antibiotic treatment regimen to reduce the complications associated with chronic conditions (Preeti Upadhayay, 2016). The success of an antibacterial therapy depends upon the achieving and maintaining the therapeutic concentration of drug at the site of infection for a prolonged duration without compromising the intraocular region. The fluoroquinolone antibiotics are a class of fully synthetic, broad-spectrum antibacterial agents with a unique mechanism of action and wide clinical use, structurally related to nalidixic acid and oxolinic acid (Żamojć et al., 2021). Their activities result from the inhibition of different kinds of enzymes that control DNA topology and are vital for chromosome function and replication (Seedher & Agarwal, 2010). Fluoroquinolones are mainly effective against both aerobic Gram (-) and Gram (+) bacterial and so very helpful in the therapy of a wide range of infections including urinary, bone-joint, enteral, soft tissue and respiratory tract infection, typhoid fever, prostatitis, blood poisoning sinusitis, gonorrhea and bacterial gastroenteritis.

Different generations of fluoroquinolone antibiotics have been already developed to increase the range of their action and to solve problems of their resistance (Żamojć et al., 2021). Since it has exited justified that miscellaneous type of fluoroquinolones depending on substituents in the structure exhibit completely various uncontaminated answers and cohesion, it enhanced very main study the material and synthetic features of these particle in miscellaneous atmospheres. Although the machine of operation of all fluoroquinolones is very akin, skilled are

many important distinctness's in their antimicrobial range of exercise pharmacokinetic traits, security characterization, transport and complexing characteristics mainly by way of a difference of working groups in medicines particles. All these result that an exact understanding of the interplay means tween medicines and different compounds is essential and excellent significance.

2.5. Norfloxacin

Norfloxacin (1-ethyl-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid) (NRF) (Scheme 1) belongs to the group of fluoroquinolones, and is a synthetic, broad spectrum antibacterial agent that exhibits high antimicrobial activity in vitro against a wide variety of Gram-negative and Gram-positive bacteria, including gentamicin resistant Pseudomonas aeruginosa and b-lactamase positive Neisseria gonorrhoeae (Bakaeean, Kabiri, Iranfar, Saberi, & Chamani, 2012). Figure 2.2 a is the chemical structure of norfloxacin. The fluorine atom at the 6^{th} position provides increased potency against Gram-negative organisms and the piperazine moiety at the 7^{th} position is responsible for antipseudomonal activity of norfloxacin (More, VR Mote, US Patil, and, & GB, 2009). Norfloxacin is used in the treatment of upper and lower respiratory tract infections, human soft tissues and skin infections, bacterial gastroenteritis, and urinary tract infections, caused by Gram-negative bacteria in particular(Lian et al., 2019). It works by stopping the growth of bacteria. Bacterial action of norfloxacin depends on blocking of bacterial DNA replication by binding itself to an enzyme called DNA gyrase, which allows the untwisting required to replicate one DNA double helix into two.

Norfloxacin has two ionizable functional groups: an acid (carboxylic group) and a basic group (amine group). In an aqueous solution, it shows three different species, which are cationic, zwitterionic and anionic. The cationic and the anionic forms predominate in acid and basic solutions, respectively. At physiological pH value (pH 7.4), the fluoroquinolone is totally or partially ionized and the predominant specie is the zwitterionic form (Batista & Teixeira, 2013).

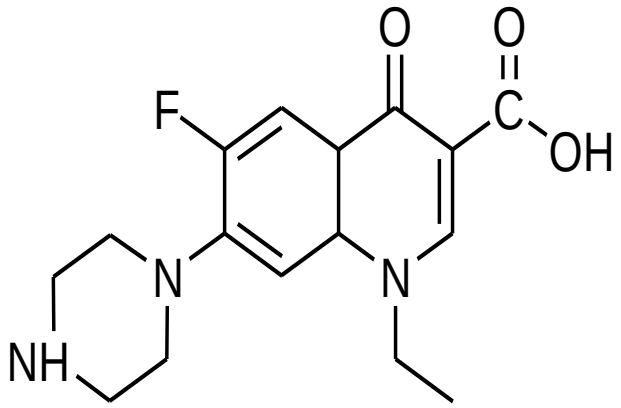

Figure 1.2 Molecular structure of norfloxacin

2.6. Interaction of Ascorbic Acid with Norfloxacin

Nowadays, drug interactions are a hot topic of study, due to the rapid development of new drugs and the increasing use of food supplements (Abraha et al., 2017). The interaction of antibiotics with bioactive ingredients is of interest because it is found in a variety of food sources and medicines. Ascorbic acid in popular drinks and foods (fruits, vegetables, etc.) is the most useful compound for human health. Moreover, they are the most commonly used drugs among all behaviorally active substances in the world. This interaction could change the way the drugs are metabolized or lead to increased toxicity.

In the presence of antioxidants such as glutathione and ascorbic acid, some research work involving the bactericidal effect of antibiotics has demonstrated that these antioxidants are protective against the action of antibiotics against E. coli, and the minimum inhibitory concentration of ciprofloxacin (MIC) significantly increased and streptomycin in the presence of antioxidants. Norfloxacin induced mutagenesis was found to be decreasing in Salmonella typhimurium in the presence of vitamins C and E (Paulraj et al., 2019). Carlsson et al. 2005 and Afzal et al. 2017 showed an increased antimicrobial activity of fluoroquinolones and aminoglycosides during AA addition(Kwiecińska-Piróg et al., 2019). Hence, as numerous ponder of the interaction between norfloxacin and ascorbic acid will offer assistance give fundamental data on the pharmacological activities, bio-transformation, bio-distribution of drugs. The authoritative marvels will moreover be valuable to clarify the relationship between the norfloxacin and ascorbic corrosive. Understanding their binding in a biological system at the molecular level is useful. The binding phenomena will also be useful to explain the relationship between the norfloxacin and ascorbic acid.

There are some popular techniques which have been used to investigate the interaction between drugs and vitamins. UV-absorption and fluorescence spectroscopy are of the powerful techniques to study molecular interactions which changes local environment of fluorophore and helps to predict the binding phenomenon of drugs to (Alsamamra, Abusharkh, & Abuteir, 2018) ascorbic acid. Fluorescence spectroscopy is becoming increasingly important as a quantitative analytical technique because of its high sensitivity and good selectivity (Mi et al., 2013) and it is the most powerful techniques to investigate the interaction between biological molecules. Fluorescence measurements can give some information on the binding mechanism of small molecule biological molecules, including binding mode, binding constants, and binding sites (MM, Ghithan, Abu-Taha, Darwish, & Abu-Hadid, 2014). Among the several physical techniques to study the interaction between biomolecules; the fluorescence quenching method is the simplest and sensitive.

2.7. Basic Concepts of Fluorescence

During the past 3 century there has been a remarkable growth in the use of fluorescence in the biological sciences. Fluorescence spectroscopy and time-resolved fluorescence are considered to be primarily research tools in biochemistry and biophysics. Fluorescence is now a dominant methodology used extensively in biotechnology, flow cytometry, medical diagnostics, DNA sequencing, forensics, and genetic analysis, to name a few (Lakowicz, 2006). Fluorescence detection is highly sensitive, and there is no longer the need for the expense and difficulties of handling radioactive tracers for most biochemical measurements.

Fluorescence is the process of photon emission as a result of the return of an electron in a higher energy orbital back to a lower orbital (Suryawanshi et al., 2012). On the atomic level, absorption of a photon by an electron in the fluorophore causes the electron to jump into an orbital further away from the atom nucleus (i.e., into a higher energy, or excited, state). However, an electron in the excited state is unstable and when it returns to its ground state, a longer wavelength photon is emitted to dissipate the excess energy – this is known as fluorescence. In excited singlet states, the electron in the excited orbital is paired (by opposite spin) to the second electron in the ground-state orbital. Consequently, return to the ground state is spin allowed and occurs rapidly by emission of a photon. Depending on the electronic structure of the molecule, fluorescence occurs for most fluorophores within 1–100 ns (Alexiev, Ulrike, and, & L, 2014). The electron states and transitions in a fluorophore are more complex

than described above, Polish physicist Professor Aleksander Jablonski in 1933 develop diagram simply illustrate electron state and transition in a fluorophore so called 'Jablonski diagram' Figure 2.3 shows a Jablonski diagram.

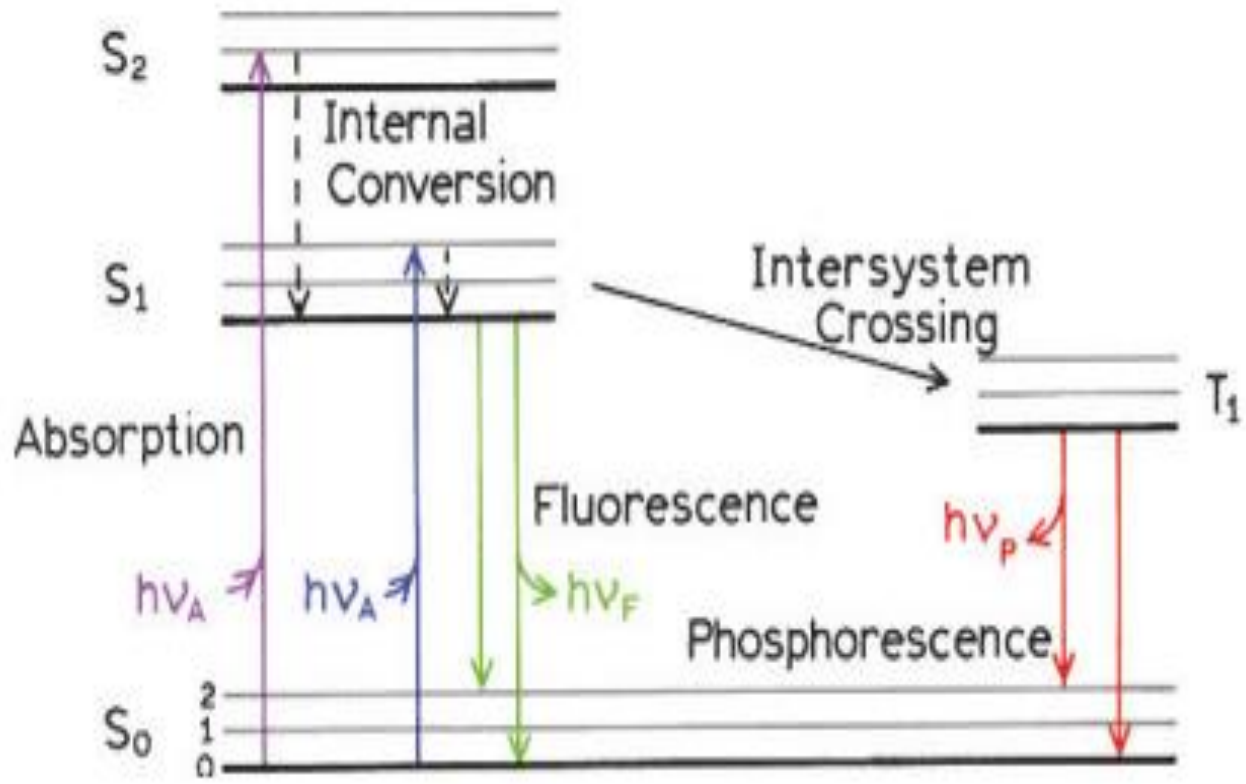

Figure 2.3. A Jablonski diagrams

Molecular vibrations, in which the inter-nuclear distances vary over time, further cause the electron to exist in discrete energy levels within each singlet state. These are known as vibrational levels. As a result of these discrete energy states, electrons can absorb photons with a range of wavelengths that can shift the electron into a higher energy level. The range of wavelengths able to induce fluorescence in this manner is directly related to the excitation spectrum of a fluorophore. Once excited, there are different ways an electron can dissipate the excess energy and return to its ground state. Two such ways, are internal conversion and vibrational relaxation. These are non-radiative energy dissipations that occur within picoseconds (10^{-12}s). In vibrational relaxation, the electron returns to the lowest energy level of its current singlet state by transferring vibrational energy to nearby molecules, whereas internal conversion

is the transition between singlet states. In efficient fluorophores, energy dissipation between exist state and the ground state result in the emission of a photon and occurs within nanoseconds (10^{-9}s). As an electron can return to any of the vibrational levels of the ground state, photons covering a range of wavelengths can be emitted. Finally, because some of the excitation energy of the electron is dissipated by non-radiative measures, the emitted photon will be of lower energy (longer wavelength) than the absorbed photon. This is called the 'Stokes shift'

2.8. The Factors That Affect Fluorescence Emission Spectra

There is different factor that influences the intensity of fluorescence of fluorophore. For example, temperature, PH (Żamojć et al., 2021), solvent polarity (Woldegiorges et al., 2021) and viscosity rate of solvent relaxation, probe conformational changes, rigidity of the local environment, internal charge transfer, proton transfer and excited state reactions, probe–probe interactions and there are some molecules that interacts with the fluorophore and reduces the intensity of the fluorescence of fluorophore, which are called a quencher. A wide variety of substances act as quenchers of fluorescence. One of the best-known collisional quenchers is molecular oxygen, which quenches almost all known fluorophores (Lakowicz, 2006).

2.9. Fluorescence Quenching

Fluorescence quenching is a phenomenon that influences the intensity of fluorescence. A molecule that interacts with the fluorophore and reduces its quantum yield or lifetime is called a quencher (Alexiev et al., 2014). A variety of molecular interactions can result in quenching, including excited state reactions, molecular rearrangements (Bhogale et al., 2013), energy transfer, ground state complex formation (Belay et al., 2017), and collisional quenching. An Increase in the concentration of quencher will mask the fluorophore, result in reduction of emitted fluorescence from the fluorophore molecules (Alsamamra et al., 2018). Usually, the quenching of fluorescence emission is caused by a static or dynamic interaction between the quencher and the luminescent molecule (Wu, Dudu et al. 2016).

Dynamic quenching is the process of fluorescence weakening caused by collision between fluorescent group and quenching agent, while static quenching is the process of fluorescence weakening caused by the formation of complex of fluorophore and quencher (Sun et al., 2020). Both those quenching behaviors may be prominent through investigating the fluorescence quenching at different temperatures. As the temperature increases, the diffusion rate

of the quencher increases providing better opportunity for the dynamic type quenching. In contrast, increased temperature is possibly to bring about decreased stability of complexes, and as a consequence decrease value of the static quenching constant. In order to explore the mechanism of fluorescence quenching of quencher to fluorophore, firstly assume that it is a dynamic quenching process. The dynamic quenching process can be described by the Stern-Volmer equation (Teir et al., 2014).

$$\frac{F_o}{F} = 1 + k_{sv}[Q] = 1 + k_q \tau_o [Q] \quad (2.1)$$

Where, F_o and F are the fluorescence intensities of fluorophore in the absence and presence of the quencher, respectively. k_q is the quenching rate constant of the biomolecule, is the Stern-Volmer dynamic quenching constant, and τ_o is the average lifetime of the biomolecule without quencher and [Q] is the concentration of quencher.

Quenching data are usually presented as plots of F_o/F versus [Q]. This is because F_o/F is expected to be linearly dependent upon the concentration of quencher. A plot of F_o/F versus [Q] yields an intercept of one on the y-axis and a slope equal to K_D. The Stern-Volmer quenching constant indicates the sensitivity of the fluorophore to a quencher. Generally, the maximum scattering collision quenching constant, k_q, of various kinds of quenchers with a biopolymer is $2 \times 10^{10} M^{-1} s^{-1}$ (Belay et al., 2017). Values of k_q smaller than the diffusion-controlled value can result from steric shielding of the fluorophore or a low quenching efficiency. Apparent values of k_q larger than the diffusion-controlled limit usually indicate some type of binding interaction (Bakaeean et al., 2012).

2.10. Binding sites and Binding Constant

Fluorescence quenching of fluorophore also provides the information on the binding constant (K) and the number of binding sites (n) between the quencher and fluorophore accordingly to the equation (Bhogale et al., 2013). When there is bimolecular binding, the equilibrium between free and bound molecules is given by (More et al., 2010).

$$\log\left(\frac{F_o - F}{F}\right) = \log k_a + n \log[Q] \quad (2.2)$$

By comparing equation (2.1) and equation (2.2) we get best equation.

$$1 + k_q \tau_o [Q] = 1 + k_a [Q]^n$$

$$k_q \tau_o [Q] = k_a [Q]^n$$

$$k_q \tau_o = k_a [Q]^{n-1}$$

$$k_a = \frac{k_q \tau_o}{[Q]^{n-1}} \quad (2.3)$$

where k_a, is binding constant

n, is binding site.

2.11. Thermodynamic Parameters and Nature of the Binding forces

The forces acting between small molecules and macromolecules are mainly hydrogen bonds, electrostatic forces, van der Waal's force and hydrophobic interaction (Belay, A., et al. 2015). Assessment of change in entropy ΔS and enthalpy ΔH and free energy change (ΔG) are of great significance for the determination of the binding mode of ascorbic acid and norfloxacin. The thermodynamic parameters, Gibb's free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) are important for confirming the binding mode assessment of change in entropy ΔS and enthalpy ΔH and free energy change (ΔG) are of great significance for the determination of the binding mode. According to the data of enthalpy change and entropy change, the model of interaction can be conclude as follows(Bardhan et al., 2009) :If enthalpy change greater than zero (ΔH>0) and entropy change greater than (ΔS >0), hydrophobic forces; enthalpy change less than zero (ΔH < 0) and entropy change less than (ΔS < 0), van der Walls interactions hydrogen bonds; enthalpy change less than zero (ΔH < 0) and entropy change greater than (ΔS > 0), electrostatic interaction. These parameters can be obtained from the Van't Hoff equation (2.4) (Xiong et al.,2015)

$$lnk_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (2.4)$$

Where k_a binding constant R is the gas constant and T is experimental temperature. The enthalpy change (ΔH) and the entropy change (ΔS) can be determined from the slope and intercept of the curve of lnk_a versus 1/T, respectively (Deepa et al., 2016).

The Gibbs free energy can be calculated from equation (2.5)

$$\Delta G = -RTlnk_a = \Delta H - T\Delta S \quad (2.5)$$

If the value change in free energy (ΔG) is negative it may indicate that the binding process occurs spontaneously while negative values of change in entropy ΔS and change in enthalpy ΔH suggest that the hydrogen bond and van der Waals forces play major roles in the binding process and the reaction is mainly enthalpy-driven (Bhogale et al., 2013). The positive values of both ΔH and ΔS indicated that the acting forces are mainly hydrophobic forces (Yousef et al., 2017).

CHAPETR THREE

3. Material and Methodology

In this chapter, the materials and methods of the experiment were discussed in detail. The various chemicals, apparatus, instruments, and solvents that are necessary to carry out this experiment was also briefly explained.

3.1. Chemicals and Solvents

Norfloxacin EP (LOT 79590, Remedica, Cyprus), were purchased from local pharmacy, L(+)-Ascorbic acid A R(515000 Guangdong Guanghua sci-Tch CO. Ltd, China) and Tri sodium citrate (Batch no.211119) analytical reagent grade taken from physics laboratory, and DDW was used as a solvent.

3.2. Apparatus and Instruments

3.2.1. Apparatus

The laboratory apparatus used for preparing solution and powder of norfloxacin and ascorbic acid were Aluminum foils, Different sizes of beakers, Digital electrical analytical balance (Model-FA2104, China), Filter papers, Gloves, measuring cylinder, mortar and pestle, pH meter (010475), Refrigerators, Sample holders and Thermometer.

3.2.2. Instruments

The laboratory instruments used were Fluorescence Spectrophotometer (Model, MY18490002, Cary Eclipse, Agilent Technologies, Malaysia), UV-Visible absorption (UV-Vis) Spectroscopy (MODEL-JASCO V-770, Japan), and Fourier Transformed Infra-Red Spectroscopy (FT-IR) (Perkin Elmer, Spectrum 65 FT-IR).

3.3. Procedure for Preparing solution and Powder of Ascorbic Acid and Norfloxacin

Norfloxacin tablet of 400mg was grinded and the stock solution of $2x10^{-4}$M norfloxacin was prepared by dissolving 2mg in 30 ml of Tri sodium citrate buffer solution which has pH 7.4 and stored in the refrigerator. Aqueous solution of ascorbic acid different molar concentrations (0.44, 0.57, 0.8, 1.33 and 2) $x10^{-3}$M was prepared and added to fixed amount $2x10^{-4}$M of norfloxacin stock solution for fluorescence quenching measurement and UV–vis. The powder of the mixed solution was obtained by filtering the solution and dry at room temperature for FTIR study.

3.4. Fluorescence Quenching Measurement

The steady state fluorescence emission measurements were performed at three different temperature 298, 303 and 310K. Excitation wavelength at 290 nm and emission spectra were measured 300–600 nm. The excitation and emission slit width is set at 5. Three sets of fluorescence measurements were recorded for different concentration of ascorbic acid. The emission spectral data was analyzed using Origin 8 software and the quenching rate constant (k_q), Stern–Volmer constant (K_{sv}) and thermodynamic properties of the interactions were determined from emission spectral by using Stern–Volmer and Van't Hoff equation respectively.

3.5. UV–vis Absorption Studies

The UV/vis absorption spectra were measured by successive addition of ascorbic acid concentrations to a fixed amount of norfloxacin solutions at room temperature (298 K). The measurements were performed with in the wavelength range of 200–500 nm. The quartz cuvette (4 cm × 1 cm × 1 cm) with 1 cm path length was used.

CHAPTER FOUR

4. Results and discussion

4.1. Fluorescence Quenching of Norfloxacin in Presence of Ascorbic Acid

There are several techniques to study the interaction between biomolecules and antibiotic, but most convenient method is to study the fluorescence quenching of Norfloxacin. Thus, the fluorescence spectra were recorded for the Norfloxacin and mixed with different concentration of ascorbic acid to study the interaction between them. The figure 4.1 shows that the Norfloxacin has a strong emission band at 415nm when excited with 290 nm wavelength.

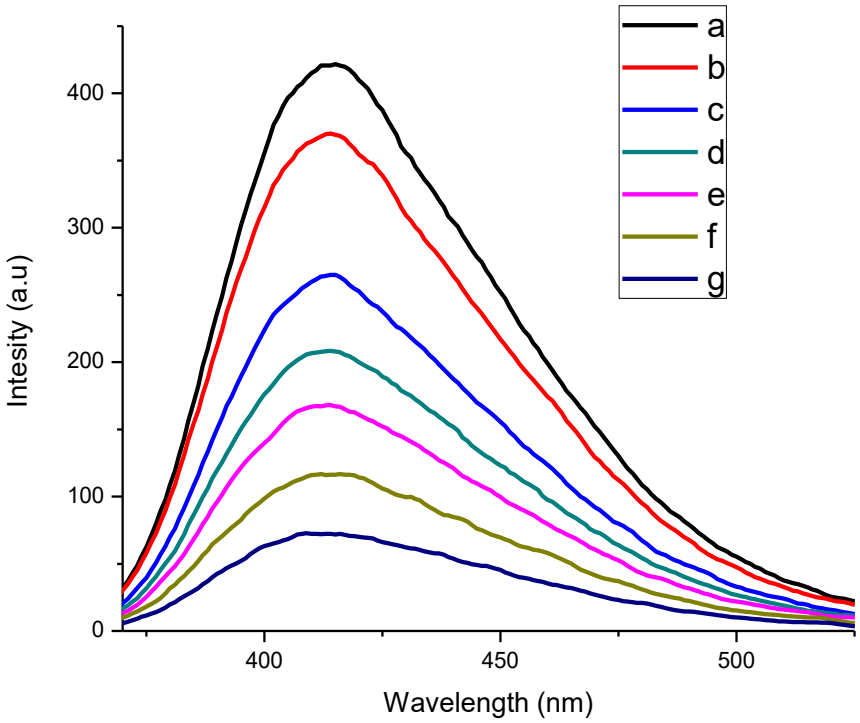

Figure 2.1. Emission spectra of NRF (concentration = $2x10^{-4}$) M and A.A (concentration a = 0, b = 0.44, c = 0.57, d=0.8, e = 1.00, f = 1.33 and g = 2.00) x 10^{-3} M

The intensity of this fluorescence band decreases gradually and the maximum of the peak is also shifted with increasing concentration of ascorbic acid. This change in the fluorescence characteristic of the NRF indicates the binding between ascorbic acid and NRF to form a certain complex

4.2. Binding Mechanisms Between Norfloxacin and Ascorbic Acid

In order to interpret the quenching mechanism of the molecules, the Stern–Volmere equation was applied for analysis of fluorescence quenching results (Eq 1). Figure 4.2. is the Stern–Volmer plots for the quenching of Norfloxacin by ascorbic acid at 298, 303 and 310 K. The plots are linear and the slope decreases with increasing temperature, which indicates that the quenching process is static types of quenching mechanism rather than dynamic(Belay et al., 2017). The rate of quenching constant Kq determined and the values are given in Table 1 by using the fluorescence lifetime τ_o Norfloxacine is about 1.2ns at Ph 7.4 (Żamojć et al., 2021).

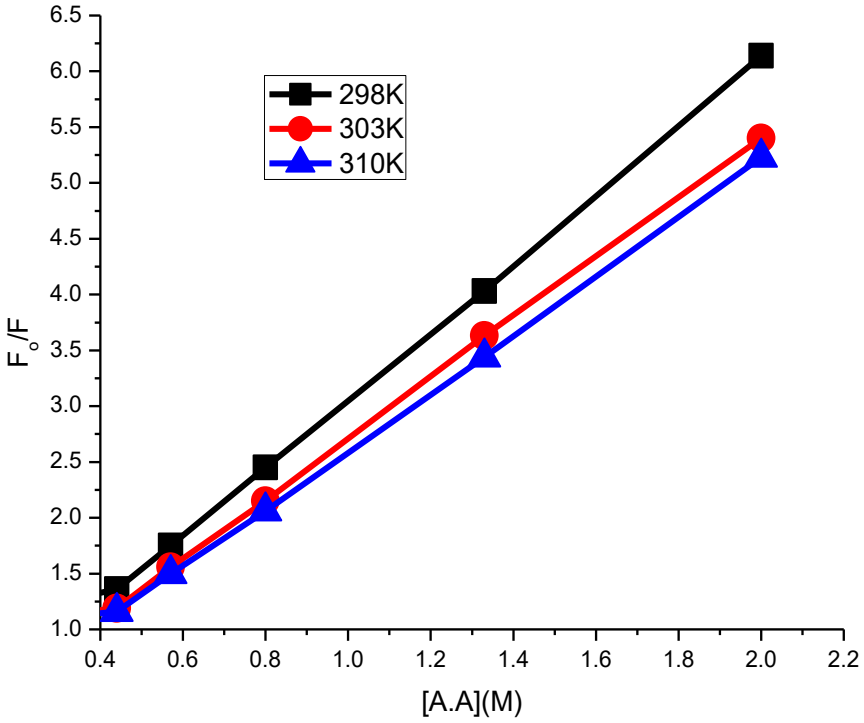

Figure 4.2. The Stern-Volmer plots for the quenching of NRF by AA at temperature of 298, 303 and 310K

The obtained Kq values are in the order of 10^{12} M^{-1} s^{-1} for ascorbic acid. The values are greater than the diffusion-controlled rate constant in aqueous solution, that mean 2.0×10^{10} M^{-1} s^{-1} (Abebe Balay et al, 2017), and this confirms that the quenching does not involve the dynamic diffusion process but occurs due to formation of the complexes of NRF–A.A. Moreover, in two temperatures the plots exhibit good linear relationship indicates the domination of static quenching mechanisms in the studied concentrations.

Table 4.1Stern-Volmer constant, quenching rate constant for the interaction of NFR with A.A at 298 and 310k

T(K)	$K_{sv}(M^{-1})$	$K_q(M^{-1}s^{-1})$	R
298	$2.72x10^3$	$2.27x10^{12}$	0.96
303	$2.37x10^3$	$1.97x10^{12}$	0.95
310	$2.27x10^3$	$1.89x10^{12}$	0.95

4.3. **Binding Constants and Binding Sites**

The values of k_a and n were determined from the slope and intercept of the linear fit of Eq. (2.2) to the experimental data. Figure 3 shows the graph of log ($\frac{F_o-F}{F}$) versus log [AA]. The values of the binding constant of AA with NRF at 298,303 and 310k are listed in table 2. The binding constants are decreased with the increasing temperature indicates the formation of unstable compound; partly decompose at relatively higher temperatures. The binding site numbers n are about 2 indicating that there are two sites for the binding of AA to NRF. Strong binding constants were obtained between AA and NRF and the values are by far much higher than the binding observed between biomolecules. From our experimental results observation, it could be inferred that NRF have high binding affinities with biomolecules.

Table 4.2. Binding constant k_a, and binding site n of NRF-AA complex at different temperature.

Temperature (k)	$\log k_a$	$k_a(M^{-1})$	N	R
298	6.90	7.9×10^6	2.29	0.96
303	5.38	0.2×10^6	1.71	0.98
310	3.89	0.07×10^6	1.37	0.99

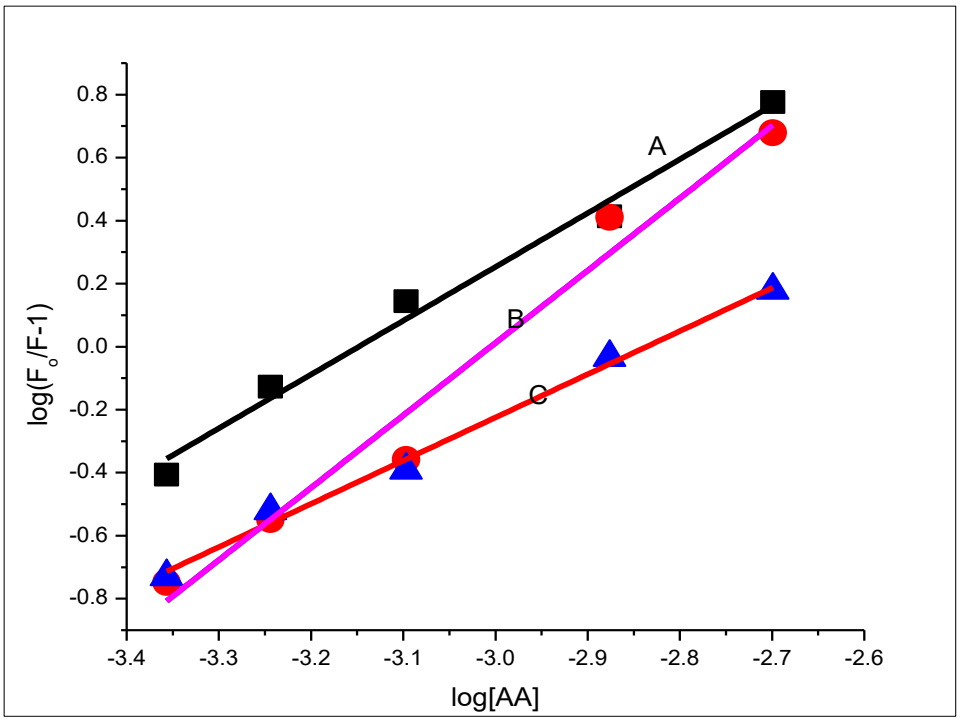

Figure 4.3. log(Fo/F-1) versus log[AA] A is 298K, B is 303K and C is 310k

4.4 Thermodynamic Parameters and Nature of the Binding Forces between Norfloxacin and Ascorbic Acid

In order to characterize the force between NRF-AA molecules, thermodynamic parameters at the specified temperatures were analyzed by using Vant's Hoff's equation. From the linear relationship between ln(ka0 and reciprocal of excremental temperature, the values of thermodynamic parameters are determined and listed in Table 4.3. The sign and magnitude of the thermodynamic parameters associated with various individual types of interaction were characterized by the obtained results from fitting of Eq. (3) to the experimental results are negative ΔH and Δ*S* indicate that hydrogen bonds and van der Waals forces played a major role in the binding process and the reaction is mainly enthalpy-driven (Bhogale et al., 2013) while the negative sign Gibb's free energy change (ΔG) for indicates the spontaneity of the binding for NRF with AA.

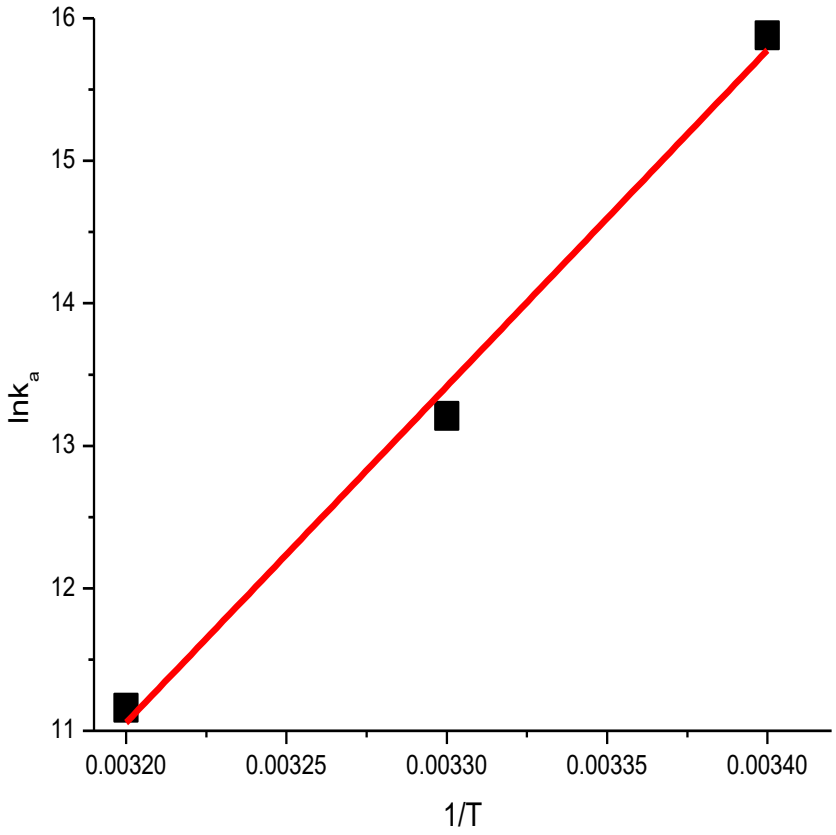

Figure 4.4. Ln(ka) versus reciprocal of excremental temperature

Table 4.3Thermodynamic properties determined by fluorescence quenching of norfloxacin by ascorbic acid

Temperature (k)	$\Delta G(kJmol^{-1})$	$\Delta H(kJmol^{-1})$	$\Delta S(Jmol^{-1}k^{-1})$
298	-35.66	-196.2	-538.74
303	-32.96		
310	-29.19		

4.5. UV/vis Absorption Spectra

The UV/vis absorption spectra of NRF and its interaction with AA were measured to confirm the interaction mechanism. Figures 4.5 the UV/vis absorption of NRF in the presence and absence of AA. Upon addition of AA solutions, decreases in the intensity at the peak were observed in NRF. In dynamic quenching, the spectra of the molecule will not change, however, in static quenching due to the formation of reaction, spectral changes were observed in the compounds (A. Belay et

al., 2015). The effects confirm the existence of binding of AA with NRF due to ground state complex formation. This is also evidence that the fluorescence quenching of the norfloxacin induced by ascorbic acid are mainly caused by static quenching.

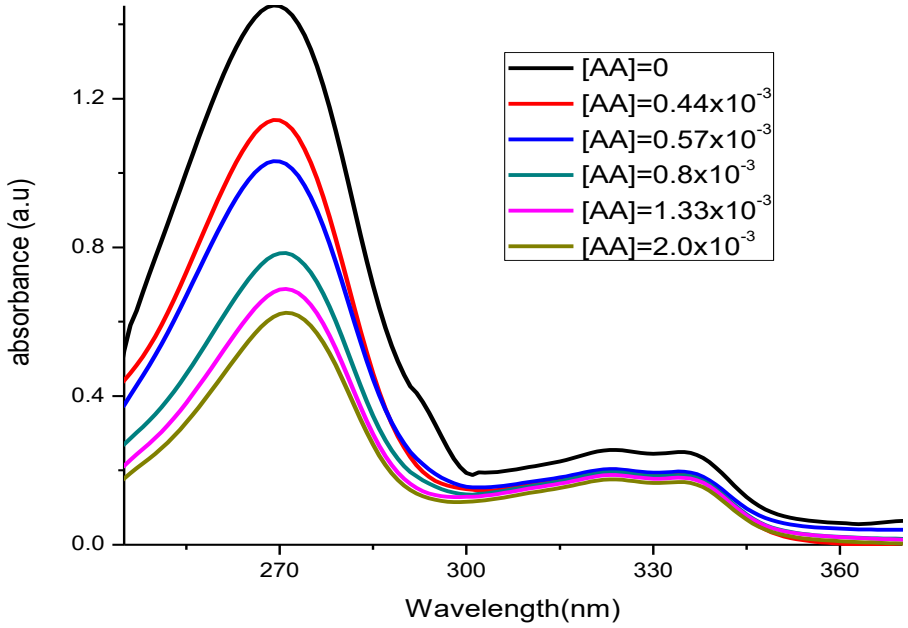

Figure 4.5. UV/Vis absorption spectra of norfloxacin in the presence of ascorbic acid ($0 – 2 \times 10^{-3}$) M

4.6. FTIR Spectroscopy

FTIR spectroscopy is another technique for understanding conformations binding of norfloxacin to ascorbic acid. Figure 4.4 shows the vibrational spectra of (A) NRF, (B) their corresponding NRF-AA. The vibrational spectra of NRF are mainly characterized by hydroxyl and carbonyl functional groups.

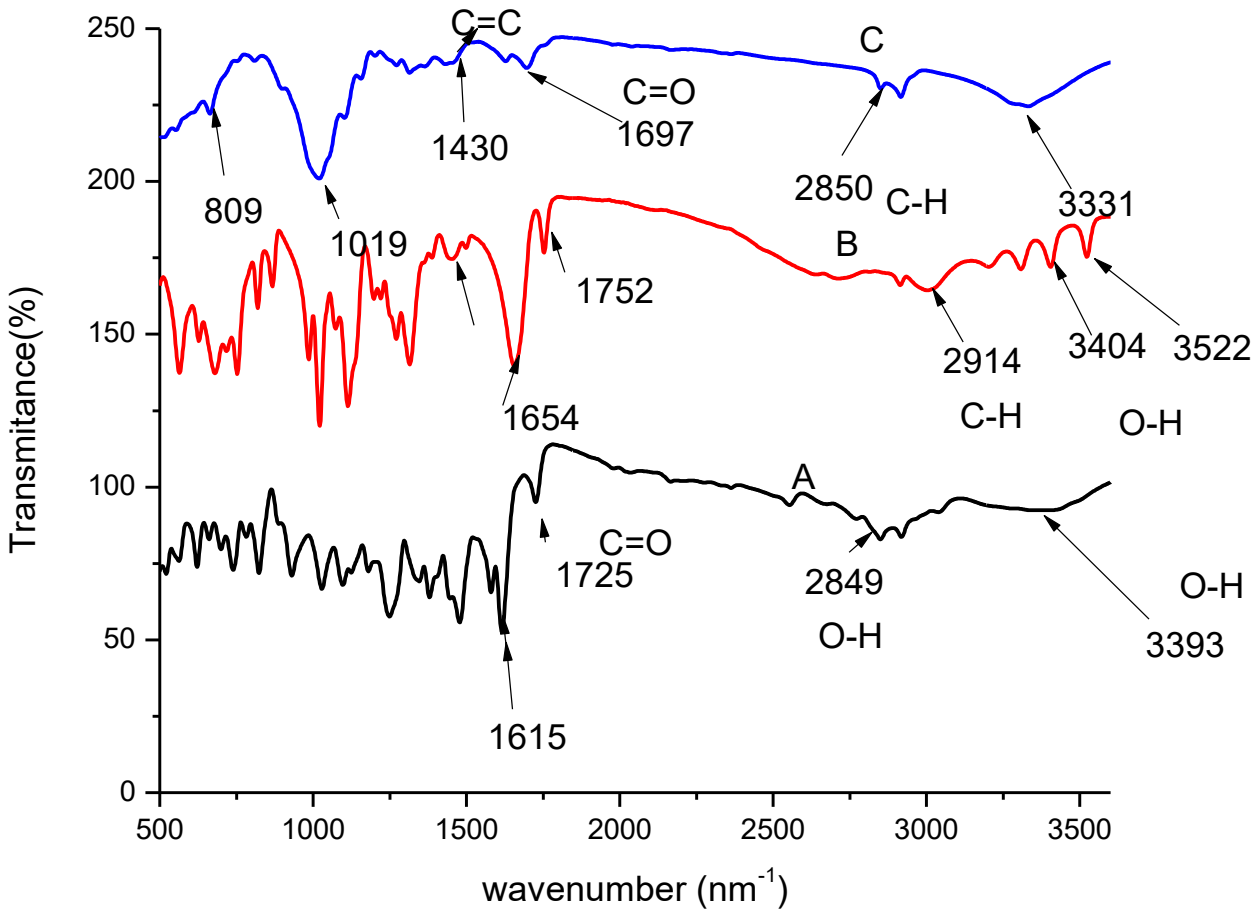

Figure 4.6. *FTIR absorption spectra of (A) NRF, (B) AA and (C) NRF-AA*

There are two strong bands at around 1000 cm^{-1} and 550 cm^{-1}. The other absorption band peaks in the region of 1600–1700 cm^{-1} are due to C=O stretching (Belay et al., 2016) (Belay et al., 2016). In addition, the peak located at 1477 cm^{-1} is due to C=C–C aromatic ring stretching. By contrast, these main peaks did not exist in the spectrum of NRF-AA due to adsorption of NRF onto the surface of AA. In the corresponding spectra for NRF-AA the disappearance of the O–H vibration attached to the benzene ring and the formation of a new broad band at 3331 cm^{-1} was observed. According to a literature report, the H-bonded OH stretch usually appeared as broad spectra in the region 3200–3570 cm^{-1} due to hydrogen bond formation(A. Belay et al., 2015). Similarly, in this study, observation of such a peak was due to the binding of norfloxacin and ascorbic acid through hydrogen bonding. This is also confirmation of the interaction between norfloxacin and ascorbic acid.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

The binding of ascorbic acid with Norfloxacin was investigated using fluorescence quenching, UV/vis and FTIR spectroscopic techniques. Study shows that ascorbic acid quench the fluorophore of norfloxacin by forming ground state complexes. The fluorescence quenching data, analyzed by the Stern–Volmer equation, permitted us to obtain the number of binding sites and binding constants at different temperatures. In particular, the calculated thermodynamic parameters suggest, negative sign the free energy change (ΔG) indicate the spontaneity of the binding for AA with NRF, while the negative values of change in enthalpy ($\Delta H = -196.2 KJmol^{-1}$) and change entropy ($\Delta S = -538.74 Jmol^{-1}K^{-1}$) indicated van der Waals forces played a major role in the reaction of AA with NRF.

5.2. Recommendations

The study results assist us to recognize the mechanisms of binding of these biological active compounds that are naturally available in different plant types with antibiotics. Thus, as the result of the study indicated, the conclusions are important in the field of pharmacology and biochemistry and are helpful for understanding the effect of vitamin C on antibiotics. Also, one of the important features of ascorbic acid is its multifactorial antibacterial action; with no risk of development of bacterial resistance the clear and quantitative information on the nature of this study may provide some information for its rational use in clinical practice and for furthering clinical research. In general, the quenching mechanism binding constants and binding sites of ascorbic acid and norfloxacin, as well as thermodynamic parameters, binding force operating between the compounds, and conformational changes from the study's fluorescence quenching spectrum analysis, will be inputs for any field of applied science, including health, chemistry, and biology.

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