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Synthesis of 1,2,4-Triazol Compounds and Their Applications on Urease Producing Proteus Miribilis Bacteria

A Thesis
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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

إِنَّا فَتَحْنَا لَكَ فَتْحًا مُّبِينًا ۝ لِّيَغْفِرَ لَكَ اللَّهُ مَا تَقَدَّمَ مِن ذَنبِكَ وَمَا تَأَخَّرَ وَيُتِمَّ نِعْمَتَهُ عَلَيْكَ وَيَهْدِيَكَ صِرَاطًا مُّسْتَقِيمًا ۝ وَيَنصُرَكَ اللَّهُ نَصْرًا عَزِيزًا ۝

صَدَقَ اللَّهُ العَظِيمِ

سورة الفتح

الأية (١-٣)

الإهداء

الى منارة العلم والامام المصطفى الى الأمي الذي علم المتعلمين الى سيد الخلق رسولنا الكريم سيدنا محمد ((صلى الله عليه واله وسلم))

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Abstract

The current study involves preparation a number of 1,2,4-Triazol derivatives by the reaction of 4-amino-5-phenyl-4H-1,2,4-triazole-3-thiol (A) with different aromatic aldehydes to form the Schiff bases (B, C, D and E). In addition preparation of different complexes (A1, A2, B1 and B2) were prepared from the reaction of A and B Ligands with Ni (II) and Cu (II) metals. All the prepared Compounds and their metal complexes were characterized using: Micro elemental analysis, Fourier transform infrared spectroscopy (FTIR), UV-visible spectroscopy, ^{1}H NMR and conductivity measurements. According to the spectral data of the complexes a tetrahedral geometry was suggested for Ni(II) complexes, Cu(II) complexes exhibit a square planer structure.

The antibacterial activities of the above prepared compounds against *Proteus mirabilis* were estimated using well-diffusion method.

The inhibition effect of the prepared compounds on urease activity were studied. Maximum velocity (Vmax), Inhibition force constant (Ki), Michael constant (Km) and percentage of inhibition (i %) values of the enzyme were calculated using line weaver Burk equation. The results showed promising inhibition effect on urease activity. All the prepared compounds showed competitive inhibition since the value maximum velocity (Vmax) was remain constant and Michael constant (Km) increased, except compound (C) which showed non-competitive inhibition since the value Vmax was decreased and Km remain constant.

Among all compounds studied in this work, the complexes were exhibit the strongest inhibition effect on urease enzyme, where the Complex (B2) has inhibition force constant (Ki = 0.017 M) and the percentage of inhibition (% i = 97.6).

Symbols & abbreviation

Symbols	Full meaning
µ	Micro
µg	Microgram
µL	Microliter
a	relative activity as fraction
Asp	Aspartate
B.A	Blood Agar
CHNS	Elemental Analysis
C^o	centigrade
Conc.	Concentration
CPS	Capsule Polysaccharide
CT	Charge Transfer
DNA	Deoxyribonucleic Acid
FT-IR	Fourier Transform Infrared Spectroscopy
His	Histidine
HIV	Human Immunodeficiency Virus
hrs.	hour
HSAB	Hard And Soft Acids And Bases Rule
I	Inhibitor concentration
i%	degree of inhibition as a percent
IC_{50}	Half Maximal Inhibitory Concentration
K_i	Strength of inhibitor
K_m	Michaelis constant for enzyme
LPS	Lipopolysaccharides
Lys	Lysine
M	Molarity
MHA	Mueller Hinton Agar
MIC	Minimum Inhibition Concentration
min	Minute
mL	Milliliter
mm	Millimeter
mM	Milmolarity
NMR	Nuclear Magnetic Resonance
ONPG	Ortho-Nitrophenyl-β-Galactoside
PMX	Polymyxin
RBC	Red Blood Cell
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
S	Substrate
Spp.	species
TE	Tetracycline

tRNA	Transfer Ribonucleic Acid
U.T.I.	Urinary Tract Infection
UV-Vis	Ultraviolet-Visible Spectroscopy
V	velocity of the enzyme reaction
Vmax	Maximum velocity for enzyme reaction

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Chapter Two

Chapter Three

CHAPTER ONE

INTRODUCTION

1.1. Urease (urea amido hydrolase)

Urease is one of the enzymes belongs to the group (hydrolases) which has classification number (E.C. 3.5.1.5) [1], and this enzyme contains nickel ion (metalo-protein) to induce urea hydrolysis that evolves ammonia and carbomate molecule and carbonic acid according to the equation [2,3], as in Figure (1-6):

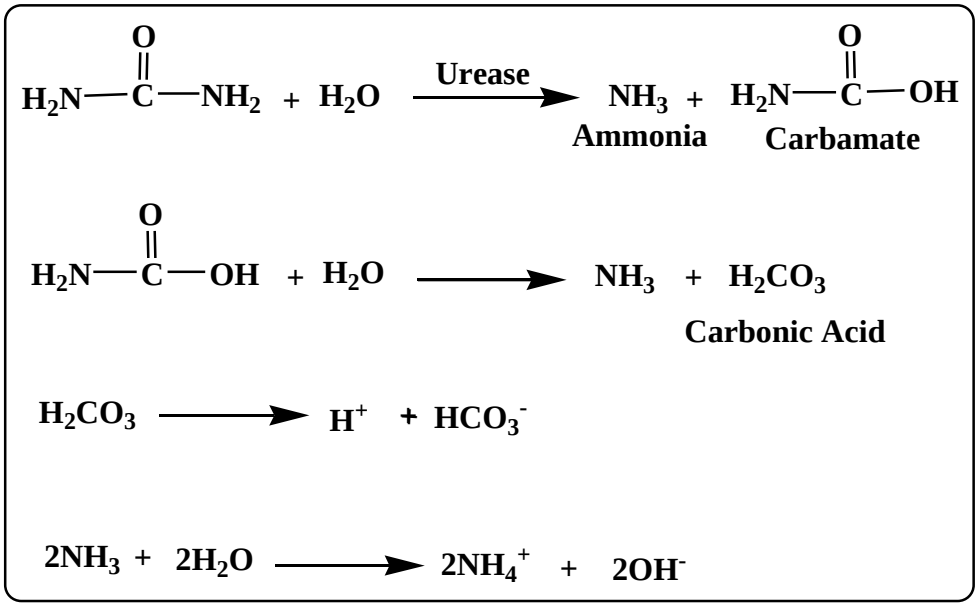


Figure (1-1) The equations of urea hydrolysis to evolve ammonia carbomate molecule and carbonic acid [28]

After equilibrium between ammonia and water the ammonium hydroxide occurs that rapidly increases pH [2]. Musculus (1876) is considered to be the first that study the bacterial urease after that Miquel (1890) diagnosed the production of urease in many microorganisms including bacteria [4,5].

Urease is produced by pathogenic or nonpathogenic bacteria [6], that helps causing disease and considered a virulence factor in many bacteria. It has been shown that this enzyme is produced by about 100 types of bacteria [2,7].The infection with the Proteus, Pseudomonas, Staphylococcus, Corynbacterium, Morganella, Micrococcus, Klibsiella, cause U.T.I. and urolithiasis [8].

All these bacteria produce urease that has a major role in urolithiasis by increasing the pH from 5 to 9 causing the mineral salts to precipitate in mucous

material [8,9], which is produced by the bacteria and entered in its cellular structure and acts as naives around which salts are precipitated to form stones. It also has been found that bacterial cells inside renal stones in proteins are treated with antibiotics [10].This enzyme is high -specific, which means that the enzyme catalyzes the hydrolysis of urea only [11].

Very high concentrations of urease are found in some cells, for example, Bacillus pasture, Aspergillus tamari, and the seeds of some Leguminosae [12]. It has been suggested that the prime function of urease in these organisms is as a storage protein[13] the breakdown of urease would provide amino acids and ammonia necessary for growth.

Urease activity is subject to a variety of regulatory mechanisms. The synthesis of urease in many bacteria appears to be repressed in the presence of a preferred nitrogen source by the nitrogen regulatory system. In other bacteria urease synthesis may be directly induced by the presence of urea. Both nitrogen repression and urea induction control urease expression in some bacteria. A third class of ureases is expressed constitutively and is unaffected by the nitrogen source or the presence of urea [12].

1.1.1. Cellular localization of urease

Urease is an extracellular enzyme[14, 15], and most studies show that urease localized in yeasts and cytoplasm parts of most bacterial strains[10], as enzyme is detected in the cytoplasm of 22 bacteria[16]. Electronic microscope shows the conjugation of the inner membrane of Staphylococcus, while in the periplasm and outer membrane of Proteus mirabilis[10].While Helicobacter pylori is the only bacteria that has enzyme in cytoplasm and on cell membrane, because of the ability of the bacteria outer membrane to absorb the enzyme that is released from adjacent cell autolysis [17].

Mobley et.al.[18] have illustrated the relationship between the production of urease and differential stages during the growth of bacterial cells. They minted that there was a difference in the urease activity in swarming property of the Proteus spp.

from the non-swarming bacteria cells, since the activity of urease in swarming cells was greater than the non-swarming bacteria cells.

1.1.2. Urease mechanisms of action

Historically the earliest Ni-containing enzyme to be described was urease from jack bean meal, which was crystallized by James Sumner in 1926. However, analytical techniques did not allow urease to be recognized as a Ni-containing enzyme until 50 years later. Urease plays a key role in nitrogen metabolism in plants and microbes whereas land dwelling animals excrete urea as the end product of their nitrogen metabolism; clearly, they do not produce urease[19].

The active site (Figure 1-7) contains two Ni ions, (~3.5 Aº) apart which are bridged by a carbamylated lysine residue. Both Ni ions are coordinated by two His nitrogen atoms, oxygen from the bridging carbamyl group and oxygen from bound water[19].

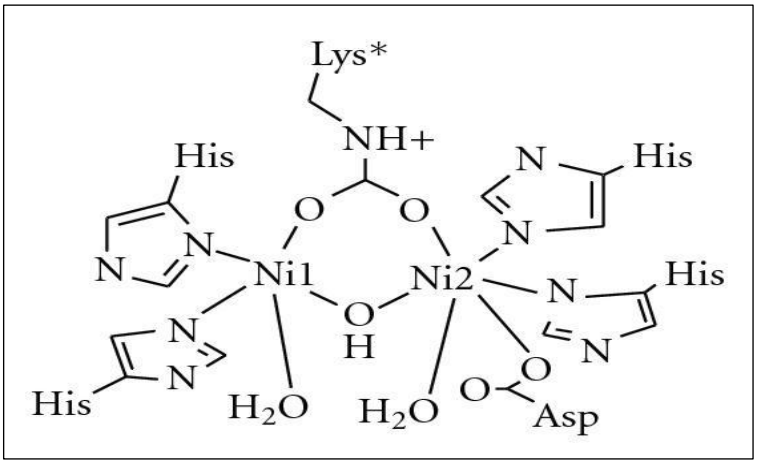


Figure (1-2) Dinuclear Ni active site of urease[4]

One of the Ni atoms in addition has an oxygen ligand from an Asp residue. CO_2 is required for formation of the carbamylated Lys bridge between the two Ni atoms, and mutation of this Lys results in loss of activity[19]. A proposed reaction mechanism is presented in Figure (1-8):

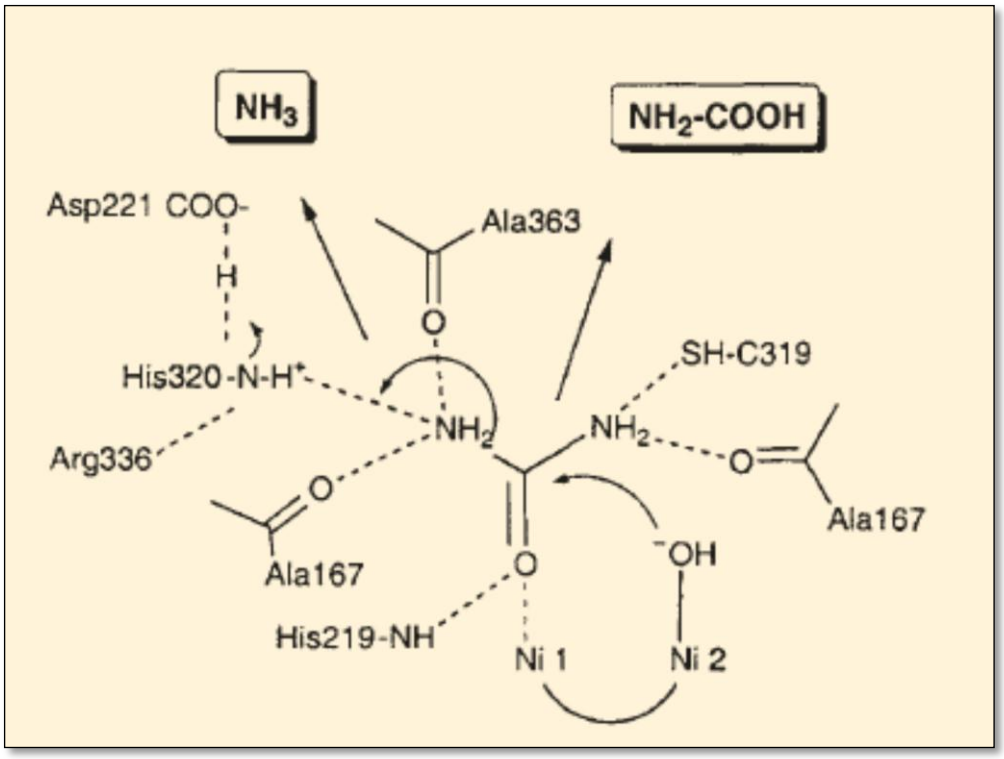


Figure (1-3) Reaction mechanism of urease [19]

The large kinetic barrier to urea hydrolysis is presumed to be lowered by:

1- Coordination of the carbonyl group of urea to the first nickel atom (Ni1), making the carbonyl more electrophilic.

2- Binding of water to the second nickel atom (Ni2) to generate an activated hydroxyl species and

3- Hydrogen bonding interactions of all four of the protons of urea with electrophilic groups of the protein. Attack of the metal-activated hydroxyl would generate a tetrahedral intermediate. Protonation of this intermediate would eliminate ammonia, leaving carbamate bridged between the two Ni atoms. Dissociation of carbamate from the dimetallic site would be followed by spontaneous hydrolysis to carbonate and second molecule of ammonia, with a protonated His residue acting as a general acid to promote ammonia release[19].

1.1.3. Environmental Urease

Urease activity is widely distributed in soil and aquatic environments, where it plays an essential role in nitrogen metabolism. For example, degradative processes involving protein and nucleotide turnover require urease activity. More importantly, effective urea fertilization requires controlled ureolysis to enhance efficiency and minimize crop damage[20].

1.1.4. Regulation of Urease production by bacteria

Urease is either induced or constitutive according to bacteria and depends on environmental factors; some studies pointed to that enzyme production is associated with nitrogen balance. Ammonia or nitrogen rich compounds like urea inhibit its production and it is inhibited when nitrogen quantity in environment is limited[21].

There are environmental factors that affect the urease production like pH, urea concentration, glucose or ammonia initiated in Proteus while Klebsiella regulates enzyme production in response to nitrogenous compound like ammonia, glutamine, and lysine[22].

In Streptococcus salivarius, enzyme production is regulated by surrounding pH; as low pH of this bacteria produce urease to increase pH of the media[23]. In Bordetella bronchioseptica urease production is regulated thermally[23].

1.1.5. Occurrence Of Urease In Microorganisms

Urease enzyme is considered as virulence factor for one kind of bacteria [24,25,3].Many studies show that urease enzyme plays a role in alimentary canal and pathogenesis (that enables the bacteria in acidic media) causing dangerous diseases like stomach cancer [26].

Proteus mirabilis is considered as coccobacilli that causes urinary tract infection and these bacteria have many virulence factors that aid in causing infections like flagella, urease, LPS in addition to protease enzyme that play important role in bacteria [27].

1.2. Proteus Bacteria

Proteus is a Greek word that Hauser (1885) called the bacteria he discovered and means "continuous changing shape" for its ability to move fast and change the colony shape. He mentioned two strains of these bacteria: mirabilis, vulgaris. He described colony of these bacteria as circular colony that change to irregular colony with amoeba like extension[28].

There are now five named species of the genus Proteus: P. mirabils, P. vulgaris, P. penneri, P. myxofaciens, and P. hauseri, along with three unnamed genotypes[29].

These bacteria are present widely in nature and play a very important role in organic substances degradation and were isolated from soil, water, home[30].

Proteus bacteria colonies appear to be in a pale yellow color in a media of MacConkey agar, because it does not fermentate lactose sugar. But it fermentates galactose, sucrose and glucose [30], and it does not produce beta-galactosides enzyme in Ortho-Nitrophenyl-β-Galactoside (ONPG) test [31].

The physiological properties of these bacteria were studied and differentiate the possibilities between these bacteria to use and ferment maltose where one strain can ferment maltose while others cannot [32].

These bacteria can utilize urea by urease and liberate ammonia and CO_2 which elevate pH of the media to basic limit [33]. This property is specific to protues which makes protues move fast and in addition to that the sea wave appearance on solid agar or what is called swarming, this can distinguish it from Morganella by producing H_2S gas and lipase enzyme in addition to urease production and swarming [34].

The motion of Proteus has a major role in Urinary Tract Infection, so Proteus bacteria invades urinary tract via the fast motion of their flagella (Figure 1-10): which increases the pathogenic of Proteus and enables it to ascend to the top of urinary system and consequently builds a colonies[35].The optimum temperature for Proteus bacterial growth is 37°C, and best pH is 7.4[36].

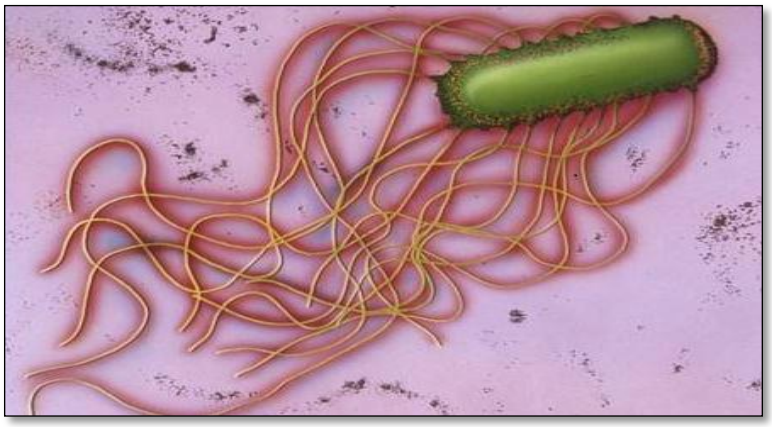


Figure (1-4) Proteus mirabils[35]

1.2.1. Clinical significance of Proteus

Proteus is considered as an opportunistic microorganism that belongs to Entrobacteriaceae for its effect on health of human and animals [37]. It causes gastrointestinal infection and genitourinary infection and its clinical significance comes after E.coli and Klebsiella pneumoni as they cause U.T.I. [38]. It has been isolated from urinary tract, wounds, skin, eyes, ears, nose, and larynx and from alimentary tract [39].

Proteus spp. causes U.T.I. in elderly patients [38], as it constitutes 15 % from bacterial infection of the urinary system [40], in addition to other surgical operations [40] .

Proteus spp. also causes nosocomial infections [41], it was clear 50 years ago that the ways of prevention of such infections inside hospital were considered as great difficulty because of emerging resistant strains of bacteria [42]. Spread of infection is either endogenous that is present on the skin, intestine and pulmonary systems from the patients in hospital or exogenous by medical staff, tools used in instrumentations like catheterization [43].

Ankler [44], shows that urinary system infections caused by Proteus occur by the movement of these bacteria upward through its ability to move fast by flagella ,and the infection may be complicated to be pyelonephritis , and in some cases these bacteria may spread in the blood stream causing septicemia [44]. This bacteria has many virulence- factors that increase the percentage of infection, as its presence in urine,

rapid growth rate, and fast movement in addition to many factors that increase the health problems especially in elderly and causes infections, renal stones, bladder stones, renal failure and urinary tract obstruction [45,38].

1.2.2. Virulence factors

Proteus has many virulence factors that help in destruction of host's defense mechanism due to the ability of secreting many enzymes and toxins in addition to have certain surface structures which increase their virulence [46].

1.2.2.1. Urease production

The Proteus bacteria have the ability to increase the pH of urine by secreting urease that splits urea to ammonia (NH_3) and carbon dioxide (CO_2) that precipitate magnesium ion (Mg^{+2}) and calcium ion (Ca^{+2}) which are present in urine [47,48].

The increase of the pH of the urine 5 to 9 increases the average of stone formation [49], and the absorption of the evolved ammonia from the decomposition of urea by urease may lead to increase the ammonia concentration in blood (hyperammonemia), and may cause death to the patient [50].

1.2.2.2. Hemolysin production

Proteus bacteria can secrete hemolysin that destructs red blood cell (RBC) and is one of factors that help in invasion[51]. Hemolysin performs many functions, one of them making pores in both RBC membranes (erythrocytes) and epithelial cells, and due to its cytotoxicity, it may lead to tissue damage[52].

Hemolysin is produced from many gram-negative bacteria, such as E.coli, Proteus, and gram-positive bacteria as well, such as Staphylococcus[52].

1.2.2.3. Flagella and swarming motility

A Proteus mirabilis posse more than 100 peritrichous flagella. These flagella are responsible for the movement of bacteria and facilitate its adherence to the cells forming the outer surface of different tissues. Then after penetration of these tissues it

initiates its pathogenicity. These flagella also give the Proteus spp. one of its features which are the swarming phenomena [53].

1.2.2.4. Fimbrial and adherence ability

These bacteria can adhere to uroepithelial cells and the presence of fimbria in large numbers on bacterial surface cause severe infection leading to pyelonephritis (inflammation of its pelvis and kidney parenchyma) due to bacteriuria [54].

1.2.2.5. Lipopolysaccharide-O-antigen endotoxin

This is one of gram negative virulence factor that help in activities of bacterial antibodies present in serum like phagocytes. LPS consist of O-specific sequence, core zone and lipid a (lipophilic zone) that act as fixation of LPS on outer membrane of Proteus spp. [55].

1.2.2.6. Natural resistance to polymyxin

Proteus bacteria resist cyclic antibiotics of (PMX) kind because of connection of positive charge present in their structure with negative charge of lipid a region [56].

1.2.2.7. Invasiveness

Infection occurs after Proteus penetration of the host that causes secretion of a protein which helps them to change their shape to small rods with fast movements and multiplication inside the host [57].

1.2.2.8. Capsule polysaccharide (CPS)

The capsule of Proteus bacteria has relation with their pathogenicity because of its ability to collect metallic magnesium ions as the result of presence of some acids like uric acid, pyruvic acid or phosphate groups that result in urolithiasis [58].

1.2.2.9. Protease production

Proteus can resist the immune system by producing of protease enzyme that splits peptide chains present in immunological activities that permit the establishment of infection [59].

1.3. Urinary tract infection (U.T.I.)

Urinary tract infection (UTI) (also known as an acute cystitis or bladder infection) is an infection that affects part of the urinary tract. When it affects the lower urinary tract it is known as a simple cystitis (a bladder infection) and when it affects the upper urinary tract it is known as pyelonephritis (a kidney infection)[60].

The urinary system consists of the kidneys, ureters, bladder, and urethra. The key elements in the system are the kidneys, a pair of purplish-brown organs located below the ribs toward the middle of the back. The kidneys remove excess liquid and wastes from the blood in the form of urine, keep a stable balance of salts and other substances in the blood, and produce a hormone that aids the formation of red blood cells. Narrow tubes called ureters carry urine from the kidneys to the bladder, a sack-like organ in the lower abdomen. Urine is stored in the bladder and emptied through the urethra[61].

The average adult passes about a quart and a half of urine each day. The amount of urine varies, depending on the fluids and foods a person consumes. The volume formed at night is about half that formed in the daytime [61] .

1.3.1. Causes of UTI

Normally, urine is sterile. It is usually free of bacteria, viruses, and fungi but does contain fluids, salts, and waste products. An infection occurs when tiny organisms, usually bacteria from the digestive tract, cling to the opening of the urethra and begin to multiply. The urethra is the tube that carries urine from the bladder to outside the body. Most infections arise from one type of bacteria, Escherichia coli (E. coli), which normally lives in the colon[62].

In many cases, bacteria first travel to the urethra. When bacteria multiply, an infection can occur. An infection limited to the urethra is called urethritis. If bacteria move to the bladder and multiply, a bladder infection, called cystitis, results. If the infection is not treated promptly, bacteria may then travel further up the ureters to multiply and infect the kidneys. A kidney infection is called pyelonephritis[62].

Microorganisms called Chlamydia and Mycoplasma may also cause UTI in both men and women, but these infections tend to remain limited to the urethra and reproductive system. Unlike E. coli, Chlamydia and Mycoplasma may be sexually transmitted, and infections require treatment of both partners[62].

The urinary system is structured in a way that helps ward off infection. The ureters and bladder normally prevent urine from backing up toward the kidneys, and the flow of urine from the bladder helps wash bacteria out of the body. In men, the prostate gland produces secretions that slow bacterial growth. In both sexes, immune defenses also prevent infection. But despite these safeguards, infections still occur[62].

1.3.2.The risk-factors for U.T.I.

The most important factors on U.T.I., in terms of the location of infection and pathogenicity are (stones, diabetes mellitus, sex, age, pregnancy, catheterization process, sircumsition, renal failure, and congenital malformations of urinary tract) [48,63]. These factors may facilitate the infection process by Proteus bacteria, and consequently U.T.I [64]. While congenital malformations are considered to be the main causes of urinary tract observation which leads to urinary stasis, and then increases the probability of U.T.I.[65,66].The formation of stones inside the bladder and kidney is one of the characteristic features of Proteus spp. which needs a surgical operation or other medical interventions [65].

1.3.3. Urinary tract stones

A kidney stone is a solid mass that consists of a collection of tiny crystals. There can be one or more stones present at the same time in the kidney or in the ureter [67].

Kidney stone may form when the urine becomes too concentrated (supersaturated urinary environment) with certain substance. These substances may create crystals that become stones[67] as illustrated in the following Figure (1-11):

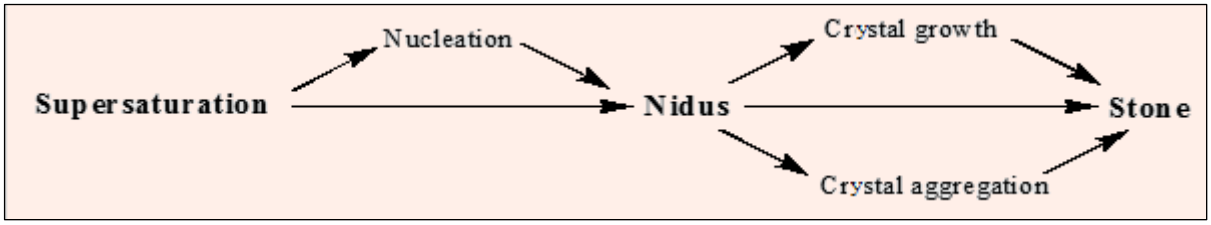


Figure (1-5) Schematic reaction to stones formation [67]

1.3.4. Types of stones

The urinary tract stones could be classified in two kinds: Figure (1-12)

1.3.4.1. stones from Metabolism

A stone that may be formed in the pure urine and could be divided into six kinds [68].

1.3.4.1.1. Calcium stones

These kinds are most common in about (65 – 80) % from other types of stones. The calcium may combine other substance such as oxalate (the most common substance), phosphate, or carbonate to form stone (sparingly soluble salts) [69]. Oxalate is present in foods. Diseases of the small intestine increase the tendency to form calcium oxalate stones [70].

1.3.4.1.2. Xanthene's stones

They rarely infect urinary tract, and it is found in (4:10000) patients, and the cause of this stone is the increase of xanthine formation in urine due to the genetic deficiency in xanthine oxidase [71].

$$\text{Xanthine} + O_2 + H_2O \xrightarrow{\text{Xanthine oxidase}} \text{Urate} + O_2^- + 2H^+$$

1.3.4.1.3. Uric acid stones

They represent about 10 % of the total stones, and are largely formed in bladder more than in kidney and commonly infect males [67]. These kinds of stones are formed due to a high consumption of animal protein [72].

1.3.4.1.4. Drugs stones

There are many drugs that facilitate the stone formation like analgesics, and anti-virus drugs (e.g. indivar) which is used for treatment HIV [73].

1.3.4.1.5. Silica stones

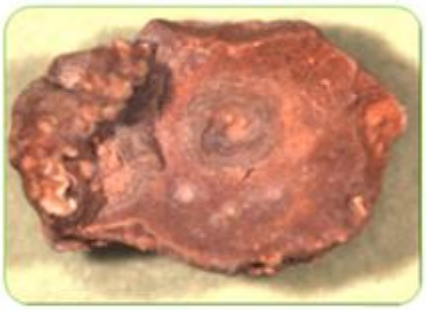
These kinds of stones are rarely found in human, but they are so common in animals. They may be formed in patients that take anti-acid drugs which contain magnesium trisilicate. When they react with any acid. They may yield silica crystals (stones) [74].

1.3.4.1.6. Cystine stones

They represent about 1 % of the total stones and they are formed due to the presence of cystinurea an autosomal recessive disorder in renal tubular and intestinal transport of dibasic amino acids, which results in increased urinary excretion of cystine, ornithine, lysine and arginine[75].

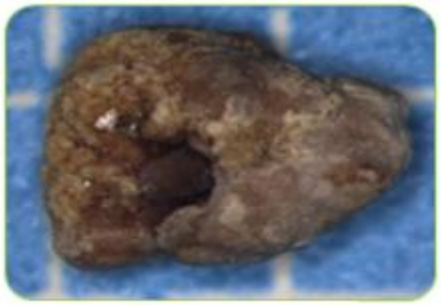
1.3.4.2. U.T.I. stones

Bacteria have a major role in formation of such type of stones and they represent 7- 31 % of the total stones [76]. It is produced from ammonium magnesium phosphate ($MgNH_4PO_4.6H2O$) and called struvite. These stones are considered a major threat more than other stones. This is so because they are continuous and bigger in size therefore it's dangerous for the kidney damage [77].



Xanthene's stones

Calcium stones



Uric acid stones

Drugs stones

Silica stones

Cystine stones

U.T.I. stones

Figure (1-6) Pictures shown different types of stone[72,77]

1.4. Biological activity

1.4.1 Mechanism of antimicrobial action

It should always be remembered that drugs are seldom the sole solution of cure but act together with the natural defenses of the body[78].

The mechanism of action of most antimicrobial drugs is not completely understood. However, these mechanisms of action can be placed under four headings[78,79]. Figure (1-1) show the overall mechanism.

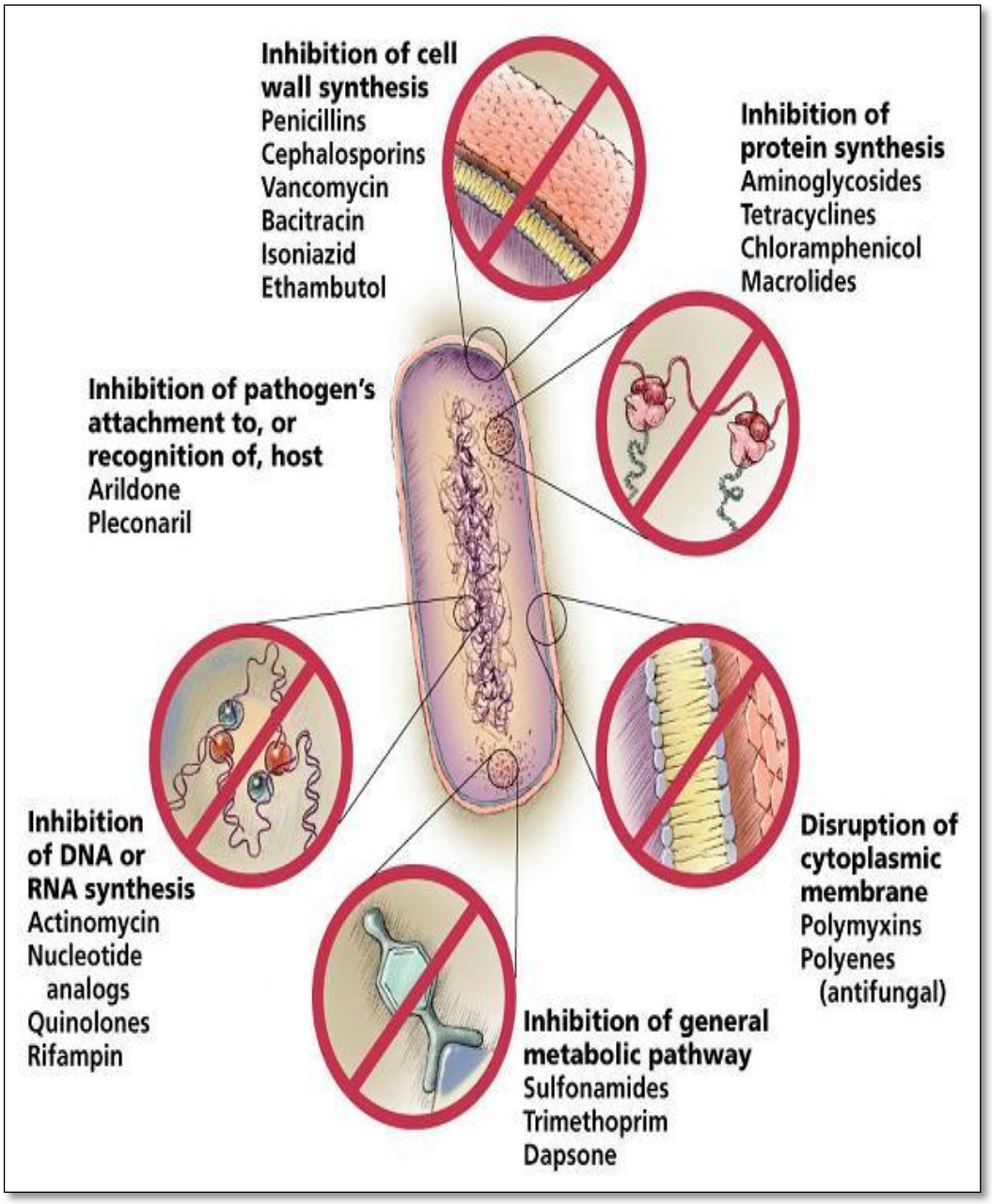


Figure (1-7) Mechanism of antimicrobial action[78]

1.4.1.1. Inhibition of cell wall synthesis

Bacteria possess a rigid outer layer of the cell wall. which maintain the shape of the microorganism and provide protection against much lower osmotic pressure of the environment (the internal pressure is three to five times greater in gram-positive than in gram-negative bacteria) [80,81],as in Figure(1-2).

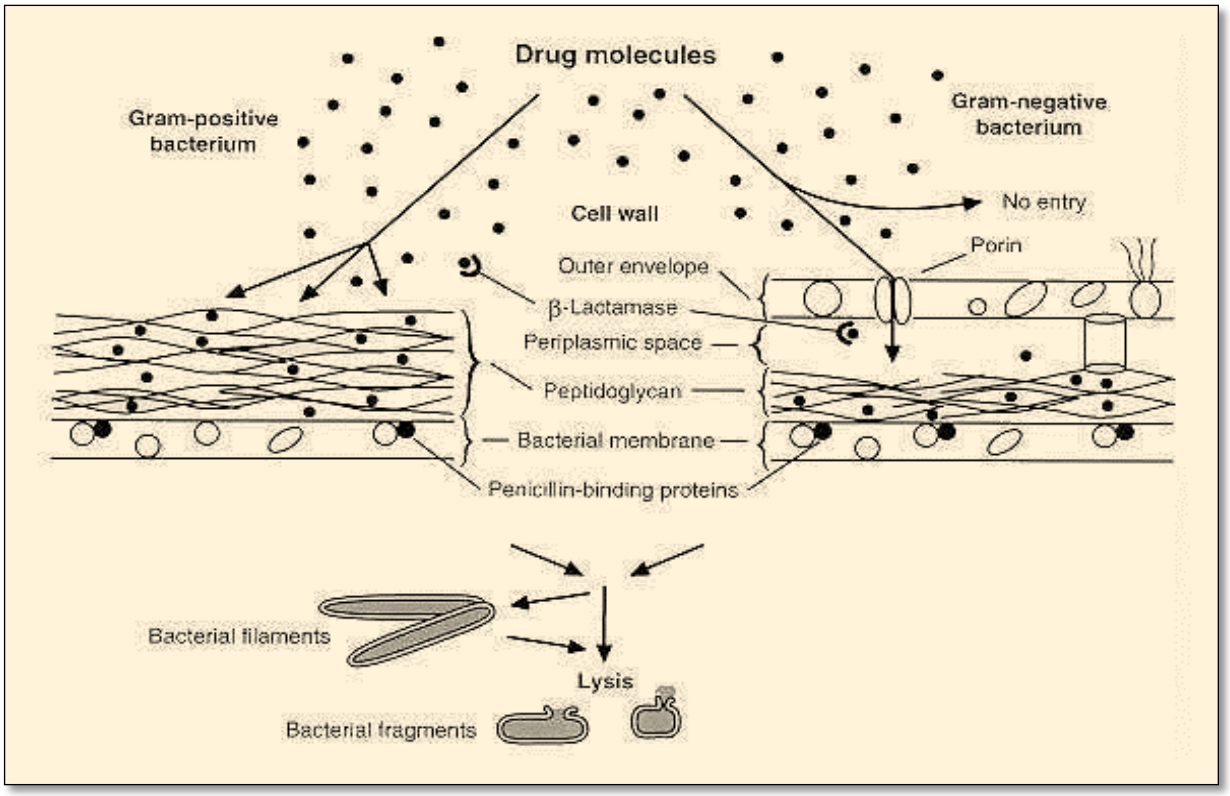
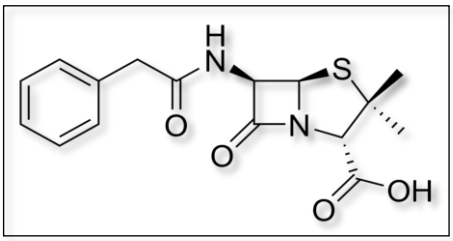


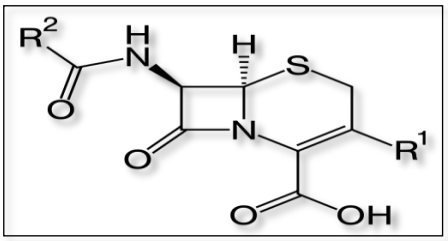
Figure (1-8) Outer wall of Gram-positive and Gram-negative species [81]

Structurally, bacteria resemble primitive plants in that the cellular contents are surrounded by an inner peptidoglycan cell wall in addition to an inner plasma membrane and, in Gram-negative bacteria, an outer lipid bilayer. Specific antibacterial interfere with the synthesis of the cell wall, weakening the peptidoglycan scaffold within the bacterial wall so that the structural integrity eventually fails. Since mammalian cells have a plasma membrane but lack the peptidoglycan wall structure, this class of antibacterial selectively targets the bacteria with no significant negative effect on the cells of the mammalian host[80].

Bacterial multiplication involves a breakdown and an extension of the wall, interference with these processes, prevents the organism from resisting osmotic pressures, so that it bursts. As these cells of higher organisms, e.g. human, these organisms do not possess this type of wall. Drugs which act here, may be especially selective, obviously the drugs are effective only against growing cells. They include (bacitracin, cephalosporins, cycloserine, penicillins, and vancomycin)[82].



Penicillin's

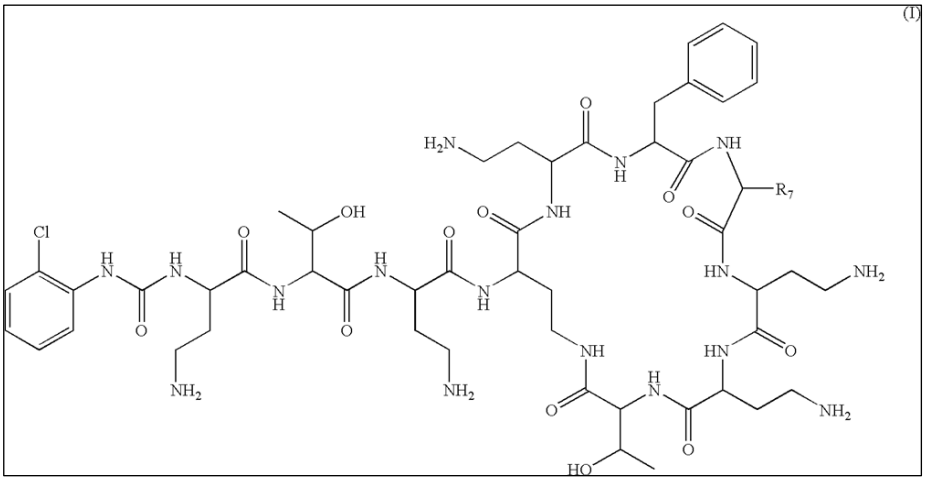


Cephalosporins

1.4.1.2 Inhibition of cell membrane function

Biologic membranes are composed basically of lipid, protein, and lipoprotein. The cytoplasmic membrane acts as a diffusion barrier for water, ions, nutrients, and transport systems. Most workers now believe that membranes are a lipid matrix with globular proteins randomly distributed to penetrate through the lipid bilayer[83]. A number of antimicrobial agents can cause disorganization of the membrane. These agents can be divided into cationic, anionic, and neutral agents. The best-known compounds are polymyxin B and colistemethate (polymyxin E). These high-molecular-weight octapeptides inhibit Gram-negative bacteria that have negatively charged lipids at the surface. Since the activity of the polymyxins is antagonized by Mg^{2+} and Ca^{2+}, they probably competitively displace Mg^{2+} or Ca^{2+} from the negatively charged phosphate groups on membrane lipids. Basically, polymyxins disorganize membrane permeability so that nucleic acids and cations leak out and the cell dies. The polymyxins are of virtually no use as systemic agents since they bind to various ligands in body tissues and are potent toxins for the kidney and nervous system.

Gramicidins are also membrane-active antibiotics that appear to act by producing aqueous pores in the membranes. They also are used only topically[83,84].



polymyxin B

The inhibition mechanism of cell membrane function by polymyxins as presented in Figure (1-3):

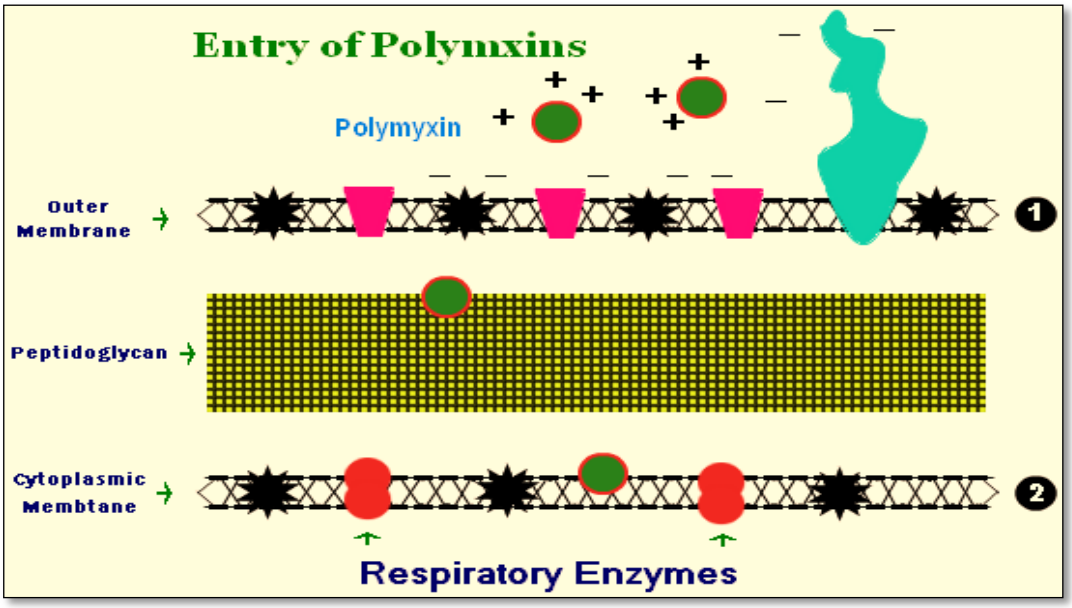
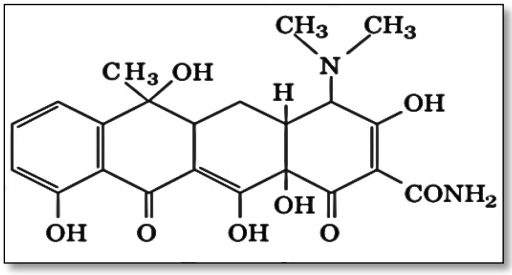


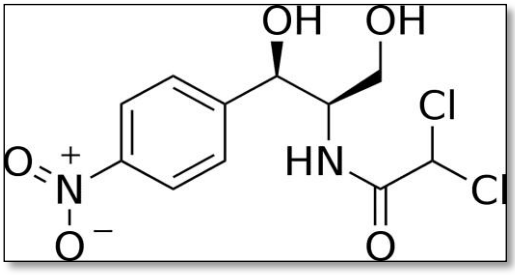
Figure (1-9) Mechanism of inhibition of cell membrane function[83]

1.4.1.3. Inhibition of proteins synthesis

It is established that (Chloramphenicol, Tetracycline, Erythromycin, lincomycin and aminoglycoside) drugs can inhibit proteins synthesis in bacteria. The precise mechanism of action is not fully establishing for these drugs [85]. Bacteria have 70S ribosomes. The subunit of each type of ribosome is different in chemical composition, and their functional specification are sufficiently different, therefore antimicrobial drugs can inhibit proteins synthesis in bacterial ribosome by inhibition of transcription and translation of genetic material without having a major effect on mammalian ribosome [86].

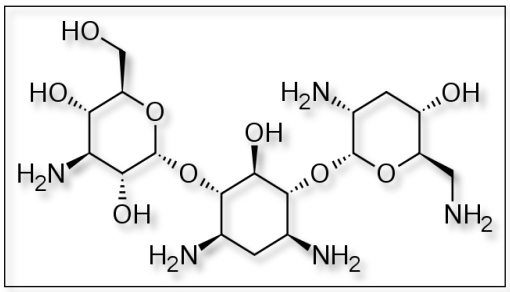


Tetracycline

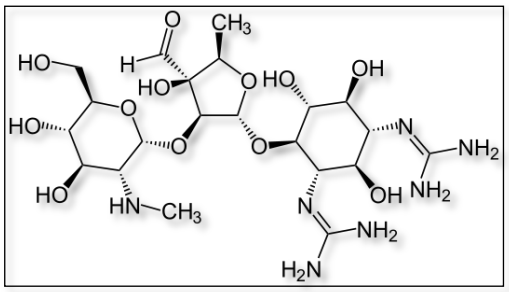


Chloramphenicol

Aminoglycosides include (kanamycin, Neomycin, Gentamycin, Tobramycin, Amikacin, Streptomycin, Sisomycin and Netilmycin) and these drugs probably all act similarly [87].



Tobramycin



Streptomycin

1. The first step is the attachments of the aminoglycoside to a specific receptor protein as on the 30S subunit of the microbial ribosome.
2. The aminoglycoside blocks the normal activity of the "initiation complex" of peptide formation.(mRNA+ formylmethionine+ tRNA).
3. The mRNA message is misread on the recognition region of the ribosome; consequently, the wrong amino acid is inserted into the peptide, resulting in a nonfunctional protein.
4. Aminoglycoside attachments result in the breakup of polysomes and their separation into monosomes incapable of proteins synthesis[81,88,89]. The Inhibition of protein synthesis by antibiotic is shown in Figure (1-4):

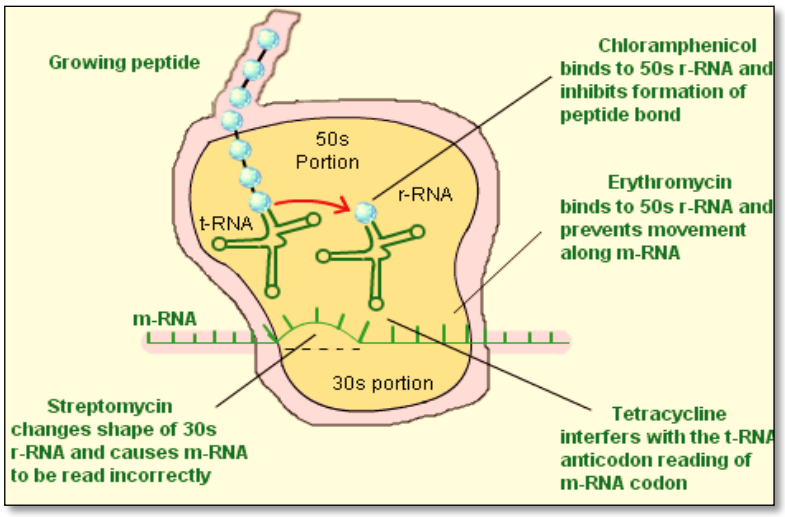
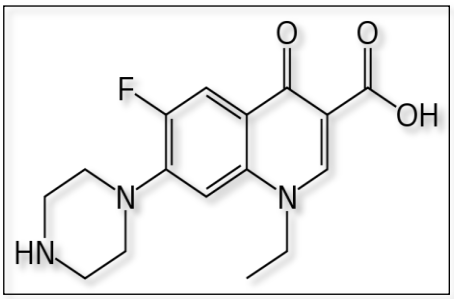


Figure (1-10) Inhibition of protein synthesis by antibiotic[89]

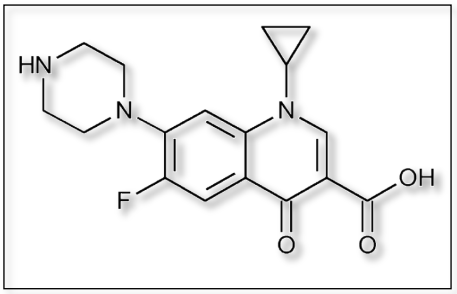
1.4.1.4. Inhibition of Nucleic Acid Synthesis

A nucleic acid inhibitor is a type of antibacterial that acts by inhibiting the production of nucleic acids. There are two major classes: DNA inhibitors and RNA inhibitors.

1. DNA inhibitors such as Quinolones are a key group of antibiotics that interfere with DNA synthesis by inhibiting topoisomerase, most frequently topoisomerase II (DNA gyrase), an enzyme involved in DNA replication. DNA gyrase relaxes supercoiled DNA molecules and initiates transient breakages and rejoins phosphodiester bonds in superhelical turns of closed-circular DNA. This allows the DNA strand to be replicated by DNA or RNA polymerases. The fluoroquinolones, second-generation quinolones that include levofloxacin, norfloxacin, and ciprofloxacin, are active against both Gram-negative and Gram-positive bacteria [90,80].

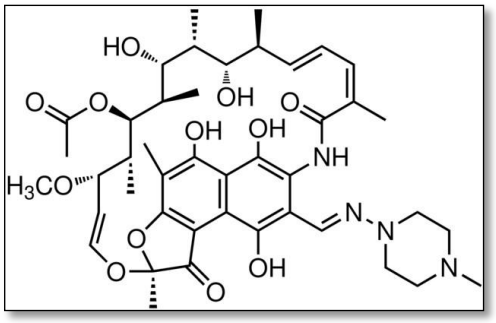


norfloxacin

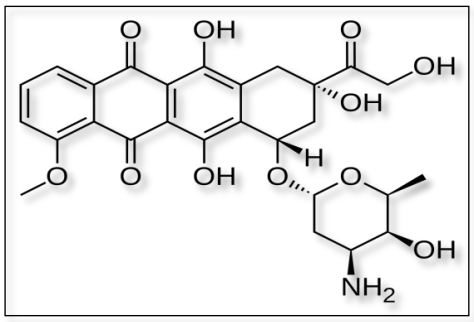


ciprofloxacin

2. RNA inhibitors such as Rifampicin blocks initiation of RNA synthesis by specifically inhibiting bacterial RNA polymerase. It does not interact with mammalian RNA polymerases, making it specific for Gram-positive bacteria and some Gram-negative bacteria. Doxorubicin and actinomycin D, are not specific for bacteria and interfere with both bacterial and mammalian systems[91,80].



Rifampicin



Doxorubicin

1.4.2. Use of antimicrobial drugs

The general rule is that selection of an antimicrobial agent for the treatment of a particular infection is largely based on the cause, location, and severity of an infection; the age, physiologic status, and immune competency of the patient; and the pharmacologic properties of antimicrobial drugs. All appropriate specimens (blood, urine, pus, spinal fluid) must therefore be taken for examination before administering any antimicrobial [(92)].

The patient's immune status is an important factor determining the success of antimicrobial therapy. Advanced age, diabetes, cancer chemotherapy, and human immunodeficiency virus (HIV) infection are among the more common causes of impaired immunity. Immunocompromised individuals should be treated with larger doses of bactericidal drugs and may require a longer duration of therapy than do immunocompetent individuals[(80)].

Many antibiotics are excreted unchanged by the kidneys, and lower doses must be used if the patient has significant renal impairment. Less commonly, hepatic insufficiency may require dosage adjustment for antimicrobial drugs that are extensively metabolized in the liver. For example, neonates cannot metabolize chloramphenicol, so their dosage of this drug per kilogram of body weight must be lower than the dosage given to older children or adults. For example aminoglycosides (Tobramycin) is used as first choice for treatment the urinary tract infection that get from (Proteus or Pseudomonas

aeruginosa) bacteria , and benzyl-penicillin for meningitis in the adult (Meningococcal or Pneumococcal) [93-95].

1.4.3. Resistance to antimicrobial drugs

Bacterial resistance for the chemical compounds may occur through many mechanisms, as:

1- Microorganisms produce enzymes that destroy the active drug , example: Staphylococci resistant to penicillin G by producing a ß-Lactamase that destroys the drug as e.g. Penicillinase destroys the ß-Lactam of penicillin and converted to inactive drugs (penicilloic acid)as more stable compound [96], as illustrate in the following reaction:

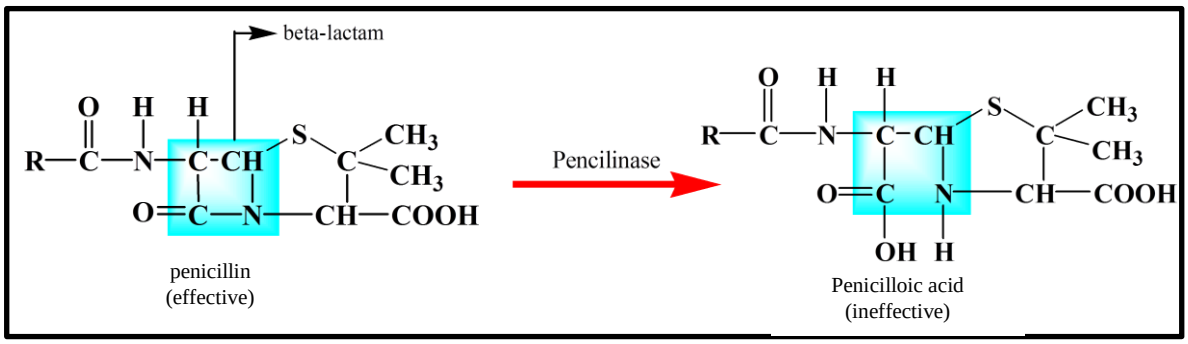


Figure (1-11) The mechanism of enzyme action (Penicillinase) [96].

Gram- negative bacteria may be resistant to Chloramphincol if they produce a Chloramphincol acetyl transferase[81].

2- Microorganisms change their permeability to the drug, example: tetracycline accumulates in susceptible bacteria but not in resistant bacterial, apparently due to an outer membrane change that impairs active transport into the cell or due to thick cell wall as it contains high lipid in the wall[97].

3- Genetic mutations, example: chromosomal mutation allows bacteria to resist aminoglycosides by alteration of a specific protein in the 30S subunit of the bacteria ribosome[97].

4- Microorganisms develop an altered metabolic pathway that by passes the reaction inhibited by the drug, example: sulfonamide resistant bacteria, do not require extracellular p-amino benzoic acid (PABA), but like mammalian cell, can utilize preformed folic acid [81, 97].

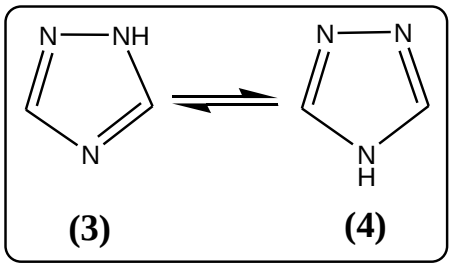
Urinary tract infection is considered one of the infections that are caused by bacteria with an increased ability for multi-drug resistance. In many studies it is noticed that researchers could isolate many spp. of bacteria of one Genus some of which are sensitive and others resistant to antibiotic [98]. It is also noticed that an increase in the occurrence of resistance to new broad spectrum antibiotics [86,99,100]. Therefor this is one of the aims to prepare novel drugs.

1.5. Triazoles

Triazoles are five membered heterocyclic compounds containing three nitrogen and two carbon atoms. This may be of two types, the 1,2,3-triazoles **(1)** and the 1,2,4-triazoles **(2)**.

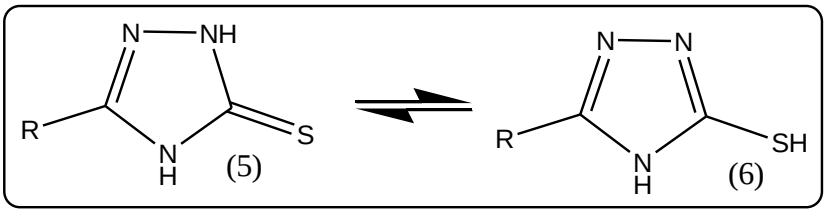
The name triazole was first given to the carbon nitrogen ring system $C_2N_3H_3$ by Bladin who described its derivatives in early 1885, although the structures reported slightly incorrect [101,102].

1, 2, 4-Triazoles exhibit two tautomeric forms namely (4*H*)-1,2, 4- triazoles **(4)** and(1*H*)-1,2,4-triazoles**(3)**.



(3) **(4)**

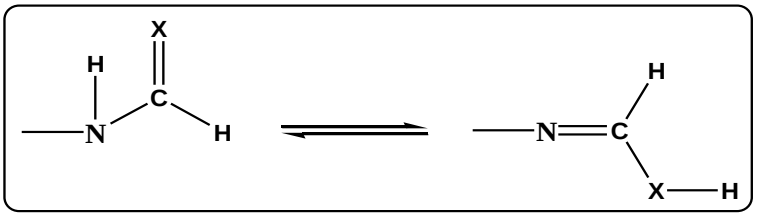
Among the substituted 1,2,4-triazoles, 3-mercapto-1,2,4-triazoles exist in two tautomeric forms, because the labile hydrogen may be attached either to the nitrogen or the sulfur atom. It exhibits thione-thiol tautomeric forms shown below. This compound exists predominantly in thione **(6)** form [(103)].



The 1,2,4-triazole is an ubiquitous feature of many pharmaceutical and agrochemical products. The substituted 1,2,4-triazole nucleus is particularly common, and can be found in marketed drugs such as fluconazole and terconazole, [(104-106)].

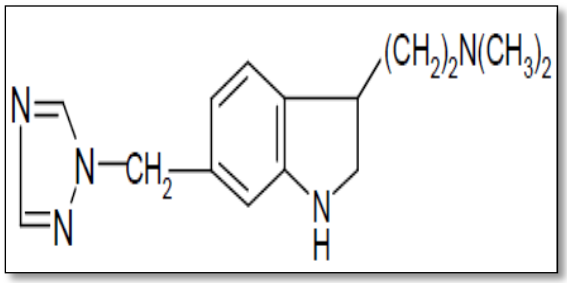
As drugs, triazole compounds are highly efficient, low poisonous and inward – absorbent. The studies on triazole derivatives are mainly concentrated on compounds with the triazole as the only active group, the reports of triazole compounds that contain both triazole group and other active group in the single molecule has rarely been found [(107)].

An important and versatile class of well-established biologically active compounds are those containing the moiety (X=N, O, S) which can exist in two tautomeric forms [108].

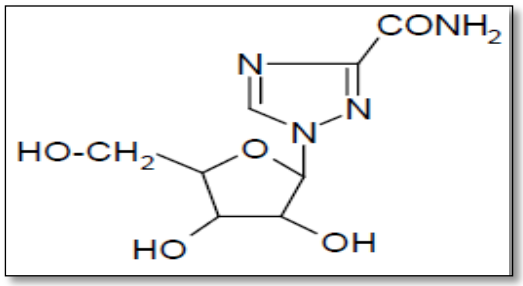


1.5.1. Biological Actions of Triazole derivatives

The derivatives of 1*H*- 1,2,4- triazole of therapeutic importance includes Rizatriptan an antimigraine agent[109] Ribavirinan antiviral agent [110].

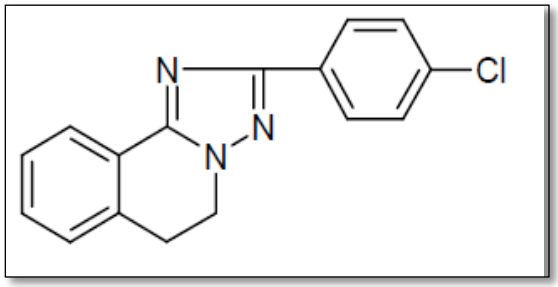


Rizatriptan

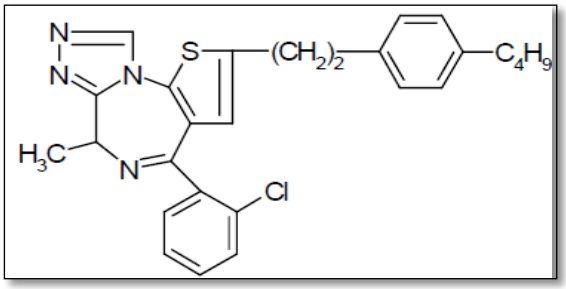


Ribavirin

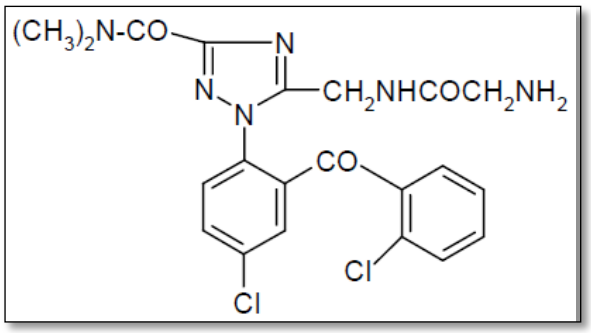
Israpafant[111] an antiasthmatic, Lotrifen[112] an abortifacient and Rilmazafone[113] a potent sedative and hypnotic agent.



Israpafant



Lotrifen



Rilmazafone

1.6. Triazol complexes

The tendency of metal ion to form a stable complex with ligands depend on many rules such as the hard and soft acids and bases (HSAB) rule for Pearson[108].Which imply that metal ion tend to coordinate with certain functional groups of the ligand to form a stable complex.

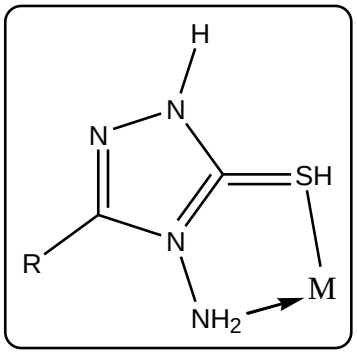
Triazoles are considered to be good coordinating ligands because they involved both hard nitrogen and soft sulfur atom as thio amide group, this ligand have doner group that coordinate with wide range of metal ions[114,115].

Metal complexes may be subjected for the design and synthesis of such possibilities having such biological activities [115-116]. The chemistry of Schiff bases and

their structural analogues has occupied a place of considerable importance [117] as they easily form stable complexes with most transition metal ions [118,119] and well-established biological properties.

1,2,4-triazoles nucleus and their derivatives emerge rapidly with the advances of modern heterocyclic chemistry, promising a variety of medical applications such as antibacterial, antifungal, anticancer, antitumor, anticonvulsant, anti-inflammatory, and analgesic properties[120]. Schiff bases of 1,2,4-triazoles find diverse applications and extensive biological activity[121].

The 4-amino-5-(substituted-phenyl)-4H-1, 2,4-triazole- 3-thiole derivatives molecule contains an S-C-N-N unit that allows for bidentate coordination to a metal ion through the amine and thione substituents to form a stable five-membered ring[122].



Many quantitave studies have confirmed that complexes of polydentate ligand are tend to be more stable than complexes derived from monodentate ligands. Furthermore, five or six membered chelates are by far the most common and are in general the most stable[117,122].

Aim of the present work

As described in (1.5) Triazoles have been found as classical biological active compounds. This feature promote me to prepare Triazole compounds and their metal complexes and study antibacterial activity against Proteus mirabilis. Then it was tended study the inhibition effect on urease enzyme. Characterization using: Micro elemental analysis, FTIR, UV-visible spectroscopy, ^{1}HNMR and conductivity measurements of the prepared compounds was important for my study.

CHAPTER TWO

EXPERIMENTAL PART

2.1. Chemicals

All of the reagents and starting materials used in the present work were of reagent grade and were used without further purifications unless otherwise noted. Table (2-1) shows all the utilized chemicals in the experimental course of the thesis.

Table (2-1) Chemicals and their manufacturers

No.	Compound	Molecular Formula	M.Wt	Purity	Supplied from
1.	Carbon-disulfide	CS_2	76	99%	Scharlau
2.	Cupper acetate	$(CH_3CO_2)_2Cu$	181.5	98%	BDH
3.	Dimethyl sulfoxide (DMSO)	$(CH_3)_2SO$	78.13	98%	Fluka
4.	Ethanol	CH_3CH_2OH	46.07	99%	Scharlau
5.	Hydrazine hydride	$N_2H_4.\ H_2O$	50.05	98%	Thomas BAKER
6.	Hydrochloric acid	ClH	36.5	99%	BDH
7.	Methanol	CH_3OH	32.03	99%	Scharlau
8.	Methyl benzoate	$C_8H_8O_2$	136.15	98%	BDH
9.	Nickel chloride	$NiCl_2$	129.5	99%	BDH
10.	*p*-chloro benzaldehyde	ClC_6H_4CHO	140.57	97%	Merck
11.	*p*-hydroxy benzaldehyde	$C_7H_6O_2$	122.12	99%	Merck
12.	*p*-methoxy Benzaldehyde	$C_8H_8O_2$	136.15	98%	Merck
13.	*p*-N,N-dimethyl amino benzaldehyde	$C_9H_{11}NO$	149.19	99%	BDH
14.	Potassium hydroxide	HOK	56	99%	Fluka

2.2.Cultures media

The cultures media used in this work were listed in Table (2-2):

Table (2-2) Cultures media with the supplied company and origin purpose

NO.	Media	Company supplied from	Using
1.	Mueller Hinton Agar (MHA)	Mumbai (India)	For determination of susceptibility of microorganism to antimicrobial agents.
2.	Nutrient Broth (NB)	Mumbai (India)	For activation the microorganism before testing.
3.	Blood Agar (BA)	Mumbai (India)	For isolation and cultivation of many fastidious pathogenic microorganism.

2.3. Instruments

2.3.1. Melting point

The melting points were determined on electro thermal capillary apparatus, *Gallenkamp*, England.

2.3.2. Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra in the range (200-4000) cm^{-1} cut were recorded as KBr disc on FT-IR.8300 Shimadzu Spectrophotometer. (Baghdad University)

2.3.3. Ultraviolet-Visible Spectroscopy (UV-Vis)

The UV-visible spectra were measured using Shimadzu UV-Vis. 160 A-Ultra-violet Spectrophotometer in the range (200-1000) nm. (AL-Nahrain University)

2.3.4. Hot plate with magnetic stirrer

Gallenkamp / England

2.3.5. Conductivity measurements

Conductivity measurements were carried out by using WTW conductivity meter. (AL-Nahrain University)

2.3.6. Nucleir Magnetic Resonance (NMR)

^{1}H NMR spectra were obtained with Bruker spectrophotometer model ultra-shield at 300 MHz in DMSO- d6 solution with the TMS as internal standard. (AL-Albait University/ Amman/ Jordan).

2.3.7. Elemantal analysis (CHNS)

Elemental C H N S analysis were carried out on a Fison EA 1108 analyzer (Ibn- Sena/ Baghdad University).

2.3.8. Autoclave

Sturdy SA- 300 VL (Taiwan). AL-Nahrain University/Biotechnology Department.

2.3.9. Incubator

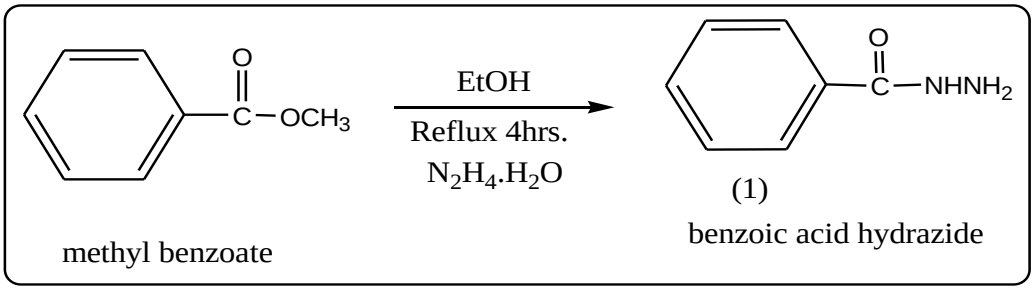
Memmert (Germany). AL-Nahrain University/Biotechnology Department.

2.4. Synthesis

2.4.1.Synthesis of benzoic acid hydrazide[123] (1).

Methyl benzoate (0.15 mol, 19 mL) in 25 mL of absolute ethanol is taken in a round bottom flask. To that (0.24 mol, 14mL) hydrazine hydrate was added and refluxed for 4 hrs.. The total volume of the solution is reduced to half and it was cooled. The solid was recrystallized from ethanol.

M.P (111-113) °C, Yield 76.5 %, color: white



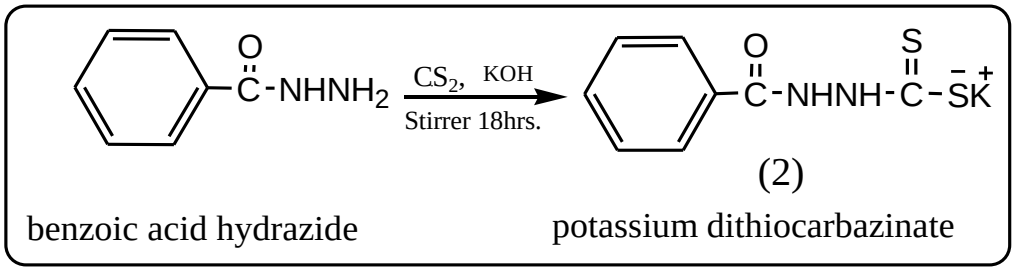
methyl benzoate

(1)

benzoic acid hydrazide

2.4.2.Synthesis of potassium dithiocarbazinate[123] (2).

Potassium hydroxide (0.03 mol, 1.68 g) was dissolved in (25 mL) of absolute ethanol. The solution was cooled in ice bath and (0.02mol, 2.94g) of benzoic acid hydrazide was added with stirring. To this mixture (0.05 mol, 5 mL) carbon disulfide was added in small portions with constant stirring. The reaction mixture was agitated continuously for 18 hrs. at room temperature. Cold ethanol (20 mL) and dry di-ethyl ether (20mL) was added to the solution and then evaporated. The potassium salt thus obtained was used in the next step without further Purification. Yield 74.1%, color: yellow



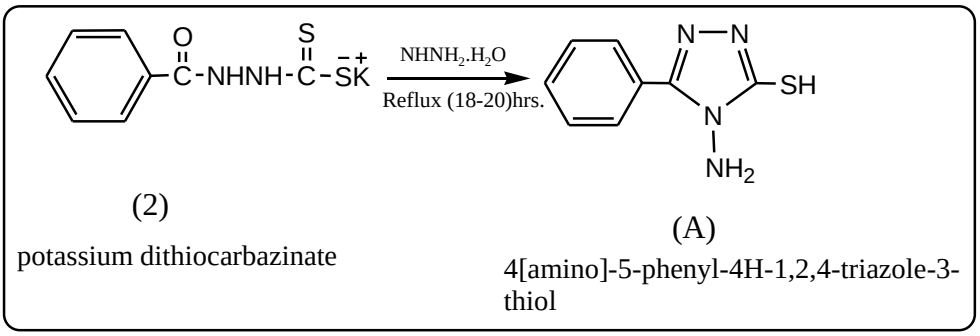
benzoic acid hydrazide

(2)

potassium dithiocarbazinate

2.4.3. Synthesis of 4(amino)-5-phenyl-4H-1,2,4-triazole-3-Thiol[123] (A)

A suspension of (0.006 mol, 1.56g) potassium dithiocarbazinate and hydrazine hydrate (98%, 0.009 mol, 0.45mL) was refluxed for 18– 20 hrs. The color of the reaction mixture changed into green with the evolution of hydrogen sulfide. The reaction mixture was cooled to room temperature and diluted with (5mL) cold water. On acidification with HCl the required triazole was precipitated out, which was recrystallized from ethanol. M.P (198-201) C°, Yield: 62 %, color: white



(2)
potassium dithiocarbazinate

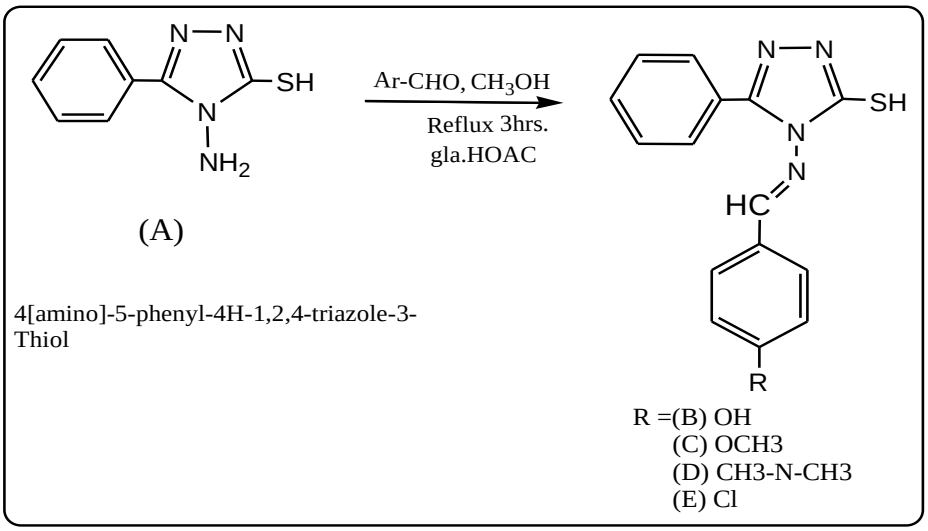
(A)
4[amino]-5-phenyl-4H-1,2,4-triazole-3-thiol

2.4.4. Synthesis of Schiff-bases[123]

A mixture of 4[amino]-5-phenyl-4H-1,2,4-triazole-3-thiol (0.25g, 0.001mol), with(0.12g , 0.001 mol) from *p*-hydroxy benzaldehyde, (0.14g, 0.001 mol) from *p*-chloro benzaldehyde, (0.13g , 0.001mol) from *p*-methoxy benzaldehyde, (0.14g, 0.001 mol) from *p*-(N,N-dimethyl) amino benzaldehyde and 2 drops of concentrated glacial acetic acid in ethanol medium was refluxed for 3 hrs. The resulting solution was cooled to room temperature and the precipitated solid was filtered under suction, washed with cold ethanol and recrystallized with hot ethanol.

Table (2-3) Melting point, Yield and color of the prepared Schiff bases

Compound	M.P	Yield	color
B	(250-253) C°	55 %	pale yellow
C	(208-210) C°	50 %	pale yellow
D	(180-182) C°	60 %	red
E	(211-213) C°	63 %	yellow



2.4.5. Synthesis of complexes (A1 and A2) with compound A as ligand [124]

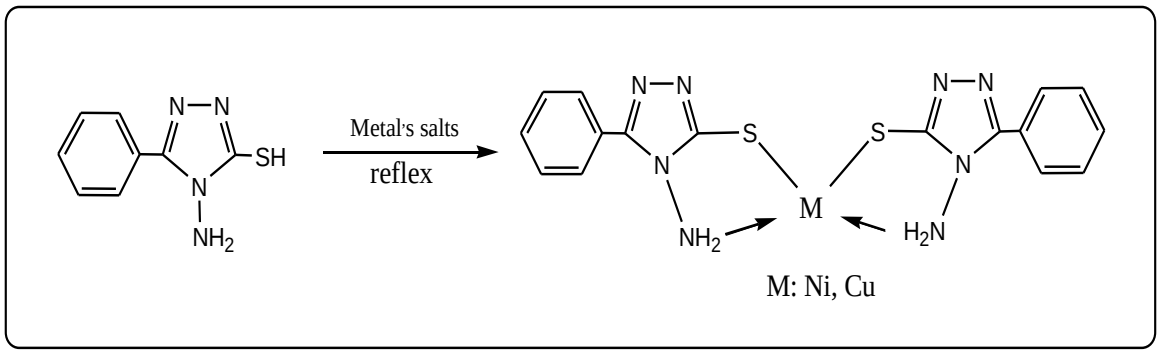
Cupper(II) acetate (0.23 g, 0.001 mol) or (0.37 g, 0.001 mol) from Nickel (II) chlori was dissolved in (5 mL) ethanol and was added to (5 mL) ethanolic solution of (0.5 g, 0.002 mol) from ligand.

The molar ratio [2: 1] from [ligand: metal] was carried out. After reflex for 3 hrs., crystalline colored precipitate was formed.

After that the precipitate was filtered and dried at room temperature. Then the precipitate was washed by hot methanol to yield a purified precipitate.

M.P (240-242) C°, Yield: 50 %, color: pale yellow of compound A1(Ni complex)

M.P (222-224) C°, Yield: 52 %, color: pale yellow of compound A2(Cu complex)



2.4.6. Synthesis of the complexes with compound B as ligand[124]

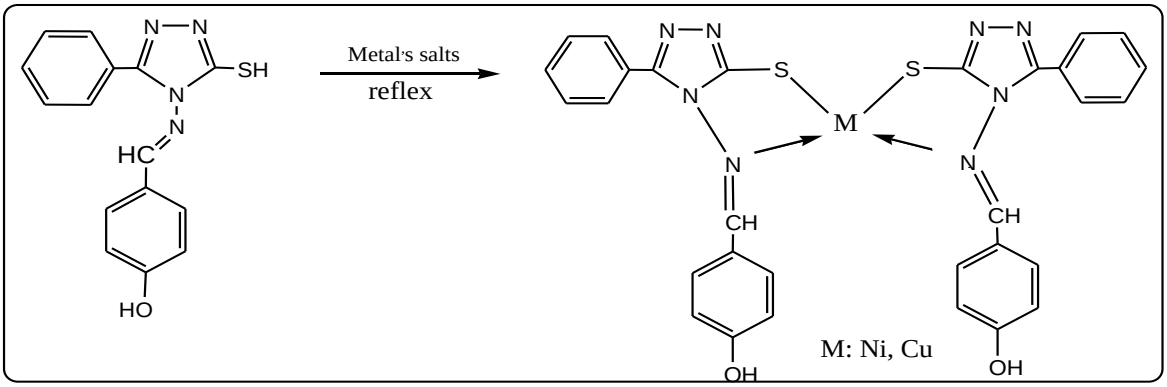
Cupper(II) acetate (0.15 g, 0.0005 mol) or (0.2 g, 0.0005 mol) from Nickel (II) chloride was dissolved in (5 mL) ethanol and was added to (5 mL) ethanolic solution of (0.5 g, 0.001 mol) from ligand.

The molar ratio [2: 1] from [ligand: metal] was carried out. After reflex for 3 hrs., crystalline colored precipitate was formed.

After that the precipitate was filtered and dried at room temperature. Then the precipitate was washed by hot methanol to yield a purified precipitate.

M.P (260-262) C°, Yield: 51 %, color: pale yellow of compound B1(Ni complex)

M.P (257-259) C°, Yield: 53 %, color: pale yellow of compound B2(Cu complex)



2.5.Biological activity

2.5.1.Antimicrobial activity

In this study, the synthesized compounds were evaluated for their in vitro antimicrobial activity against the pathogenic bacteria the gram negative bacteria species was Proteus mirabilis. This microorganism was supplied by Biology department / Baghdad University.

2.5.2. Identification of bacteria

This was achieved by following steps for identification the bacteria by appearance of colonies (culture morphology) and by Indole test

2.5.2.1. Primary identification

Colonies of the bacteria were identified primarily depending on culture characters as seen on the MacConkey agar, and to notice: swarming phenomenon clearly on the blood agar [125].

2.5.2.2. Indole test[126]

The indole test is a biochemical test performed on bacterial species to determine the ability of the organism to convert tryptophan into indole. Pure bacterial culture must be grown in sterile tryptophan for 24–48 hrs. before performing the test. Following incubation, 5 drops of Kovac's reagent (isoamyl alcohol, para-Dimethyl amino benzaldehyde and concentrated hydrochloric acid) was added to the culture broth. A positive result was indicated by the presence of a red or red-violet color in the surface of the alcohol layer of the broth. While a negative result appears yellow.

2.5.2.3 Urease production test

One of the most important tests for Proteus is a urease production test, according to the following steps:

1-The tube of urea broth base (containing urea and pH indicator as phenol red that is yellow-orange at the initial pH of 6.8 but change to pinkish-red at pH 8.4) was inoculated by the taken isolate using a sterile cotton swab.

2- The tube was incubated at 35 C^0 for 24 hrs. before examination for color change to distinguish species of Proteus [127]. As in following Figure (2-1):

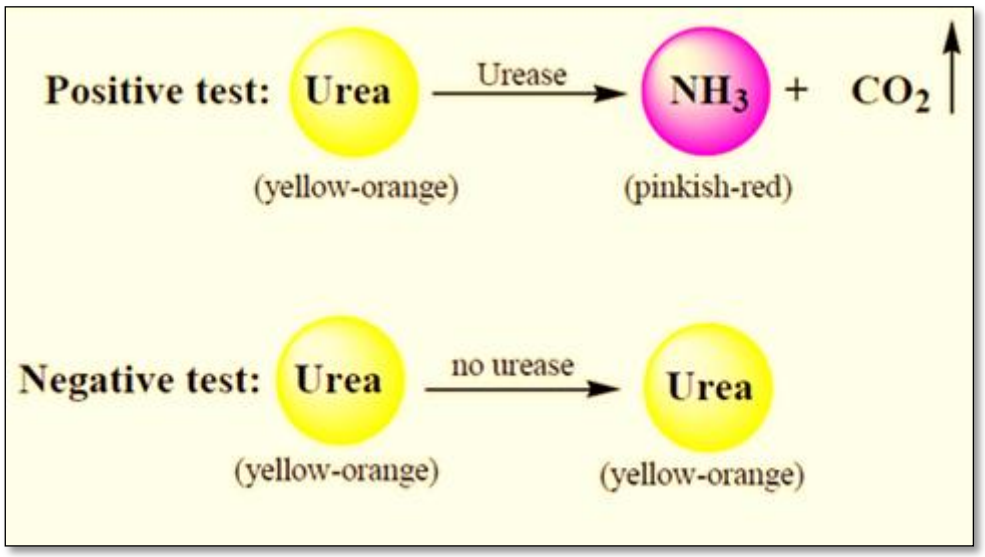


Figure (2-1) Urea utilization test results [128]

2.5.3. Bacterial Sensitivity Test of the Prepared Compounds

Well diffusion method was used to determine the inhibiting power of the prepared compounds against the pathogenic bacteria[129,130]. Tetracycline (TE) was used to compare the power of inhibition.

2.5.3.1. Well- Diffusion Method

This method depends upon the variation in concentration of the prepared compounds. A series of concentrations were made from the already prepared compounds with the range from (5,10, 25, 50 and 100 µg/ml). The following steps were done to measure the biological activity:

1. Nutrient broth and Mueller Hinton preparation

Nutrient broth and Mueller Hinton were prepared by dissolving (28 g.) Mueller Hinton in (1 litter) distilled water and sterilized by autoclaving at 121°C/ 1.5 atmosphere for 15 min. Then it was cooled to (40-45) min. Then put it in plate (20 mL) Mueller Hinton for each plate. Then left at room temperature till it became solid. The plates were placed in an incubator at 37°C for 24 hrs. to ensure that plates are sterilized and to get rid of excess humidity. The plates were kept in refrigerator till used.

2. Stimulation of Bacterial isolates

A touch of colony was taken by a loop to the test tube that contains 5 mL of the sterilized nutrient broth; it was shaken well and incubated in incubator for 24 hrs. at 37°C. The loop was sterilized by a flame before using so that the planted bacteria were not contaminated.

3. Bacterial Culture

Medium Inoculated bacteria suspension were diluted by 1/100 by using normal-saline solution with concentration of (0.85%) to prevent crowded growth. (0.1 mL) of bacteria diluted suspension was transported to each plate and spread by using sterilized cotton spreader on test medium surface. The Plates were left for (15-20) min. at 37°C to dry.

4. Well Distribution over the Plate

The plates of Mueller Hinton agar were inoculated by Proteus bacteria , by taking a swab and put it in bacterial solution and then spread it on a surface of medium and left for 10-15 min. . In each medium four pores were made by the use of a sterile dry rod with a diameter of 5 mm , these pores were made with equal distance between each other, and also between pores and plate margins by the use of a prepared diagram on a paper to be put under the dish then the

solutions of different concentrations of the prepared compounds were added using fixed amount of 0.5 mL from each concentration in one pore , and then these plates were incubated at $37C^0$ for 24 hrs. finally the inhibition diameter was measured for each pore using a ruler in a millimeter (mm). The zone of inhibition is defined as the translucent area which surrounds the disc including the diameter of the disc that lacks bacterial growth.

2.6. Determination of Urease Activity for Proteus mirabilis by Indophenols assay

Reagents

These reagents were prepared as they were mentioned in (131,132) .

a. Reagent A (Indophenol solution)

Phenol (5g) and sodium nitroprusside (0.025g) were dissolved in (500 mL) distilled water.

b. Reagent B (Hypochlorite solution)

Sodium hydroxide (2.5g) and sodium hypochlorite (4.2mL) were dissolved in (500 mL) of distilled water.

c. Urea (stock solution)

Urea (0.3g) was dissolved in 10 mL distilled water it was freshly prepared and used.

d. Buffer solution (Phosphate solution)

These reagents were prepared as they were mentioned in (133)

Mixed in volumetric flask (0.174 g) of K_2HPO_4 and (0.0185 g, 1 mM) of EDTA and were dissolved in (50 mL) of distilled water. The pH was fixed at 7.5 (7.5 is the optimum pH for the urease activity).

2.6.1. Measurement of Urease activity with inhibitors by Indophenols assay

Different concentrations of the prepared compounds (10, 25, 50, and 100) µM dissolved in (DMSO) were used with constant substrate concentration to know the rate of inhibition and then calculate IC_{50} values. The following scheme describe the procedure performed in this worke.

Inhibition Rate = ((Abs. at 625nm of control – Abs. at 625nm of test)/ Abs. at 625nm of control) *100

Abs. = Absorbance

	Sample µL	**Blank µL**	**Enzyme µL**
Buffer pH=7.5	**205**	**215**	**215**
Inhibitor	**10**	**10**	**_**
Urea	**25**	**25**	**25**

Incubate in water bath at 37C° for (10 min).

Enzyme	**10**	**-**	**10**

Incubate in water bath at 37C° for (15 minute).

Reagent A	**5ml**	**5ml**	**5ml**

It should be well shaken.

Reagent B	**5ml**	**5ml**	**5ml**

It should be well shaken.

Then it was incubated in water bath at 37C° for (20 min.).later on the absorbance was read at 625 nm; the enzyme activity and inhibition percentage were calculated.

2.6.2. Preparation of different substrate (urea) concentration

The (1, 5, 10, 20 and 30 mM) urea concentrations were prepared from serial dilution of the 500 mM of stoke solution (0.3 g urea in 10 mL distilled water) the concentration of the highest inhibition percentage of the previous step was used. This step was used to identify the kind of the inhibition to calculate Michaelis constant for enzyme (K_m), Maximum velocity for enzyme reaction (V_{max}) and Strength of inhibitor (K_i) values by the Line weaver Burk equation. The following described the steps were used to measure the enzyme activity with inhibitor and without inhibitors.

2.6.2.1. Measurement the activity without the inhibitors (This is Control)

(Different concentrations of substrate)

	Sample µL	**Blank µL**
Buffer pH=7.5	**215**	**225**
Urea	**25**	**25** **(Maximum of concentration)**
Enzyme	**10**	**-**

Incubate in water bath at 37C° for (10 min).

Reagent A	**5ml**	**5ml**

It should be well shaken.

Reagent B	**5ml**	**5ml**

It should be well shaken.

It was incubated in water bath at 37°C for (20 min.).Then the absorbance was read at 625 nm by spectrophotometer. The enzymatic activity was calculated and the relations between urea concentrations and enzyme activity were drew.

2.6.2.2 The Method with inhibitor

(different concentrations of substrate)

	Sample µL	**Blank µL**
Buffer pH=7.5	**205**	**215**
Inhibitor **(Concentration of maximum inhibition)**	**10**	**10**
Urea	**25**	**25** **(Maximum of concentration)**

Incubate in water bath at 37Cº for (10 minute).

Enzyme	**10**	**-**

Incubate in water bath at 37Cº for (15 minute).

Reagent A	**5ml**	**5ml**

It should be well shaken.

Reagent B	**5ml**	**5ml**

It should be well shaken.

It was incubated in water bath at 37°C for (20 min.).Then the absorbance was read at 625 nm by spectrophotometer. The enzymatic activity was calculate and the relations between Urea concentrations and enzyme activity were drew.

2.7 Calculations

1. From the linear Line weaver Burk equation, V_{max} and K_m were calculated [134]

$$1/V = (K_m/V_{max}) \times 1/[S] + 1/V_{max} \quad \text{.....(2-1)}$$

V=velocity of the enzyme reaction (µmole/min/ml)

K_m=Michaelis constant for enzyme (mM)

S= substrate concentration (mM)

V_{max}= Maximum velocity for enzyme reaction (µmole/min/ml)

2. Calculation K_i value for Competitive and noncompetitive inhibitors by the following equation: [134]

$$K_{m\ app.} = K_m\ (1+ [I]/K_i) \text{..... Competitive inhibitor}$$

$$V_{max\ i} = V_{max} / (1+ [I]/K_i) \text{..... non-Competitive inhibitor} \quad \text{.....(2-2)}$$

$K_{m\ app.}$ = Michaelis constant for inhibitor (mM)

K_m=Michaelis constant for enzyme (mM)

[I]= Inhibitor concentration (mM)

K_i= Strength of inhibitor (mM)

3. Calculation according to the following equation[134]

$$\% i = 100(1-a) \quad \text{……..(2-3)}$$

%i=degree of inhibition as a percent

a= relative activity as fraction =Vi/V◦

Vi and V◦ were calculated by :

$V_o = [S]\ V_{max} / K_m + [S]$……. For enzyme initial velocity

$Vi = [S]\ V_{max} / ([S]+K_m)\ (1+[I]/K_i)$ …….initial velocity of non- compotitive inhibitor

$Vi = [S]\ V_{max} / K_m\ (1+ [I]/K_i) + [S]$…….. initial velocity of compotitive inhibitor

...(2-4)

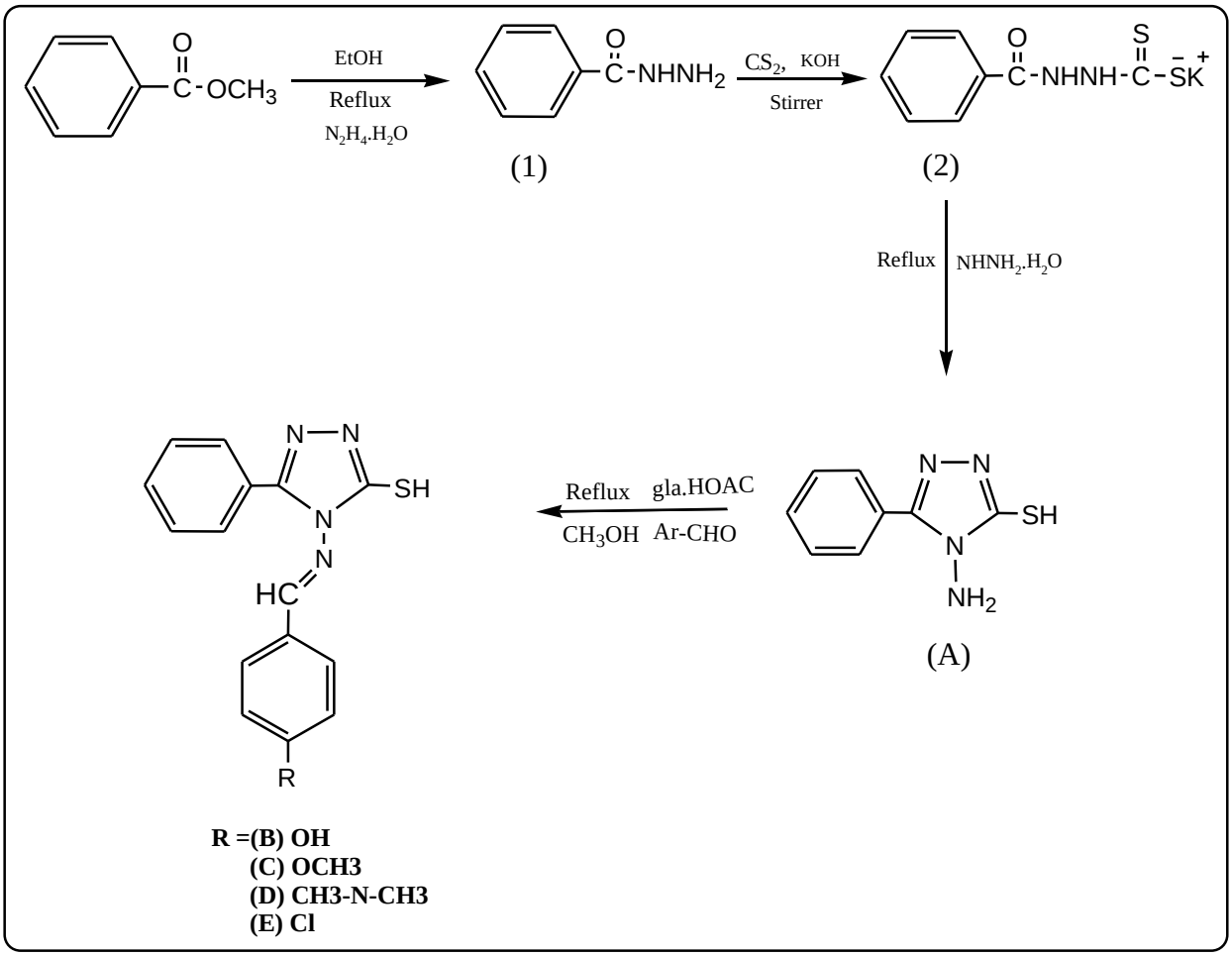
CHAPTER THREE

RESULTS

& DISCUSSION

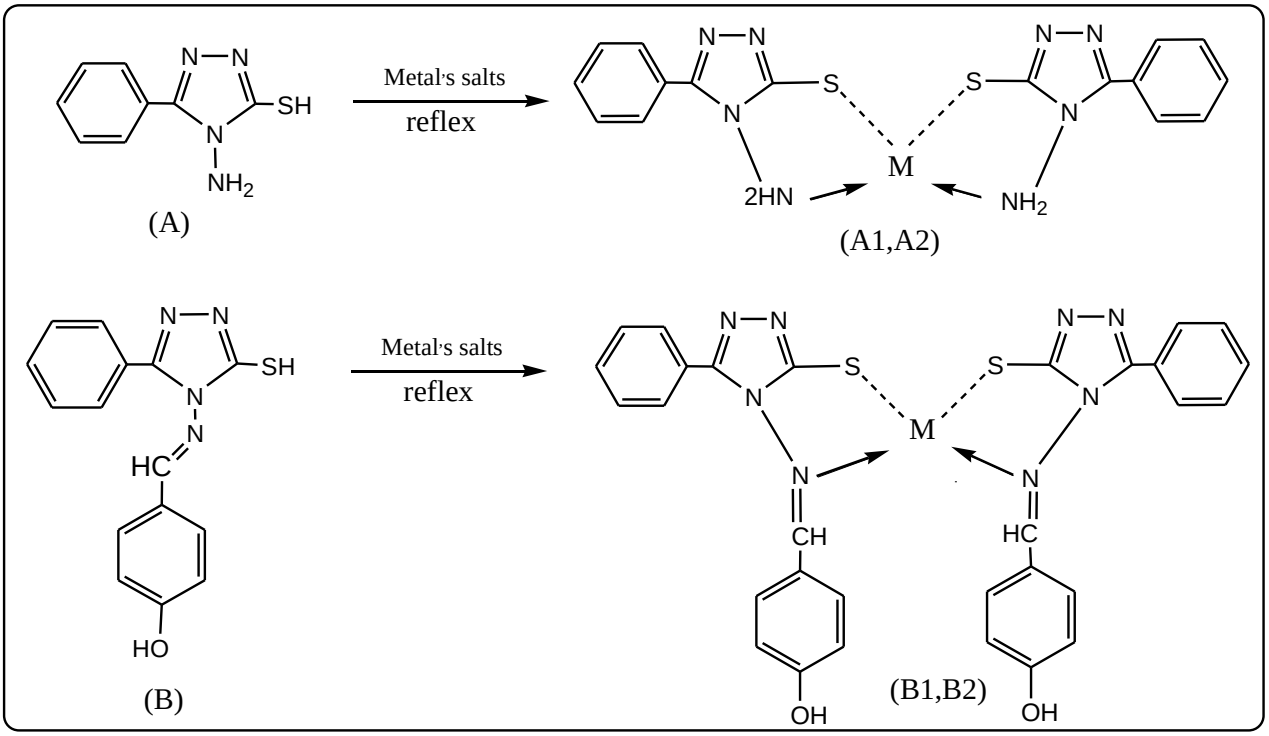
3.1 Identification of the prepared compounds

The Chemical steps for synthesis of compounds (A,B,C,D,E) are shown in Scheme (3-1).



Scheme (3-1) The chemical steps for synthesis of Compounds (A, B, C, D and E)

The steps of preparation of the complexes can be shown in Scheme (3-2):



Scheme (3-2) The chemical steps for synthesis of Complexes

3.1.1 The Physical properties of prepared compounds

Melting points and physical properties of all the compounds studied are tabulated in Table (3-1). The data of CHNS were obtained using flame atomic absorption technique. The calculated values were in a good agreement with the experimental values.

Table (3-1) Physical data(color, melting point and elemental analysis) of prepared compounds

Compounds	Color	M.P. C°		Elemental analysis theoretical (Experimental)					
			% C	% H	% N	% S	%O	%Cl	% M
A	White	198-201	50 (49.23)	4.16 (5.29)	29.16 (30.07)	16.66 (15.41)	-		-
$Ni(L)_2$ A 1	Green	240-242	43.56 (43.15)	3.17 (4.29)	25.42 (24.12)	14.52 (13.82)	-	-	13.31 (14.62)
$Cu(L)_2$ A 2	Dark green	222-224	43.09 (44.15)	3.14 (2.45)	25.14 (24.24)	14.36 (15.98)	-	-	14.25 (13.18)
B	Pale yellow	250-253	60.81 (59.75)	4.05 (5.30)	18.91 (19.80)	10.81 (10.32)	5.40 (4,83)	-	-
$Ni(L)_2$ B1	Green	260-262	55.51 (54.23)	3.39 (4.67)	17.26 (16.90)	9.68 (10.31)	4.93 (5.11)	-	9.04 (8.77)
$Cu(L)_2$ B2	Dark green	257-259	55.08 (55.10)	3.36 (4.43)	17.14 (17.02)	9.91 (8.99)	4.89 (4.86)	-	9.71 (9.60)
C	Pale yellow	208-210	61.93 (61.58)	4.51 (3.17)	18.06 (18.77)	10.32 (11.30)	5.16 (5.18)	-	-
D	Red	180-182	63.15 (62.17)	5.26 (5.98)	21.67 (22.29)	9.90 (9.56)	-	-	-
E	yellow	211-213	57.23 (57.67)	3.49 (3.35)	17.81 (16.88)	10.17 (10.57)	-	11.28 (11.52)	-

M: Metal, L: Ligand

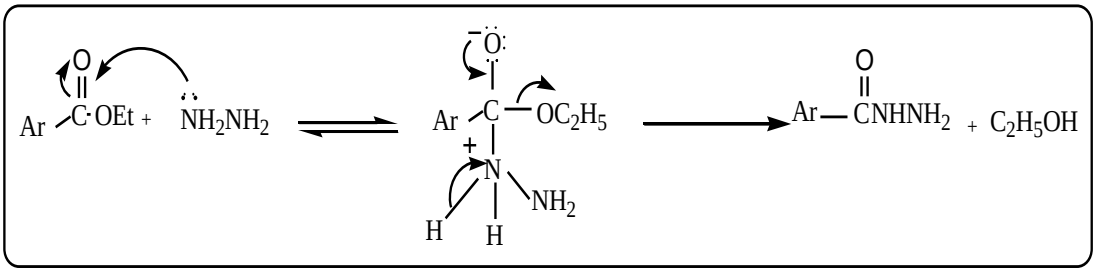
3.1.2 Characterization of the prepared compounds by Infra-Red spectroscopy

The infra-red spectrophotometer technique was used to characterize the prepared compounds through the assignment of stretching vibration bands.

3.1.2.1 Synthesis of benzoic acid hydrazide (1)

Benzoic acid hydrazide was prepared through the reaction of methyl benzoate with hydrazine hydrate in abs. ethanol. Compound (1) was characterized by its melting point and F.T.I.R spectroscopy.

The FTIR spectrum of this compound shows appearance of two stretching bands of NH_2 asymmetric and symmetric at (3301 and 3214cm^{-1}), carbonyl of amide group was also seen at 1661 cm^{-1}. FTIR Figure (3-2). The mechanism reaction for the synthesis of compound (1) was illustrated in scheme (3-3)



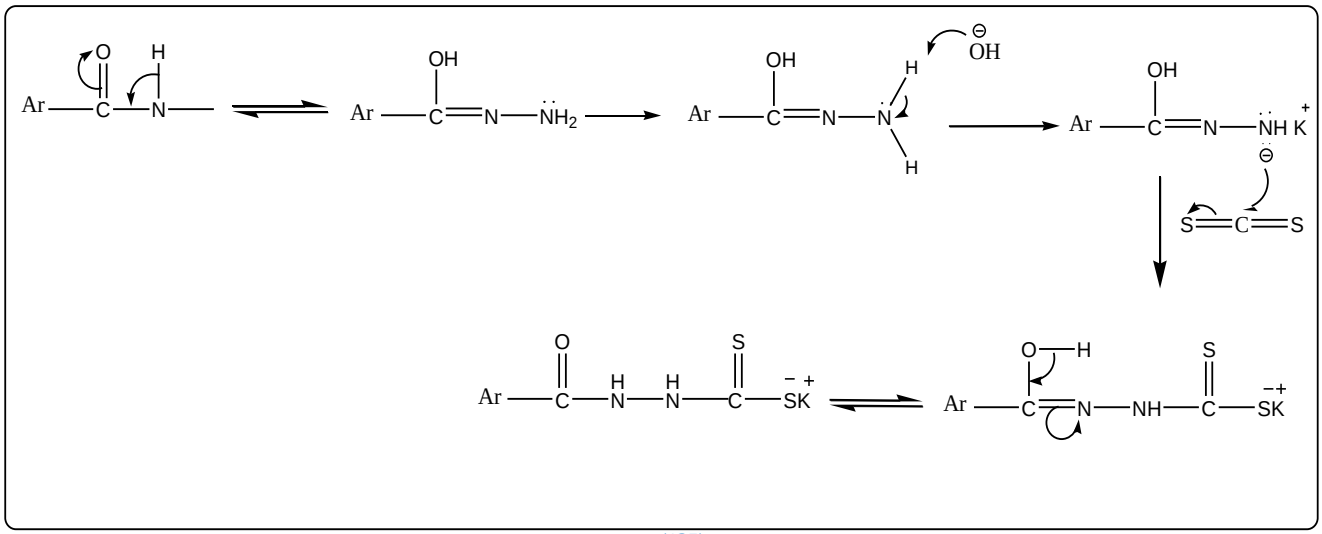
Scheme (3-3) The mechanism of the reaction [135]

3.1.2.2 Synthesis of potassium dithiocarbazinate (2)

Potassium dithiocarbazinate was prepared through the reaction of compound (1) with CS_2 in the presence of potassium hydroxide in abs. ethanol. Compound (2) was characterized by its melting point and F.T.I.R spectroscopy.

The FTIR spectrum shows a shifting in carbonyl group to 1631 cm^{-1} and appearance of C-S band at 600 cm^{-1}. FTIR Figure (3-3). The mechanism of the

synthesis reaction for the synthesis of compound (2) was illustrated in scheme (3-4).



Scheme (3-4) Mechanism of the reaction [135] for potassium salt formation

3.1.2.3 Synthesis of 4[amino]-5-phenyl-4H-1, 2, 4-triazole-3-thiol (A)

4[amino]-5-phenyl-4H-1, 2, 4-triazole-3-thiol was prepared through the reaction of compound (2) with Hydrazine hydrate was added and refluxed for 18-20 hrs.. Acidification of the solution by HCl produced a white precipitated .The compound was characterized by its melting point and FTIR spectrum.

The FTIR spectrum of the compound (A) showed some characteristic stretching bands at: 3244 and 3300, 2696, 1620, 663 assigned to NH_2, S-H, C=N of triazole ring, and the last one is for stretching of C-S bond, respectively[136,137]. FTIR Figure(3-3). In this spectrum there are two other characteristic bands at 3047.53cm^{-1} and 2696.48 cm^{-1} due to (N-H, thione form) and (S-H) stretching vibrations, respectively. This means That tatumerisum form could occur in triazole ring[138] is existing in the thiol and thione form Figure(3-1). The suggested mechanism for the cyclization of potassium salt is illustrated in Scheme (3-4).

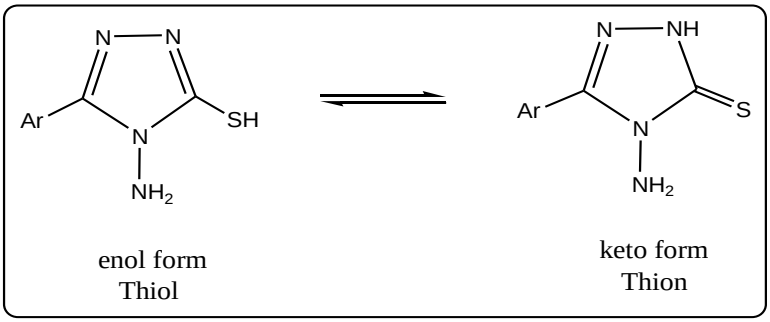
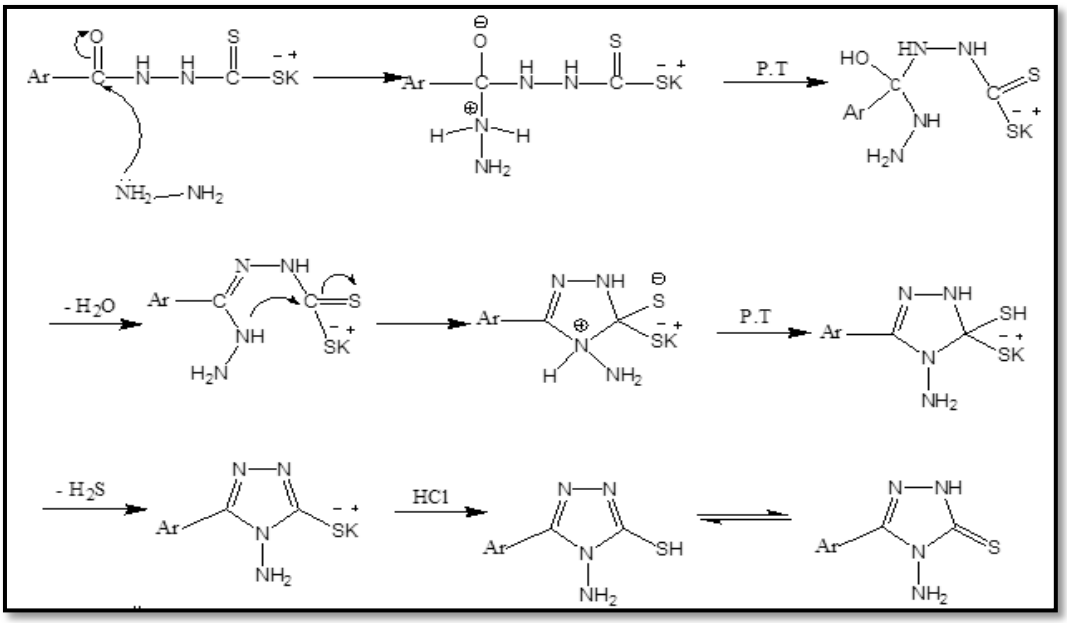


Figure (3-1) tatumerisum form in triazole[138]



Scheme (3-5) The suggested mechanism for the cyclization of potassium salt[135]

3.1.2.4 Synthesis of 4-[(3-Mercapto-5-phenyl-[1,2,4]triazol-4-ylimino)-methyl]-phenol(B)

The reaction of compound (A) with *p*-hydroxy benzaldehyde produced the imine (B). The compound was characterized by its melting point and FTIR spectrum.

The FTIR spectrum showed the disappearance of NH_2 stretching band of compound [A] at (3244cm^{-1} and 3300 cm^{-1}) ,and appearance of a broad weak band representing intramolecular hydrogen bonding of OH group at (3305cm^{-1}).

A weak band due to =CH stretching appeared at (3167 cm^{-1}), *v*(C=N) of the imine appeared at (1608 cm^{-1}). Bands of *v*(S-H) , *p*-substituted and *v*(C-S) appeared at (2692 cm^{-1}) ,(844 cm^{-1}) and (682 cm^{-1}) respectively. FTIR Figure (3-5).

3.1.2.5 Synthesis of 4-[(4-Methoxy-benzylidene)-amino]-5-phenyl-4H [1,2,4]triazole-3-thiol(C)

The reaction of compound (A) with *p*-methoxy benzaldehyde produced the imine (C). The compound was characterized by its melting point and FTIR spectrum.

The FTIR spectrum showed the disappearance of NH_2 stretching band of compound (A) at (3244cm^{-1} and 3300 cm^{-1}) ,and appearance of band due to =CH stretching appeared at (3186 cm^{-1}), *v*(C=N) of the imine appeared at (1624 cm^{-1}). Bands of *v*(S-H) , *p*-substituted , *v*(C-O) and *v*(C-S) appeared at (2607 cm^{-1}) ,(833cm^{-1}),(1165 cm^{-1}) and (621 cm^{-1}) respectively. FTIR Figure (3-6).

3.1.2.6 Synthesis of 4-[(4-Dimethylamino-benzylidene)-amino]-5-phenyl-4H-[1,2,4]triazole-3-thiol(D)

The reaction of compound (A) with *p*-Dimethylamino benzaldehyde produced the imine (D). The compound was characterized by its melting point and FTIR spectrum.

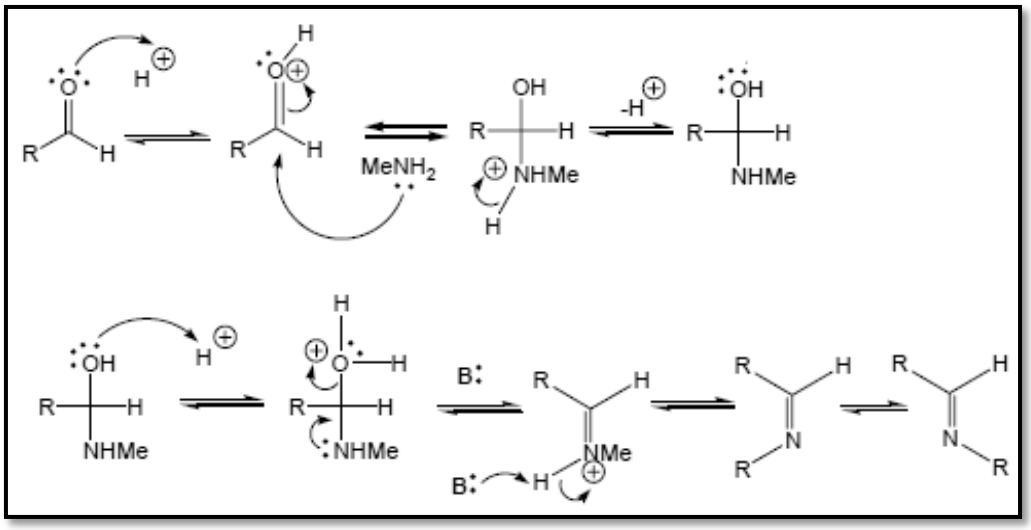
The FTIR spectrum showed the disappearance of NH_2 stretching band of compound (A) at (3244cm^{-1} and 3300 cm^{-1}) ,and appearance of band due to =CH stretching appeared at (3105 cm^{-1}), *v*(C=N) of the imine appeared at (1600 cm^{-1}). Asymmetric and symmetric absorption bands of aliphatic CH appeared at (2908cm^{-1}) and (2800cm^{-1}) respectively. Bands of *v*(S-H) , *p*-substituted and *v*(C-S) appeared at (2600 cm^{-1}) ,(810 cm^{-1}) and (682 cm^{-1}) respectively. FTIR Figure (3-7).

3.1.2.7 Synthesis of 4-[(4-Chloro-benzylidene)-amino]-5-phenyl-4H-[1,2,4]triazole-3-thiol(E)

The reaction of compound (A) with *p*-Chloro benzaldehyde produced the imine (E). The compound was characterized by its melting point and FTIR spectrum.

The FTIR spectrum showed the disappearance of NH_2 stretching band of compound (A) at (3244cm^{-1} and 3300 cm^{-1}) ,and appearance of band due to =CH stretching appeared at (3100 cm^{-1}), *v*(C=N) of the imine appeared at (1600 cm^{-1}). Bands of *v*(S-H) , *p*-substituted , *v*(C-Cl) and *v*(C-S) appeared at (2603 cm^{-1}) , (833 cm^{-1}),(524 cm^{-1}) and (617 cm^{-1}) respectively. FTIR Figure (3-8).

The reaction mechanism for the synthesis of compounds (B,C,D and E) was illustrated in Scheme (3-6).



Scheme (3-6) The reaction mechanism [139] for the synthesis of compounds (B,C,D and E)

3.1.2.8 Synthesis of complexes (A1,A2-B1,B2)

These complexes are produced from the reaction of compound (A) , compound (B) as ligand with Cupper(II) acetate or Nickel (II) chloride. The compounds were characterized by their melting points and FTIR spectrum.

The FTIR spectra of the complexes have been compared with those of the free ligand in order to determine the coordination sites that may get involved in chelation. By comparing, it was found that azomethine group frequency *ν*(N=CH) and amino group (NH_2) is present in the free ligands at 1620 cm-1, (3244-3300)cm−1, respectively . This band is shifted to the lower frequency by 5–20 cm−1 in the spectra of the complexes, indicating coordination of azomethine nitrogen and amino nitrogen towards the metal ion (M–N) [140-142]. The band of S-H in the ligand was disappeared in the spectra of the metal complexes indicating deprotonation and complexation through sulphur. The bands of C-S also shifted to the lower frequency due to complexation of the metal ion to the ligand through sulfate (M-S) [143,141,124]. the other bands such as C=C (1520-1620) cm^{-1}, C-H aromatic (3010-3080) cm^{-1} were didn't show any shifting because they are notw participate in the complexation [144]. FTIR Figure [(3-9)-(3-12)].

The major FTIR bands and their probable assignment are given in Table (3-2).

Table (3-2) key infrared data of complexes

Complexes	υ NH_2	υ C=N	υ C-S	υ M-N	υ M-S
A1(Ni)	3280, 3228	-	624	555	459
A2(Cu)	3286, 3233	-	609	540	432
B1(Ni)	-	1597	617	551	432
B2(Cu)	-	1600	632	524	455

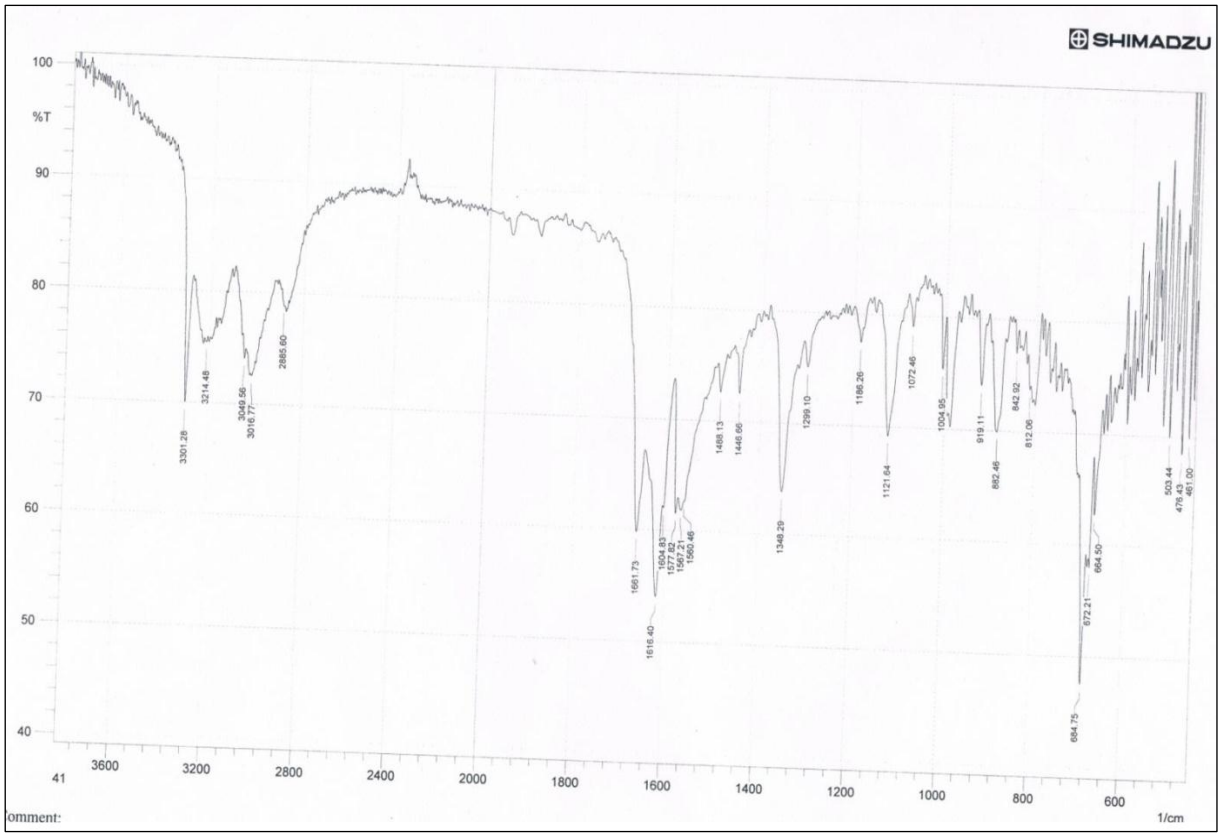


Figure (3-2) FTIR spectrum of compound (1)

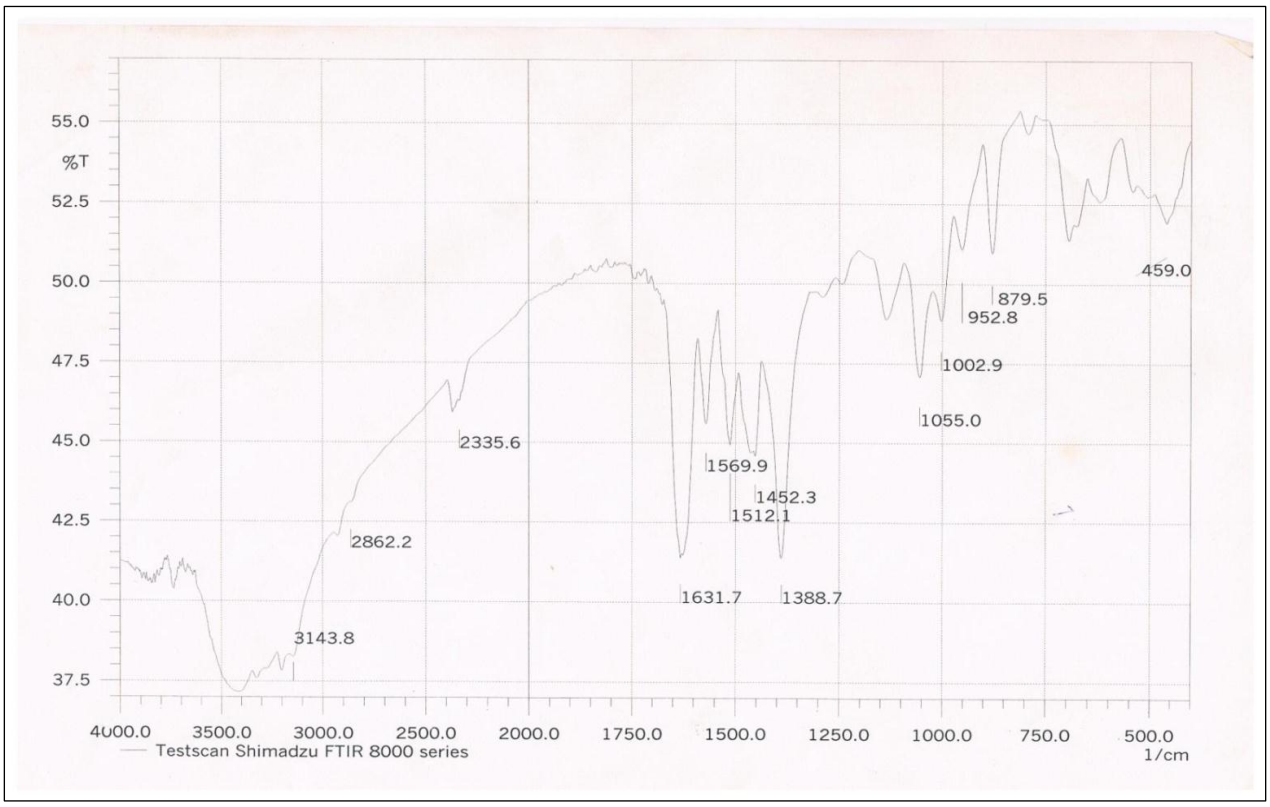


Figure (3-3) FTIR spectrum of compound (2)

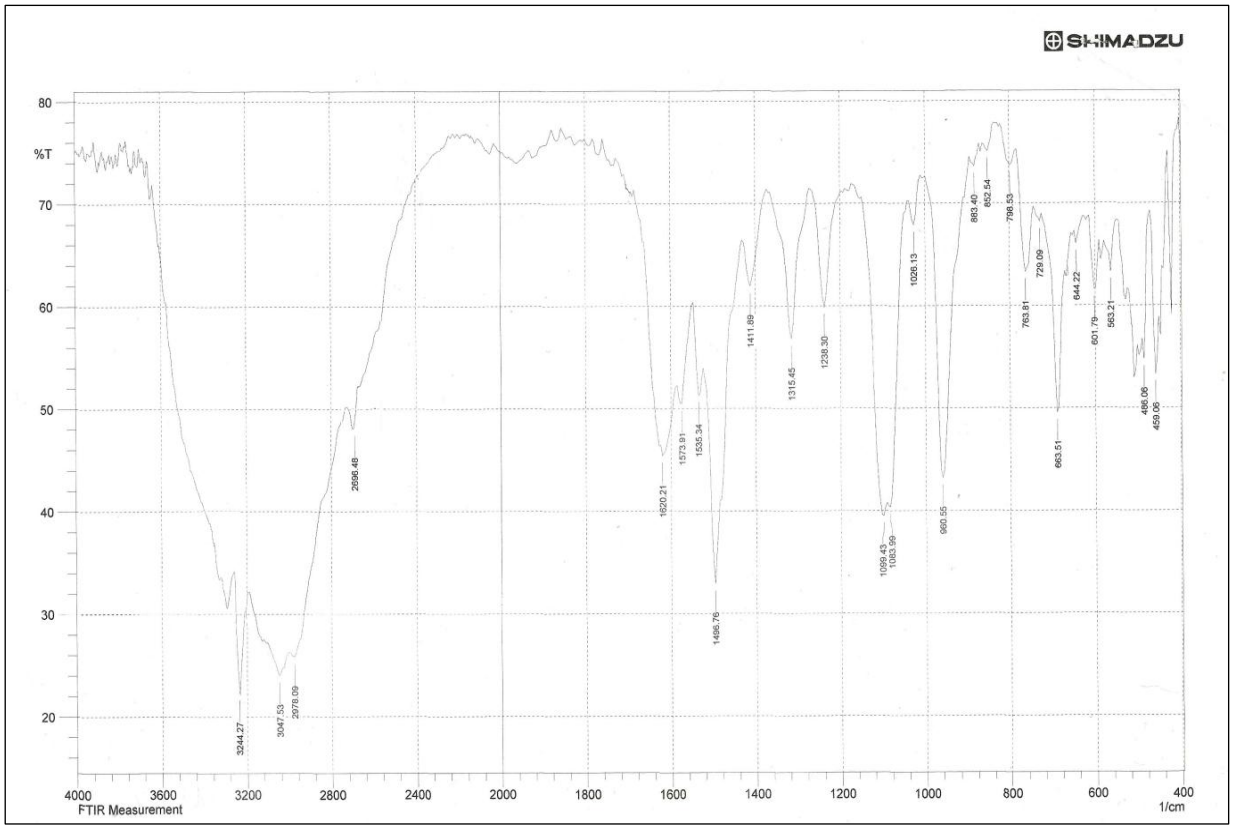


Figure (3-4) FTIR spectrum of compound (A)

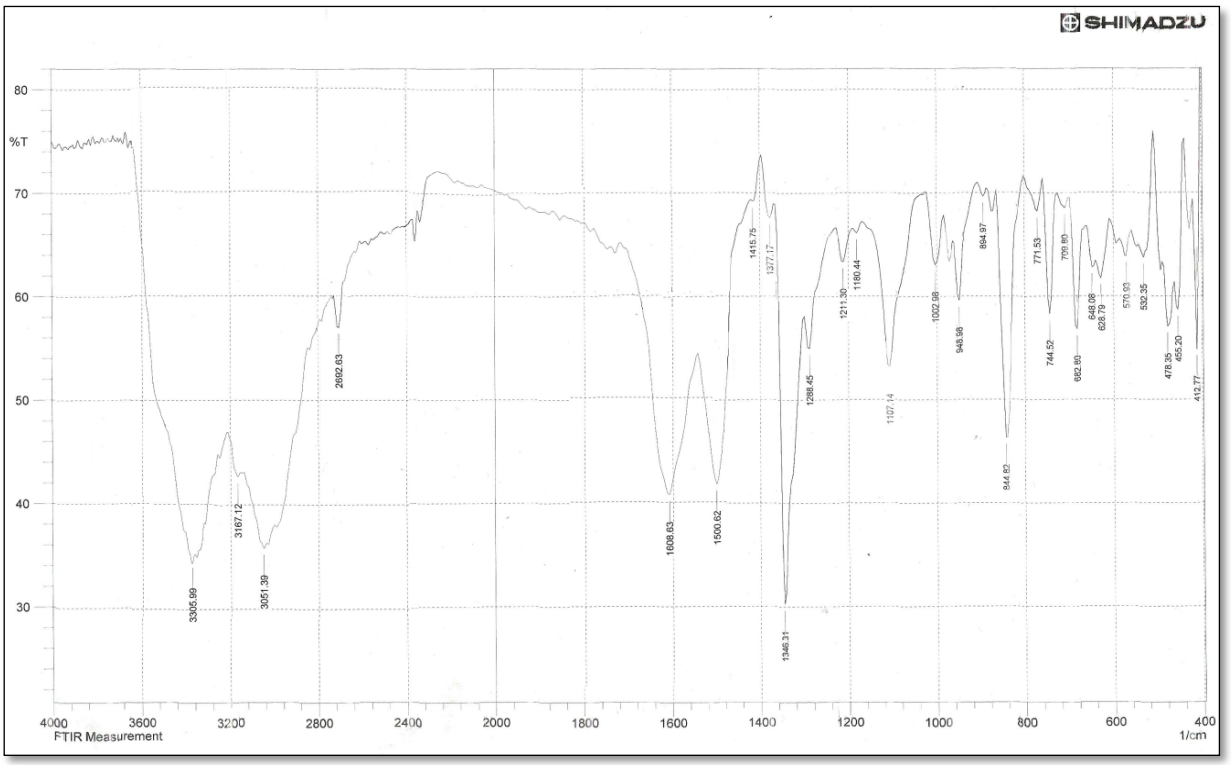


Figure (3-5) FTIR spectrum of compound (B)

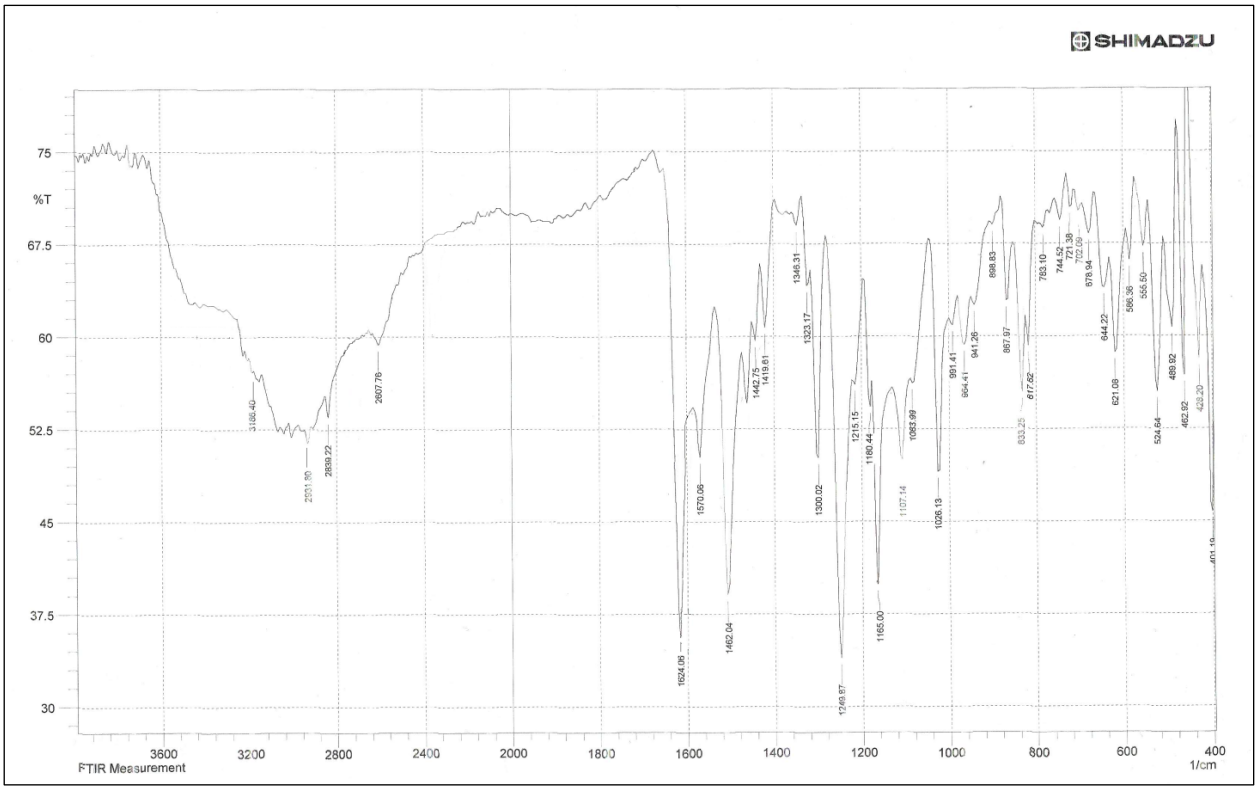


Figure (3-6) FTIR spectrum of compound (C)

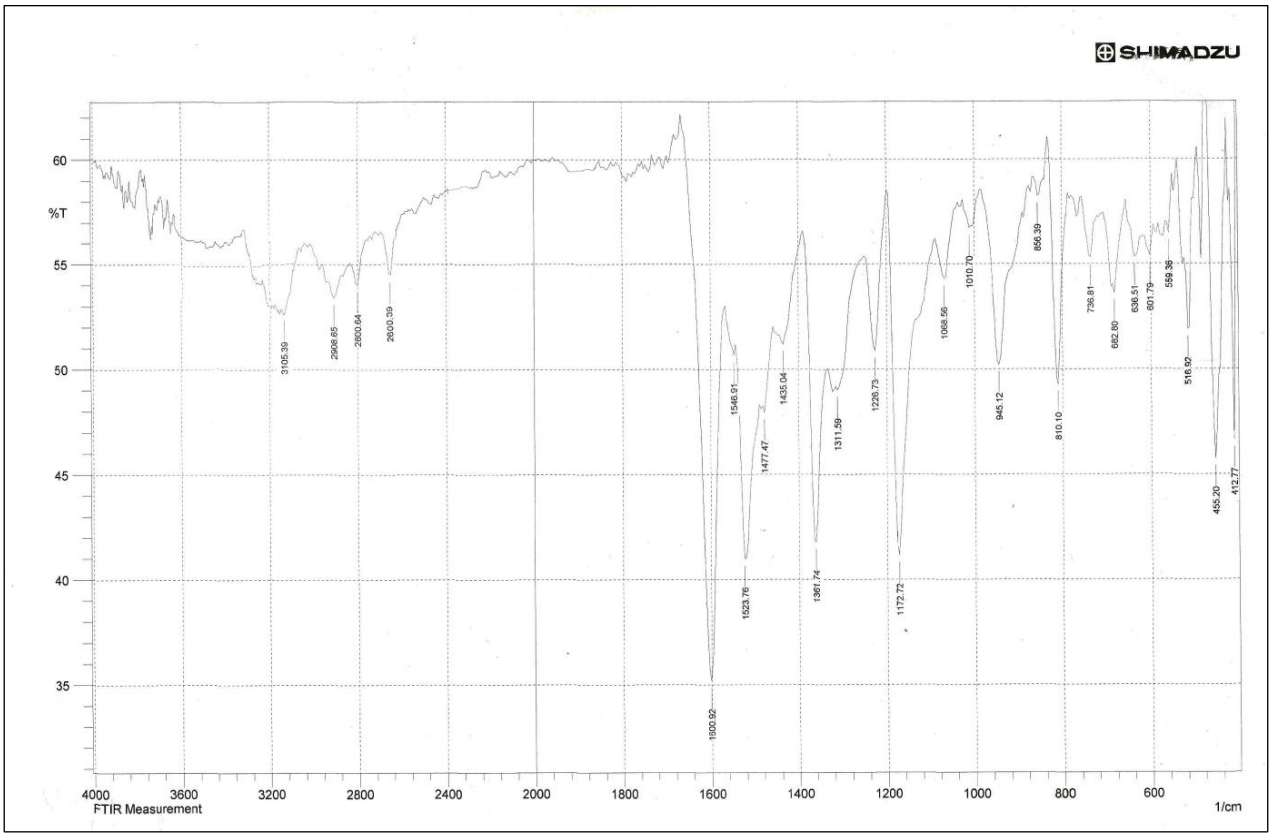


Figure (3-7) FTIR spectrum of compound (D)

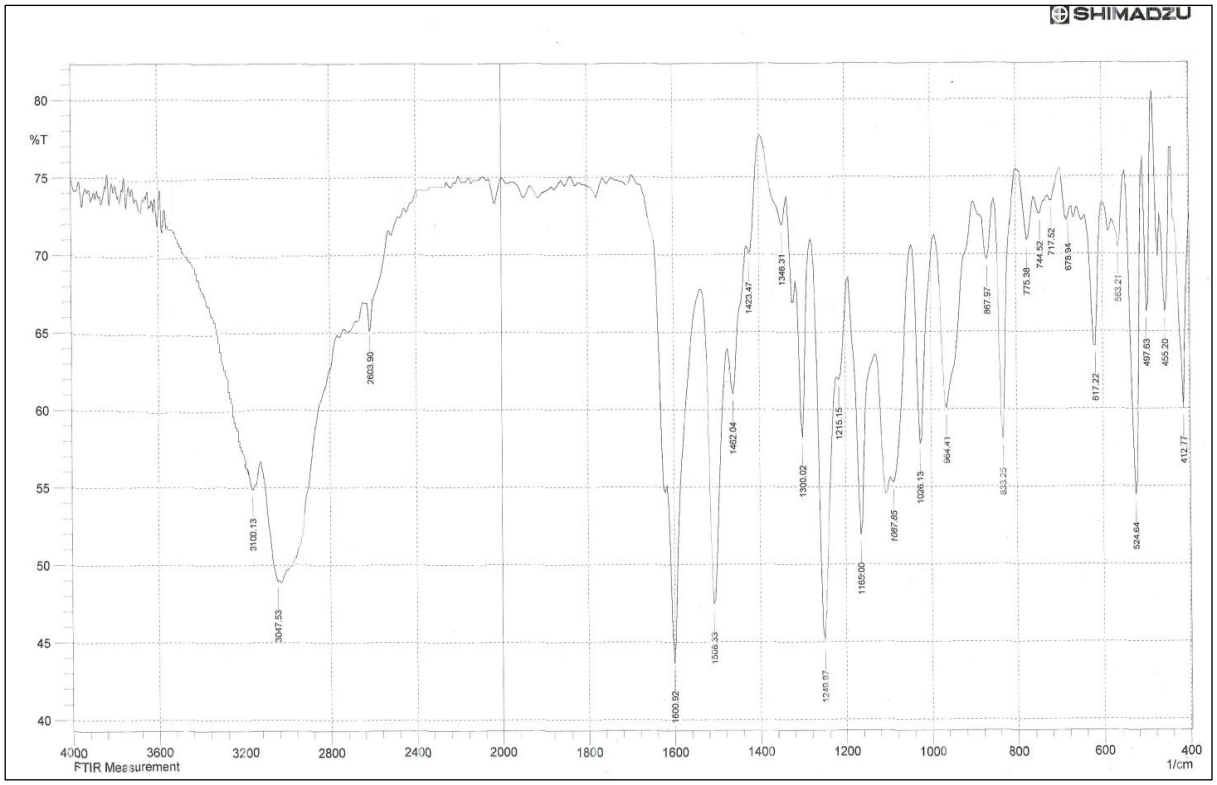


Figure (3-8) FTIR spectrum of compound (E)

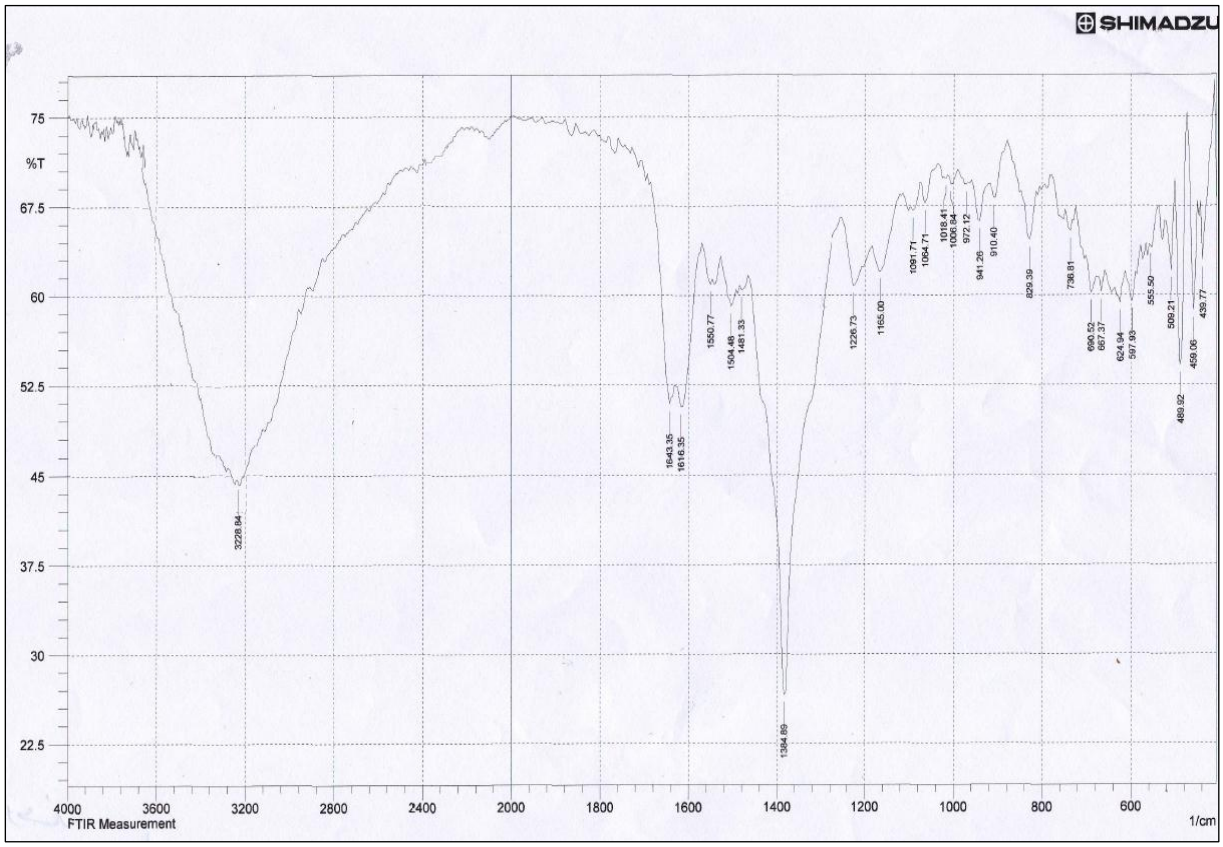


Figure (3-9) FTIR spectrum of compound (A1)

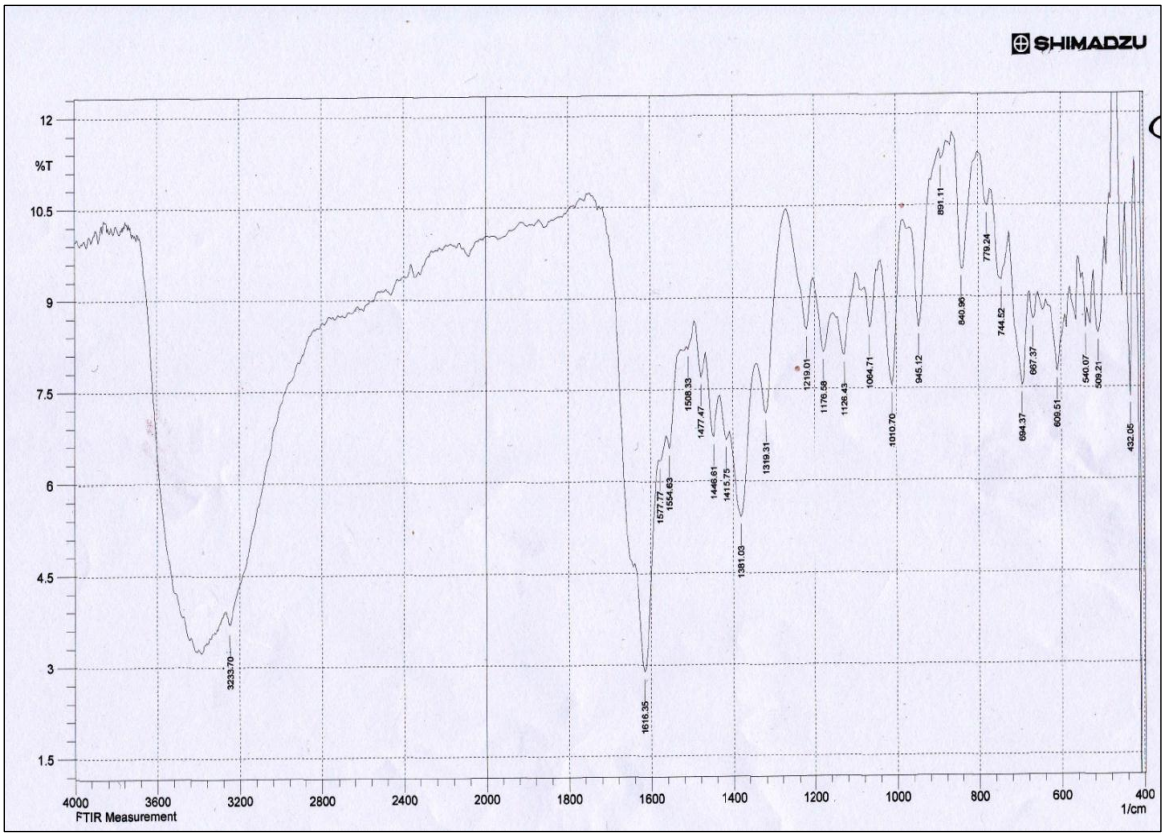


Figure (3-10) FTIR spectrum of compound (A2)

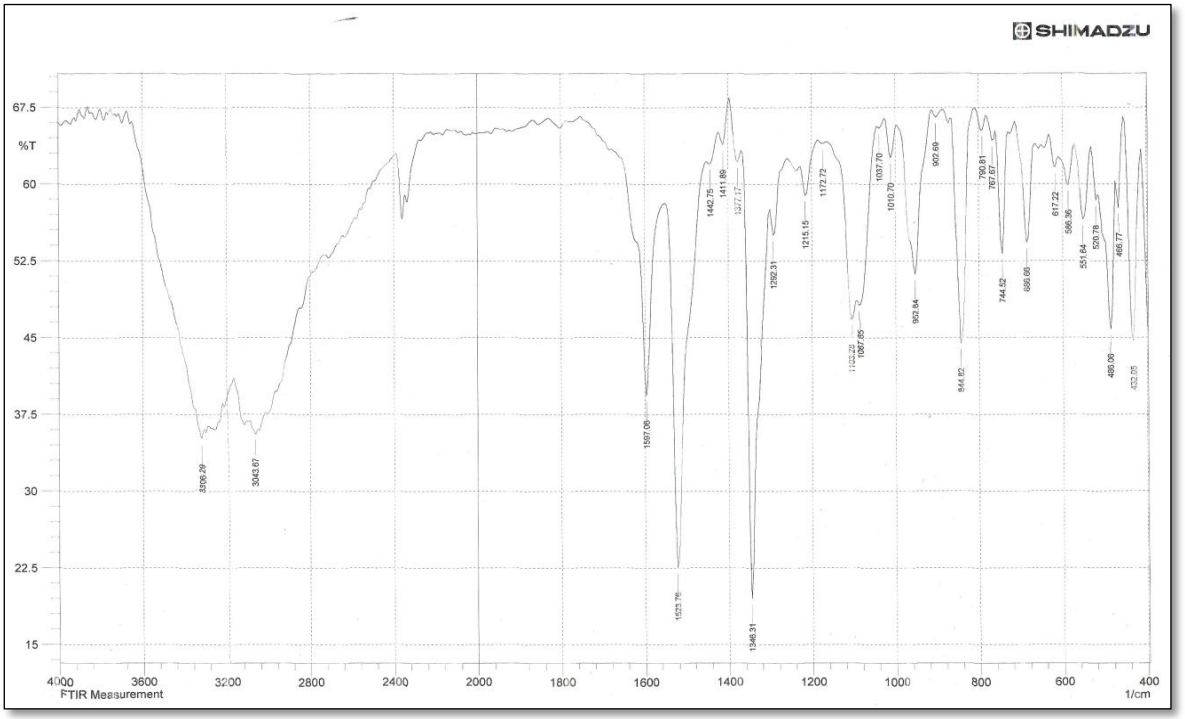


Figure (3-11) FTIR spectrum of compound (B1)

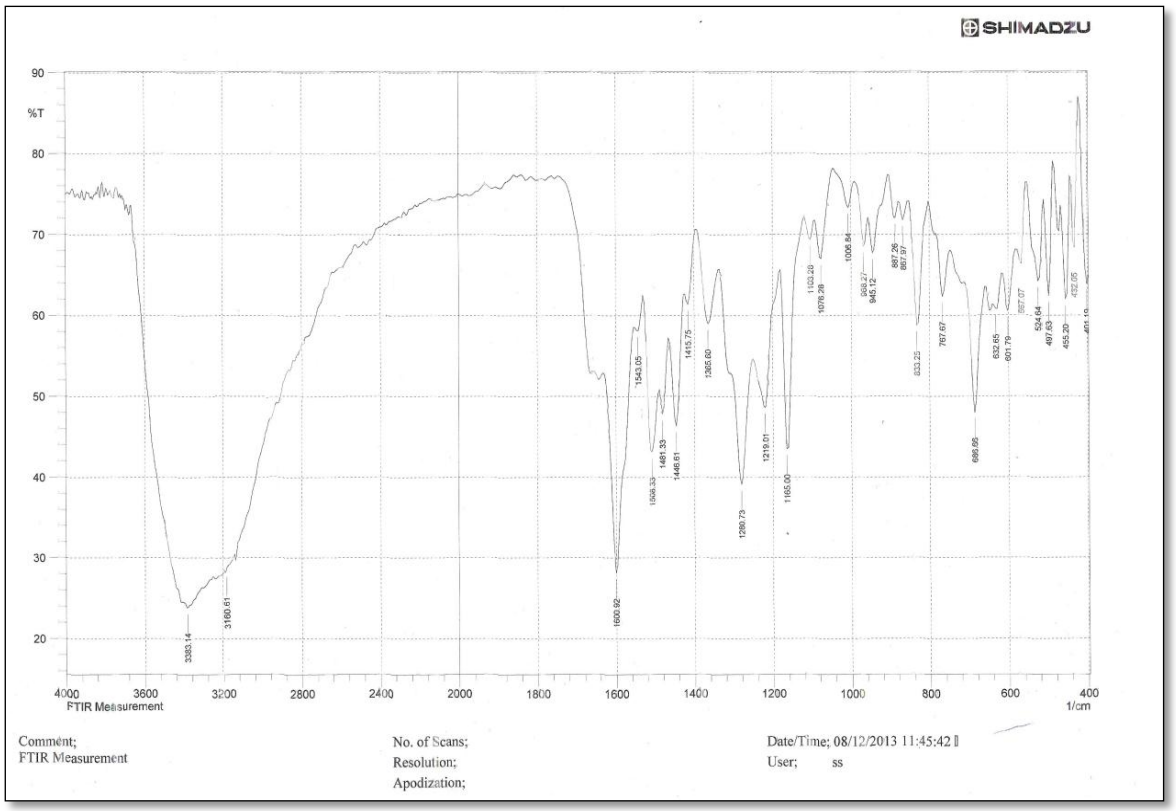


Figure (3-12) FTIR spectrum of compound (B2)

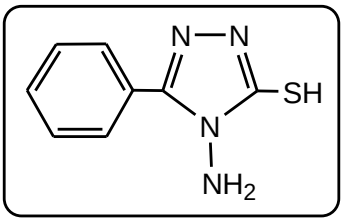
3.1.3 Characterization of prepared compounds by Nuclear magnetic resonance [145,146]

The data of ^{1}HNMR of 4[amino]-5-phenyl-4H-1, 2, 4-triazole-3-thiol and their derivatives and their complexes displayed good solubility in DMSO.

Note: for all ^{1}H NMR spectra, the peaks at 2.5 and 3.33 are for the solvent (DMSO-d6) and dissolved water in (DMSO-d6) respectively.

Note: (s = singlet, d = doublet and m= multiplet)

Compound A:



^{1}H NMR data (ppm), δ_{H}(300 MHz, DMSO-d6): Figure (3-13) shows signals at 5.276 (2H, s, NH_2), 7.567-7.825 (5H, m, CH aromatic ring) and 12. 852 (1H, s, SH).

Compound B:

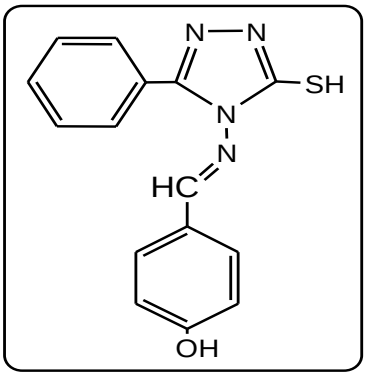


Figure (3-14) shows signals at 9.292 (1H, s, N=CH), 7.492-8.192 (9H, m, CH aromatic ring), 4.745 (1H, s, OH) and 13. 189 (1H, s, SH).

Compound C:

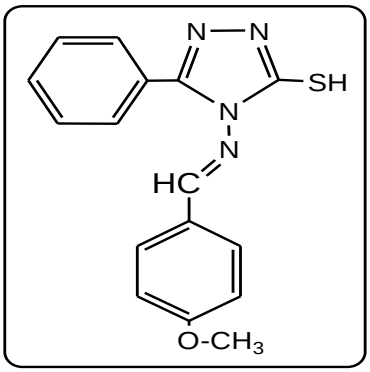


Figure (3-15) shows signals at 8.933 (1H, s, N=CH), 7.043-7.928 (9H, m, CH aromatic ring), 3.341 (3H, s,O-CH_3) and 13.311 (1H, s, SH).

Compound D:

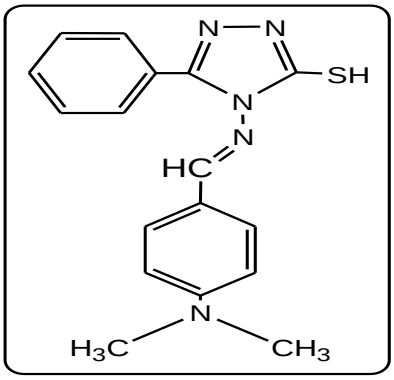


Figure (3-15) shows signals at 8.918 (1H, s, N=CH), 6.728-7.928 (9H, m, CH aromatic ring), 3.010 (6H, s, N-$(CH_3)_2$) and 13.258 (1H, s, SH).

Complex A1:

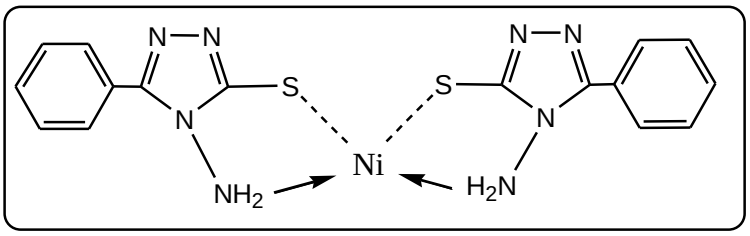


Figure (3-16) shows the signals at 3.367 (4H, s, NH_2) (this peak is shifted to lower field due to its attachment to the Nickel atom) and 7.620-7.956 (10H, m, CH aromatic ring).

Complex B1:

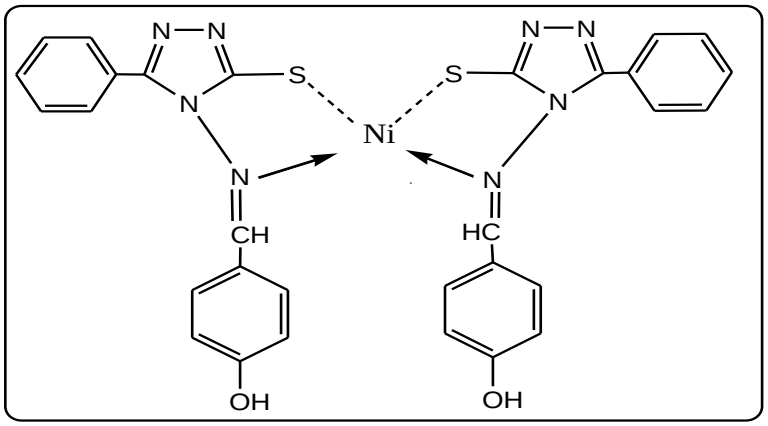


Figure (3-17) shows the signals at 8.992 (2H, s, N=CH) (this peak is shifted to lower field due to its attachment to the Nickel atom), 7.151-8.099 (18H, m, CH aromatic ring), 5.474(2H, s, OH) .

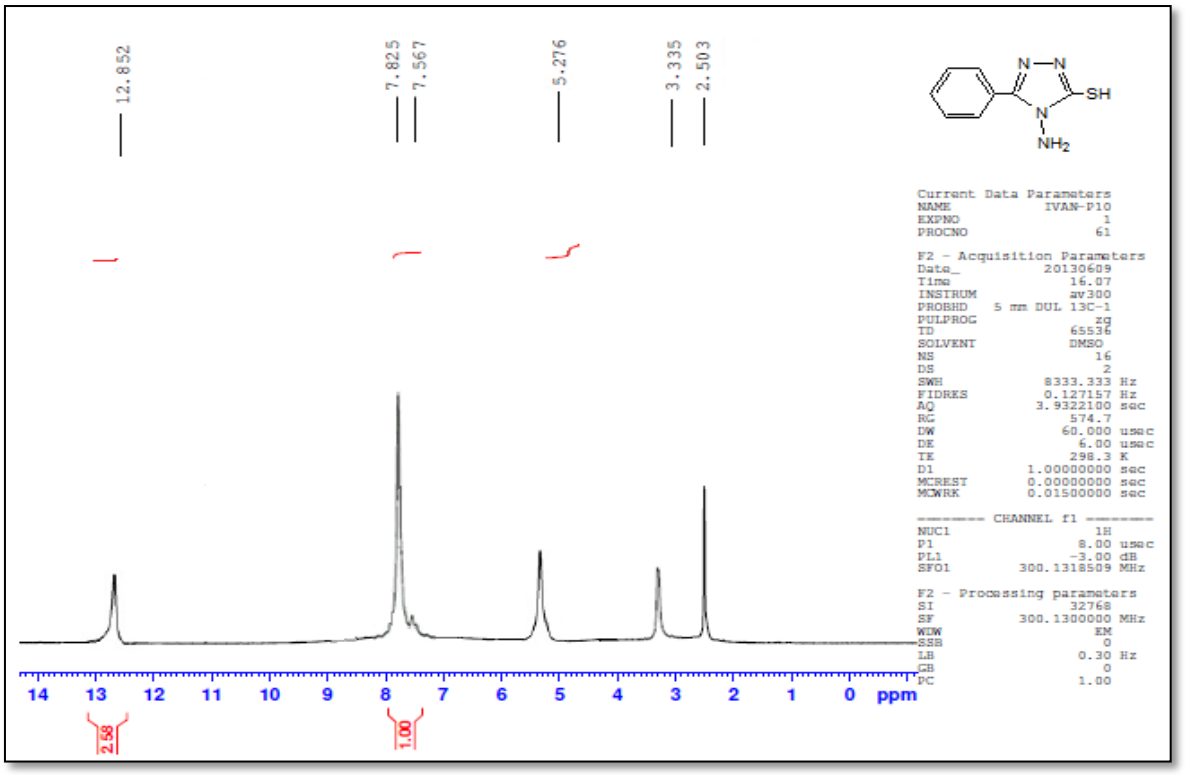


Figure (3-13) ^{1}H NMR spectrum of Compound (A)

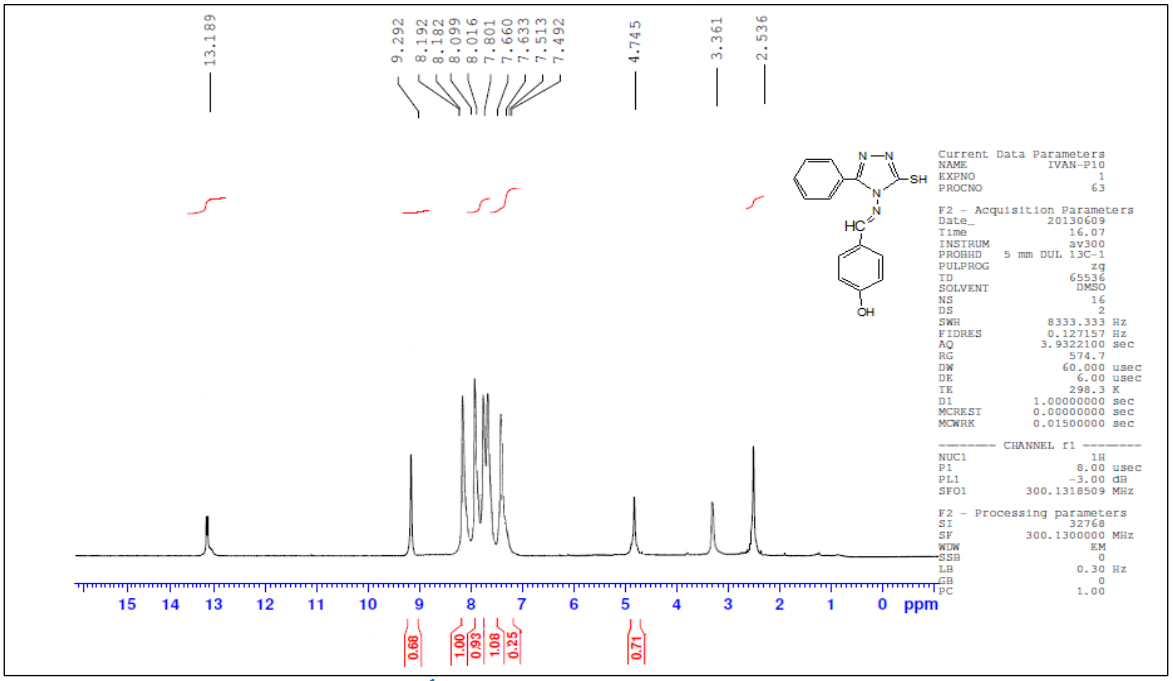


Figure (3-14) ^{1}H NMR spectrum of Compound (B)

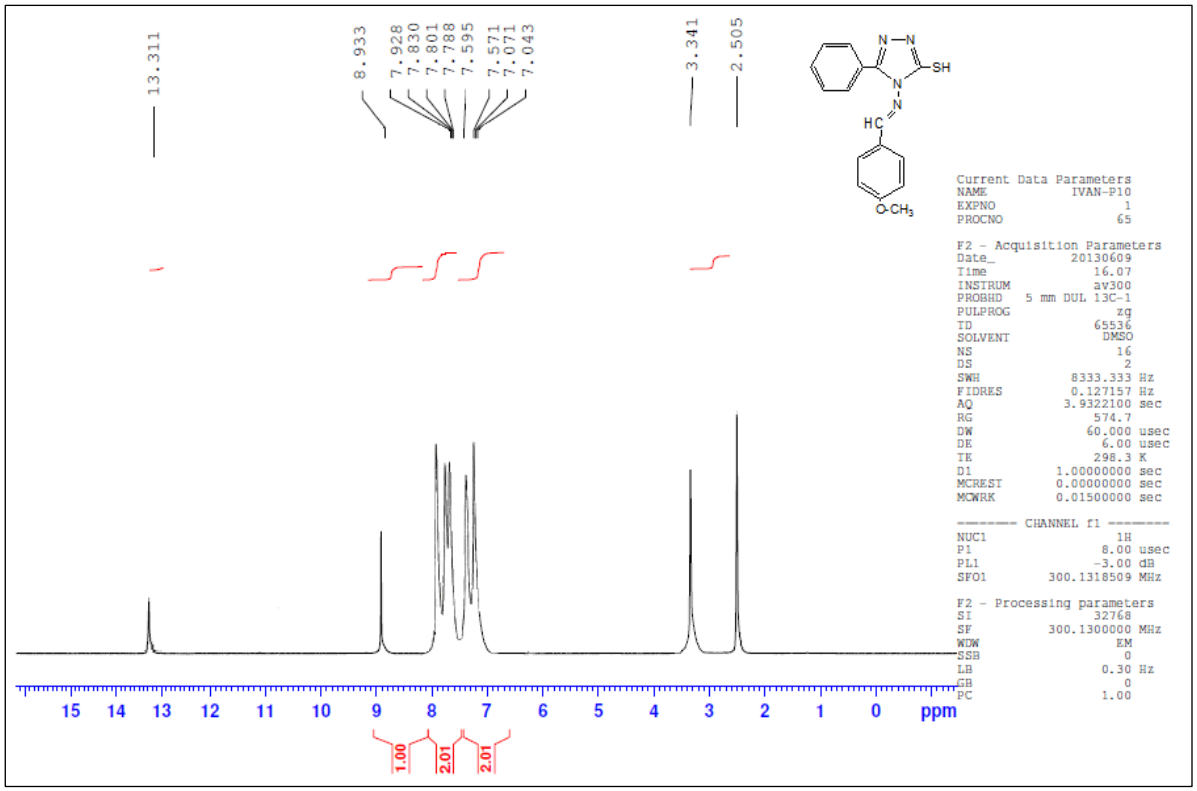


Figure (3-14) ^{1}H NMR spectrum of Compound (C)

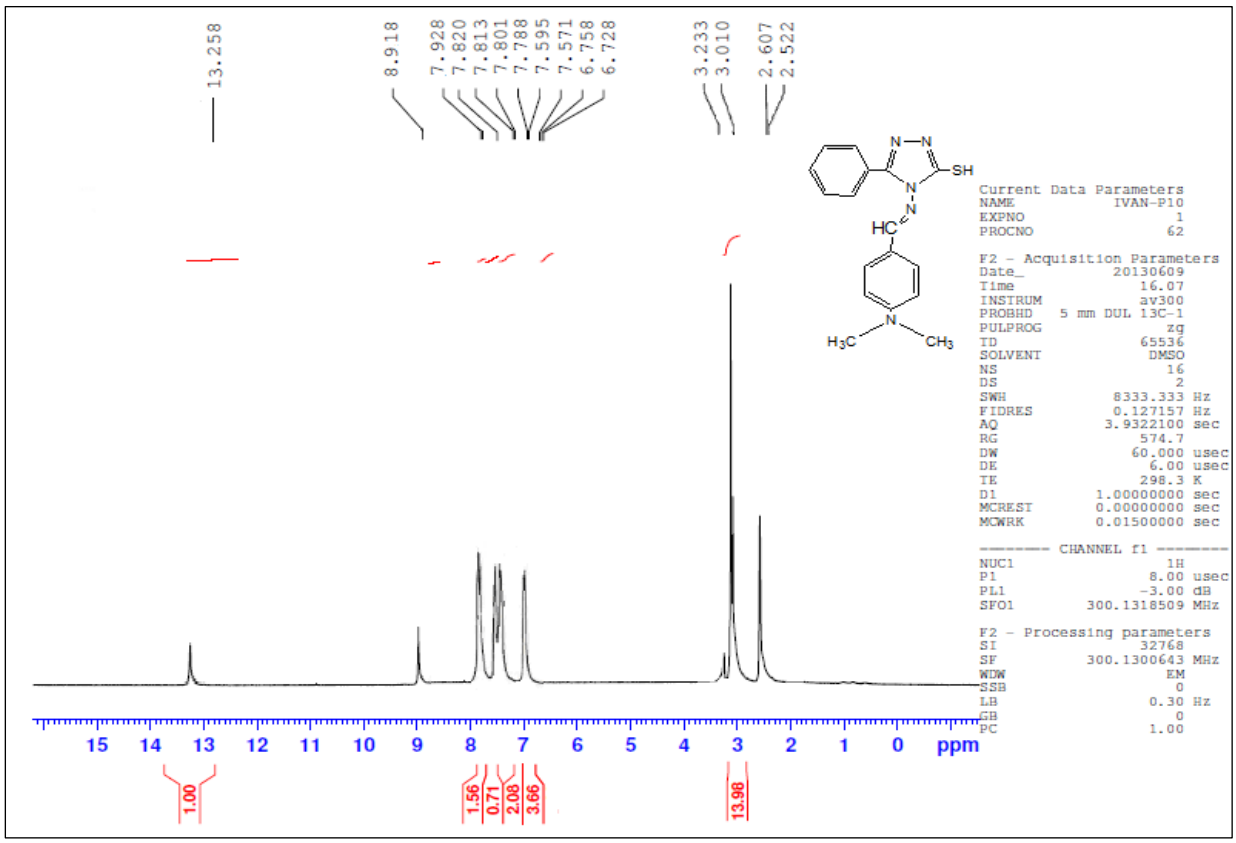


Figure (3-15) ^{1}H NMR spectrum of Compound (D)

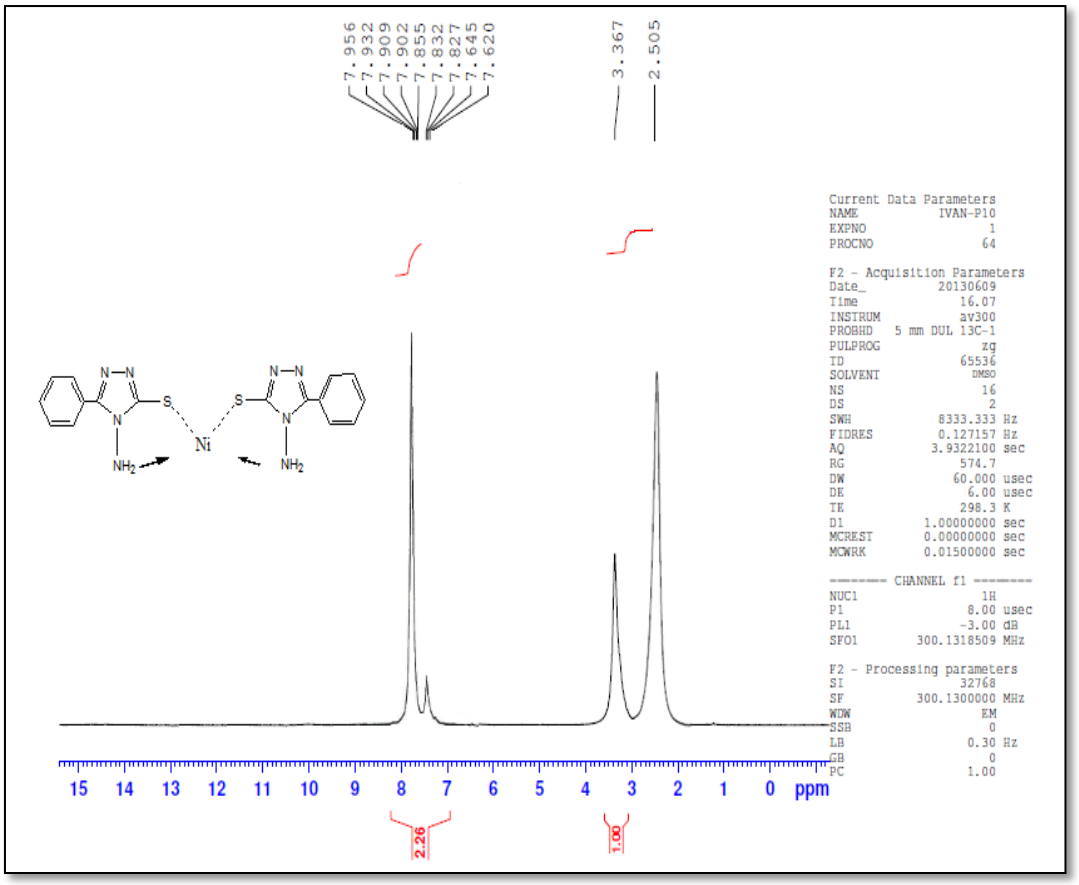


Figure (3-16) ^{1}H NMR spectrum of Complex (A1)

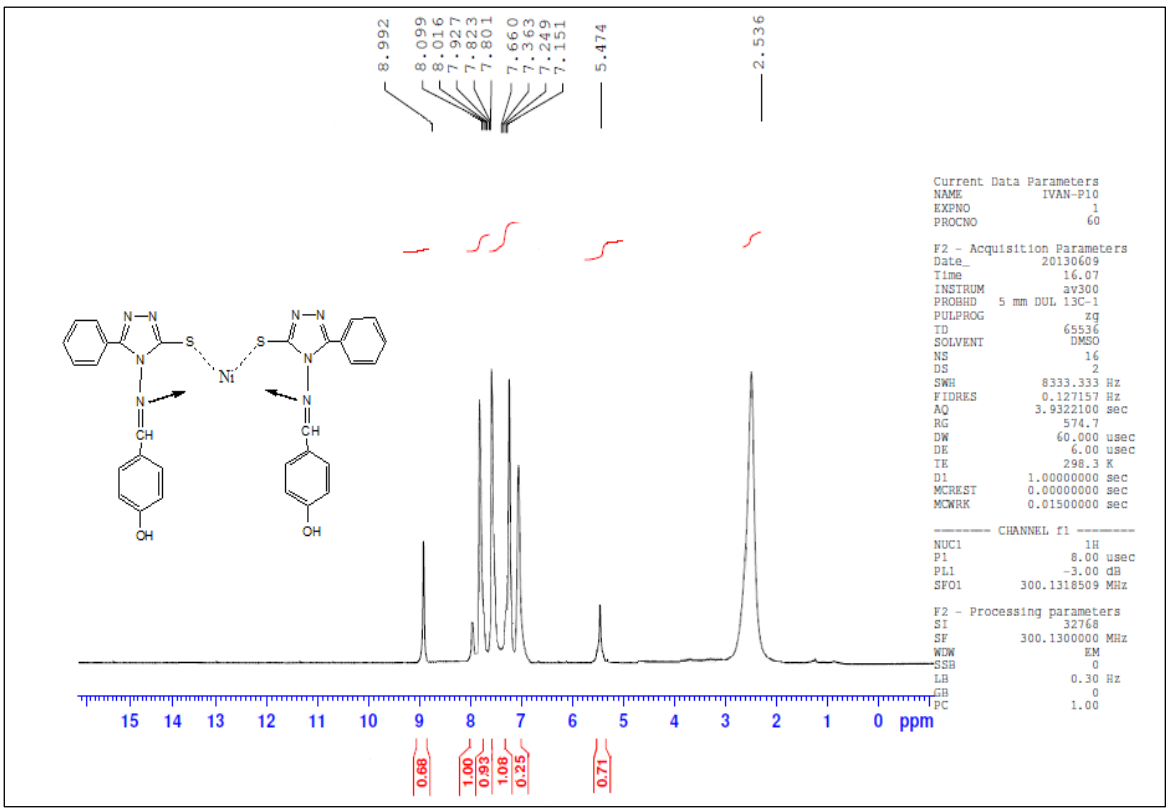


Figure (3-17) ^{1}H NMR spectrum of Complex (B1)

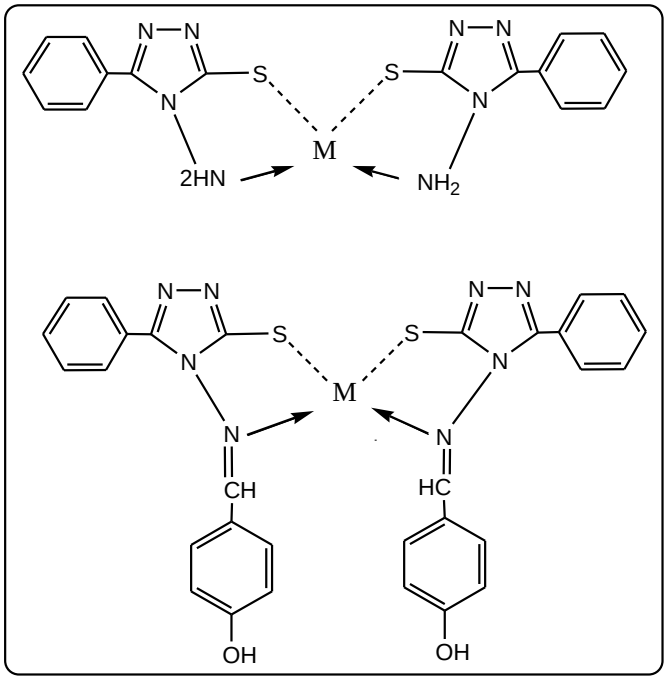
3.1.4 Conductivity measurements

The conductivity measurement of these complexes was recorded as a solution in ethanol solvent by dissolving 10^{-3}g in 5 ml ethanol. This measurement gives an idea if a solution is electrolyte or not. Table (3-3) shows the molar conductivity measurements of the complexes , all the prepared complexes were found to be non-electrolyte[147].

Table (3-3) Conductivity measurement of complexes

Complexes	Conductivity (µS/cm)
A1	3
A2	1
B1	9.3
B2	12

Based on the spectral study, complexes **A1,B1** exhibited distorted tetrahedral geometry while complex **A2,B2** showed distorted square planar [148]. The proposed structures of complexes are shown below, See Figure (3-18).



Where M= Ni(II), Cu(II)

Figure (3-18) The proposed structure of Complexes

3.1.5. Characterization of prepared complexes by Ultraviolet-Visible spectroscopy

The absorption spectra of the ligands (A and B) and its complexes were recorded in DMSO solvent in range of 250-900 nm. The electronic spectra of ligands and their complexes were illustrated in Table (3-4). The electronic spectrum of ligand (A) showed 3 bands at (263, 302, 309) nm and ligand (B) showed 2 bands at (268, 310) nm due to intraligand transition (π-π*), (n-π*) electronic transitions, respectively. From Table (3-4), complexes A1, A2, B1 and B2 also showed the similar electronic transition but with shifting in comparison with their ligands.

For complexes A1 and A2, the electronic transitions of the metal *d* orbitals (*d-d* electronic transition) observed in the Ni (II) and Cu (II) located in the visible region. There are three *d-d* electronic transitions in Ni complexes as in Orgeal diagram assigned to the $^{3}T_{1(F)} \rightarrow {}^{3}T_{2(F)}$, $^{3}T_{1(F)} \rightarrow {}^{3}T_{1(P)}$ and $^{3}T_{1(F)} \rightarrow {}^{3}A_{2(F)}$ transitions, respectively[149-150]. But in this study there is one transition appeared at 620 nm. While for Cu(II), the bands appeared at 280, 300, 312 and 451 nm were attributed to (π-π*), (n-π*), charge transfer and $^{2}T_{2} \rightarrow {}^{2}E_{2}$ respectively[151-152].

For complexes B1 and B2, the electronic transitions of the metal *d* orbitals (*d-d* electronic transition) observed in the Ni (II) and Cu (II) located in the visible region. The electronic transitions for B1 which appeared at 263, 312 and 610nm assigned to (π-π*), (n-π*) and (d-d electronic transition). But in Complex B2 there were 4 bands appeared at 264, 311, 313 and 445nm assigned to (π-π*), (n-π*), charge transfer and (d-d electronic transition) respectively.

Table (3-4) electronic spectra of prepared compounds

Compounds	λ_{max} nm	Transition
A	263, 302 309	π-π* n-π*
$Ni(L)_2$ A1	262 620	π-π* $^3T_{1(F)} \rightarrow {}^3T_{1(P)}$
$Cu(L)_2$ A2	280 300 312 451	π-π* n-π* L→ Cu(CT) $^2T_2 \rightarrow {}^2E_2$
B	268 310	π-π* n-π*
$Ni(L)_2$ B1	262 312 610	π-π* n-π* $^3T_{1(F)} \rightarrow {}^3T_{1(P)}$
$Cu(L)_2$ B2	264 311 313 445	π-π* n-π* L→ Cu(CT) $^2T_2 \rightarrow {}^2E_2$

The UV. visible spectrum of ligand and its complexes was shown in Figures (3.19) - (3.24).

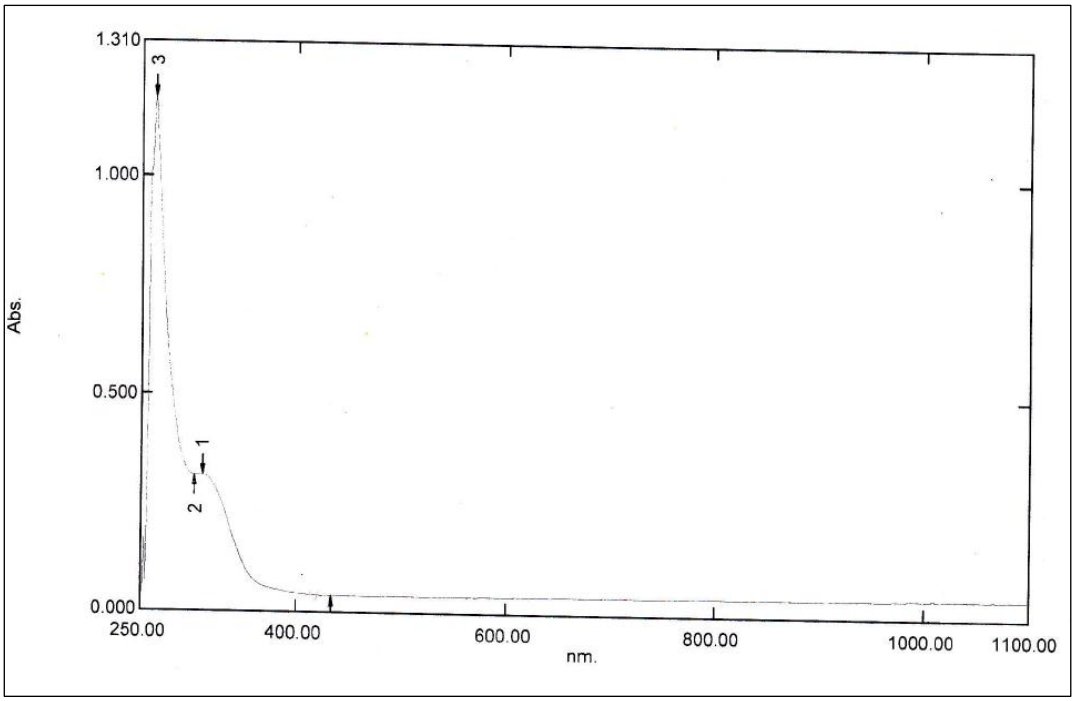


Figure (3-19) The ultraviolet visible spectrum for the A in DMSO solvent

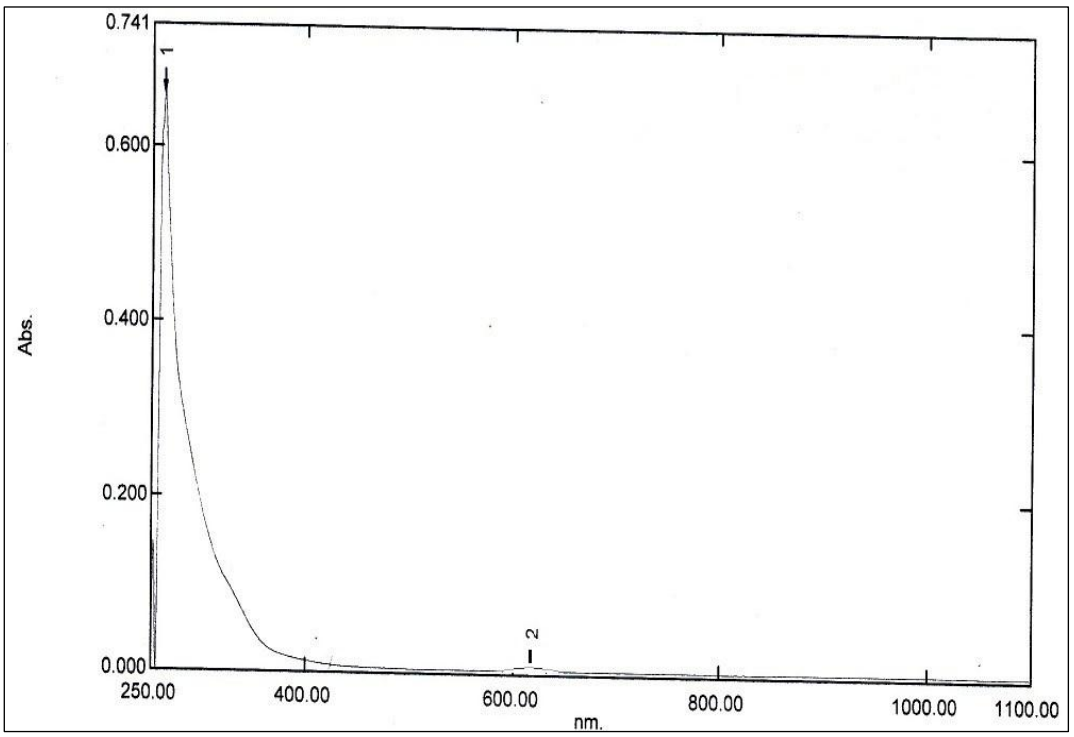


Figure (3-20) The ultraviolet visible spectrum for the A_2 in DMSO solvent

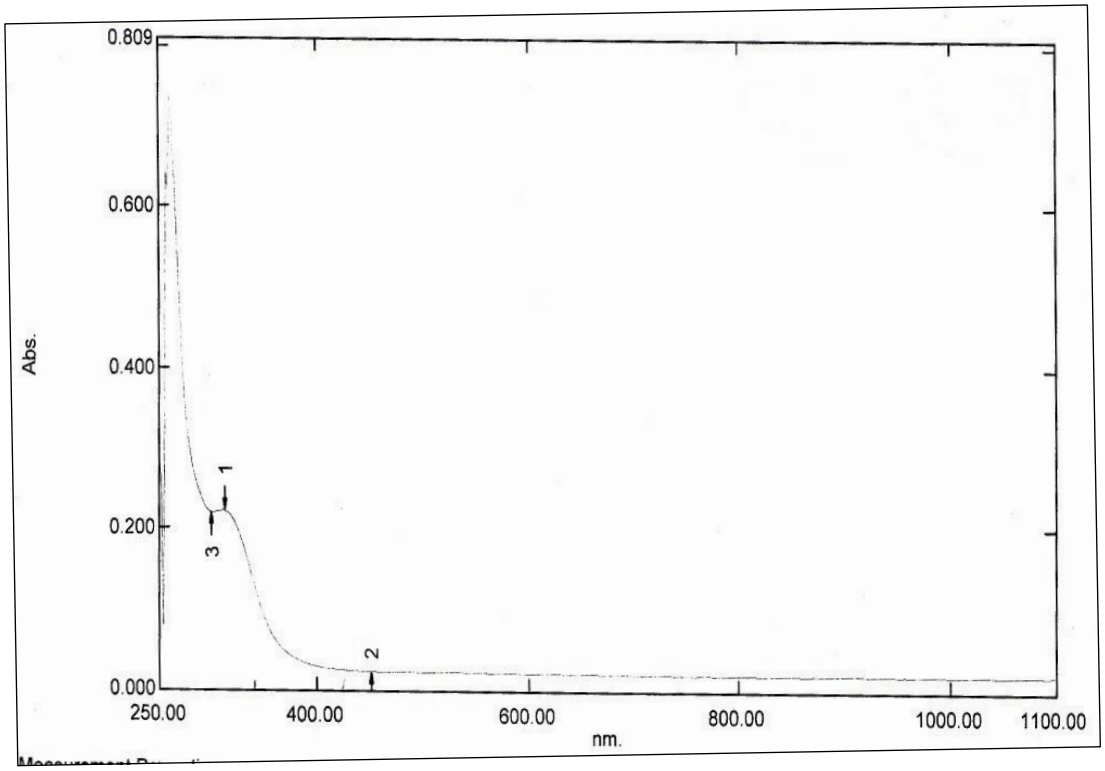


Figure (3-21) The ultraviolet visible spectrum for the A_2 in DMSO solvent

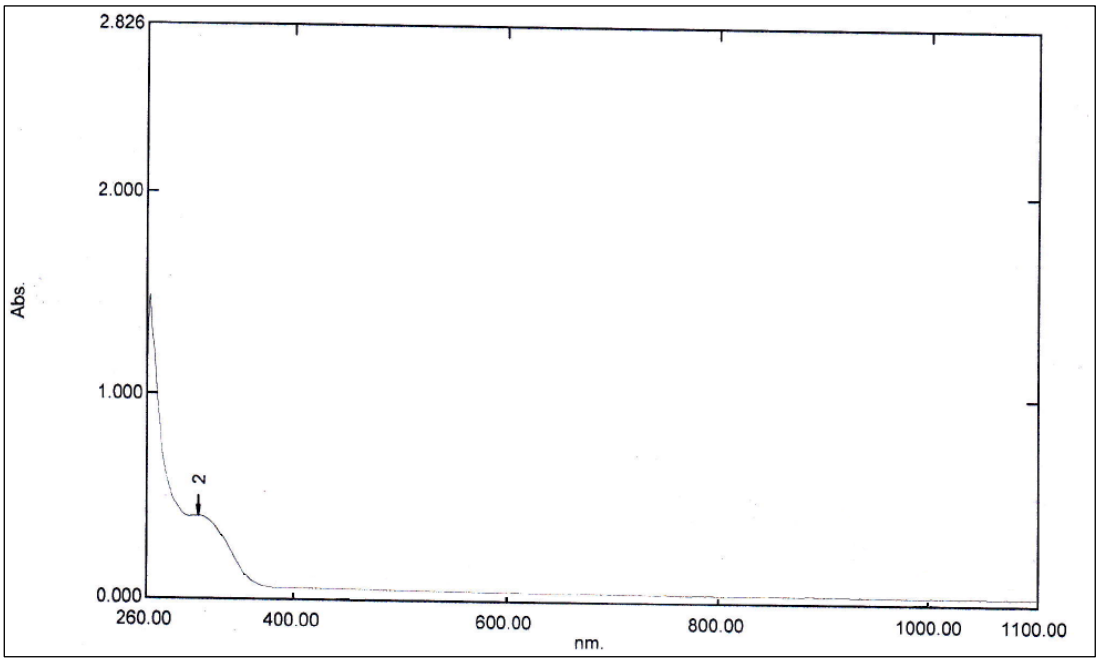


Figure (3-22)The ultraviolet visible spectrum for the B in DMSO solvent

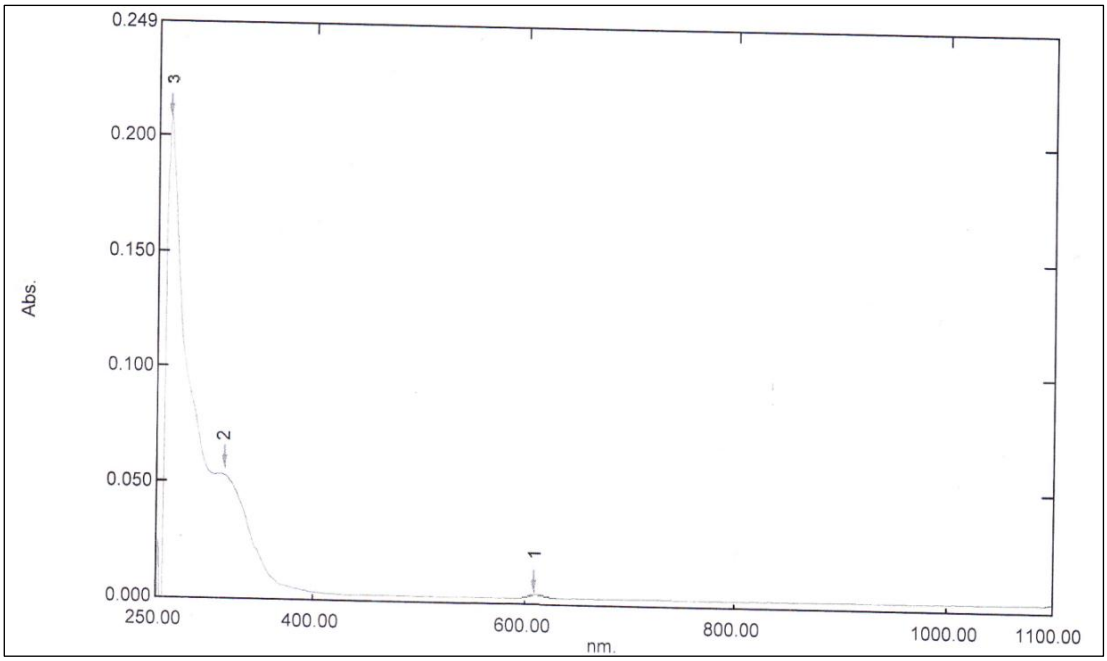


Figure (3-23) The ultraviolet visible spectrum for the B1 in DMSO solvent

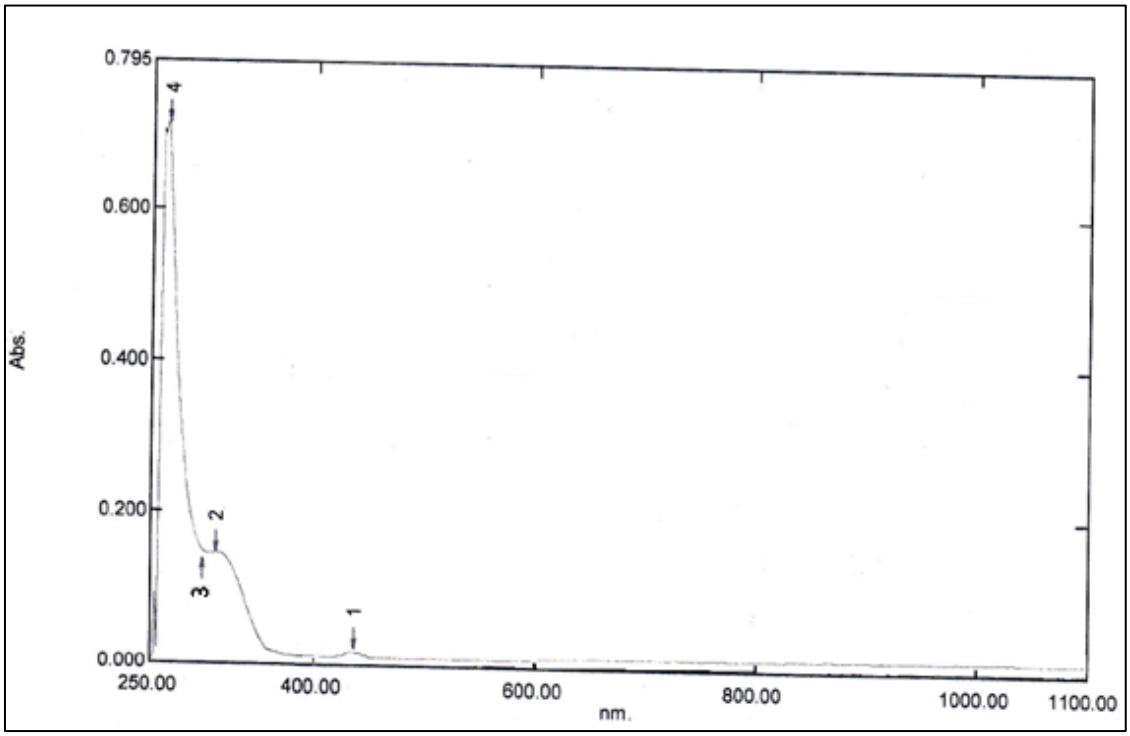


Figure (3-24) The ultraviolet visible spectrum for the B2 in DMSO solvent

3.2 The microbiologic study

3.2.1 Identification

Depending on the morphology of the colonies of the bacterial growth on MacConkey's agar and Blood agar, show the swarming phenomenon and the presence of the odor of fish on blood agar, also by the pale color of colonies on MacConkey's agar which indicates the absence of the ability to ferment lactose by the Proteus[153,154].

When the indole test gives negative result it's indication that this bacteria is Proteus mirabilis [155] Figure (3-25) shows the indole test for the used bacteria.

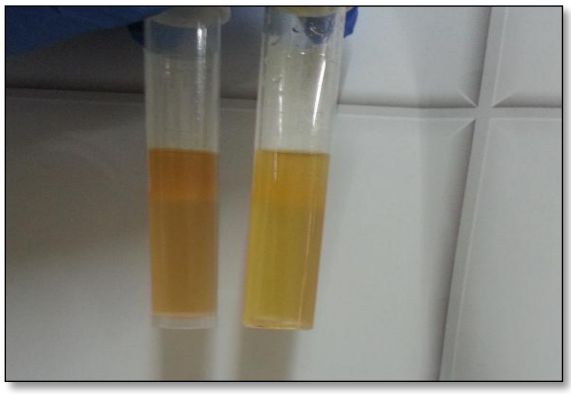


Figure (3-25) Indole test for proteus mirabilis

Proteus was also identified by its ability to change the color of urea broth base in tubes from the yellow-orange to the pinkish-red color. This is because of the ability by Proteus to produce urease which elevate the degree of the pH of the broth base in turn effects phenol-red reagent [155,156].

3.2.2 Biological study Results

3.2.2.1 Biological Activity

Pathogenic microorganisms cause different kinds of diseases to human and animals. Discovery of chemotherapeutic agents played a very important role in controlling and preventing such diseases. Chemotherapeutic agents are isolated either from living organisms known as antibiotics like penicillin and

tetracycline….etc., or they are chemical compounds prepared by chemists such as the sulpha drugs. Microorganisms have the ability to develop resistance to these chemotherapeutic agents and such strains which are resistant causing major problem in treatment of microbial infections. For this reason searching for new antimicrobial agents is continuous process and great efforts have been employed to find new antibiotics or new compounds with good antimicrobial activity which might be suitable to be used as chemotherapeutic agents[157].One microorganism was isolated and identified to be used in vitro techniques, this microorganism was:

Proteus mirabilis, (gram negatives rods that are distinguished from other members of enterobacteriaceae by their ability to produce the enzyme phenyl alanine deaminase in addition to urease, that causes UTI [158], the technique was the (Well sensitivity test), this method involves the exposure of the zone of inhibition toward the diffusion of microorganism on agar plates. A standard (5mm) diameter of wells impregnated with a specified volume of the compound solution representing the minimum inhibition concentration (MIC), this well was placed on an agar plate cultured by the test organism. The plates were incubated for (24 hrs. at 37 °C). The zone of inhibition of bacterial growth around the well was observed [159].

3.2.2.1.1 Inhibitory ability of prepared compounds against gram negative bacteria

The inhibition zones caused by the various compounds on the Proteus mirabilis bacteria were examined. (The prepared compounds showed inhibitory activity in a concentration of 100 µg/ml). The results were listed in Table (3-5) and Figures from (3-26) to (3-37). The result of IC_{50} were listed in Table (3-5) and Figures from (3-38) to (3-47).

Note : (1)=100 µg, (2)= 50 µg, (3)= 25 µg, (4)= 10 µg.

Table (3-5) The inhibition zones in mm for compounds (A, A1, B, B1, C, D and E) Minimum inhibitory concentration (MIC) (in µg/mL) and IC_{50} µM of compounds.

Compound	Concentration µg/ml	Inhibition zones in mm	Inhibition Rate%	IC_{50} µM	MIC in µg/ml
A	100	17	50.75	108.78	50
	50	7	5.02		
	25	6	4.01		
	10	5	2.51		
A1	100	21	80.42	32.54	10
	50	19	73.88		
	25	16	55.2		
	10	12	21.6		
A2	100	35	94.5	20.9	10
	50	26	83.4		
	25	18	51.75		
	10	14	35.6		
B	100	19	76.8	41.8	25
	50	17	65.82		
	25	15	51.75		
	10	6	9.54		
B1	100	22	82.43	25.26	10
	50	20	65.86		
	25	19	50.25		
	10	19	40.23		
B2	100	36	95	3.33	10
	50	27	85.4		
	15	23	57.28		
	10	20	50.25		
C	100	19	63.81	67.6	25
	50	12	47.23		
	25	10	32.16		
	10	6	9.54		
D	100	20	78.39	40.9	25
	50	17	66.83		
	25	17	55.77		
	10	5	12.06		
E	100	16	48.72	112.7	100
	50	6	4.02		
	25	5	3.51		
	10	5	2.11		
TE	100	19	60.2	68.77	10
	50	16	50		
	25	14	35.4		
	10	10	10.1		

Figure (3-26) inhibition zone of compound A on *Proteus mirabilis*

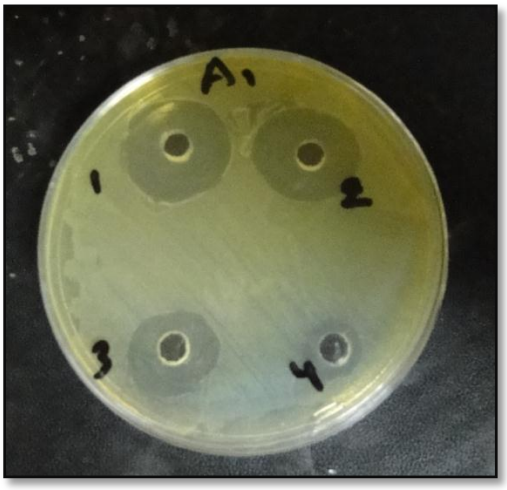


Figure (3-27) inhibition zone of compound A1 on *Proteus mirabilis*

Figure (3-28) inhibition zone of compound A2 on *Proteus mirabilis*

Figure (3-29) inhibition zone of compound B on *Proteus mirabilis*

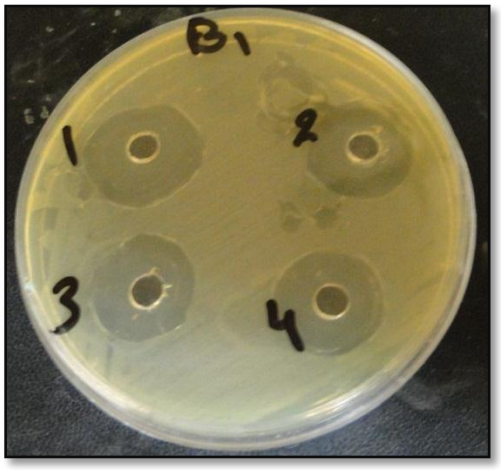


Figure (3-30) inhibition zone of compound B1 on *Proteus mirabilis*

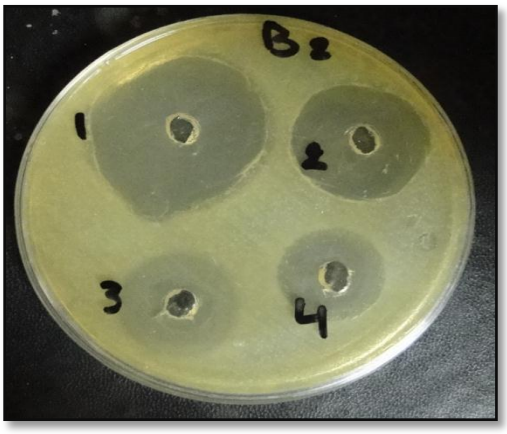


Figure (3-31) inhibition zone of compound B2 on *Proteus mirabilis*

Figure (3-32) inhibition zone of compound C on *Proteus mirabilis*

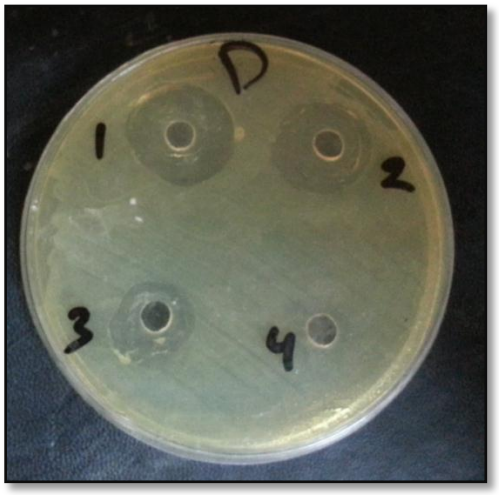


Figure (3-33) inhibition zone of compound D on *Proteus mirabilis*

Figure (3-34) inhibition zone of compound E on *Proteus mirabilis*

Figure (3-35) inhibition zone of compounds (B2, B1, A1 and A2)on *Proteus mirabilis* at concentration 5µg/ml

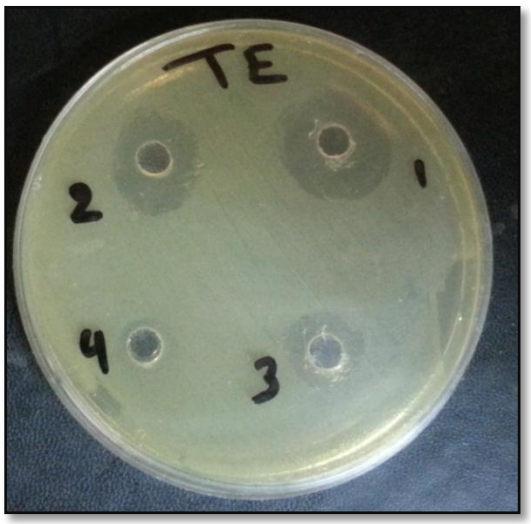


Figure (3-36) inhibition zone of standard drug Tetracycline on *Proteus mirabilis* at concentration (100,50.25and 10) µg/ml

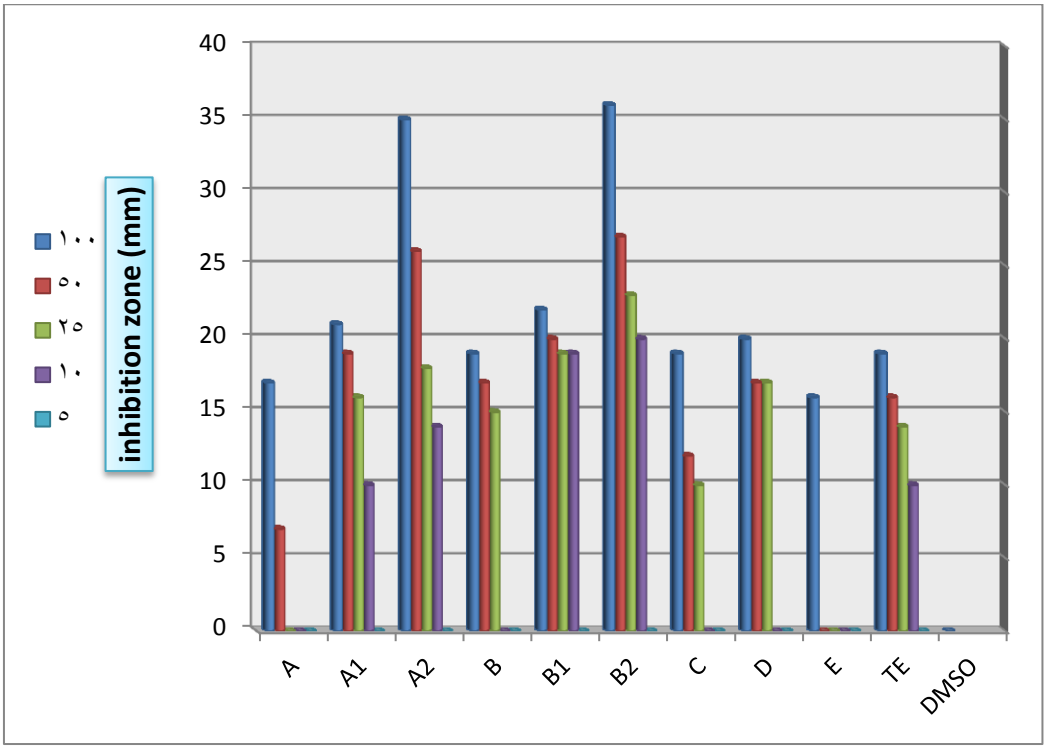


Figure (3-37) The effect of compounds (A, A1, A2, B, B1, B2, C, D and E) and (tetracycline) on *Proteus mirabilis* in concentration 100, 50, 25, 10, 5(µg/ml) dissolved in DMSO at 37C◦ for 24 hrs.

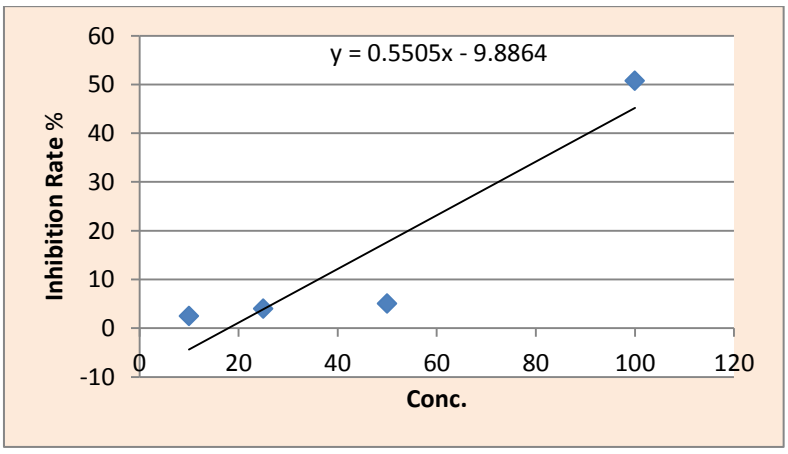


Figure (3-38) Linear relation between inhibition rate and concentration of Compound (A)

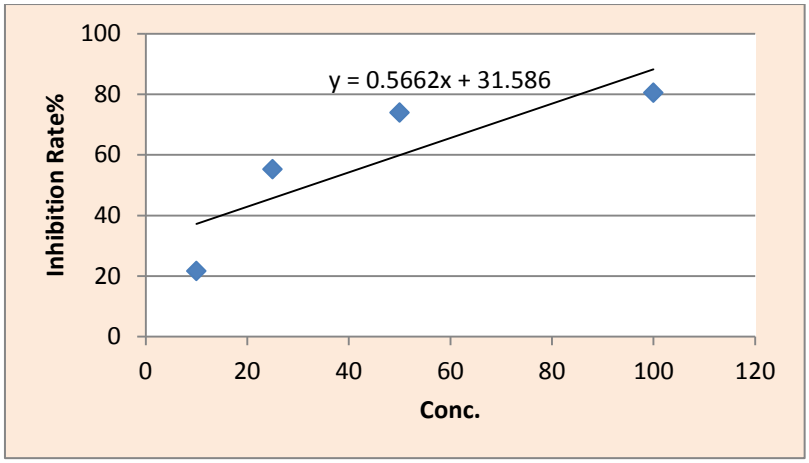


Figure (3-39) Linear relation between inhibition rate and concentration of Compound (A1)

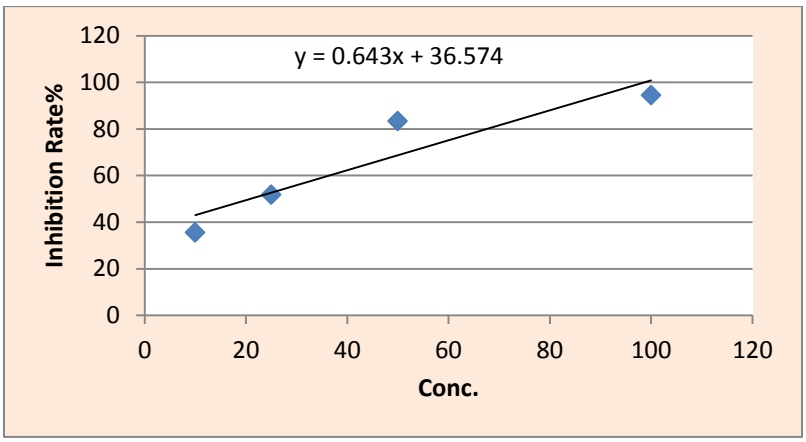


Figure (3-40) Linear relation between inhibition rate and concentration of Compound (A2)

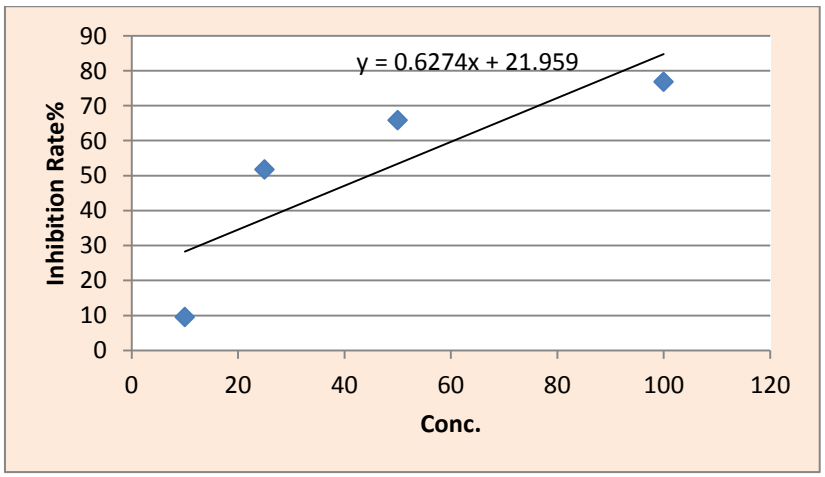


Figure (3-41) Linear relation between inhibition rate and concentration of Compound (B)

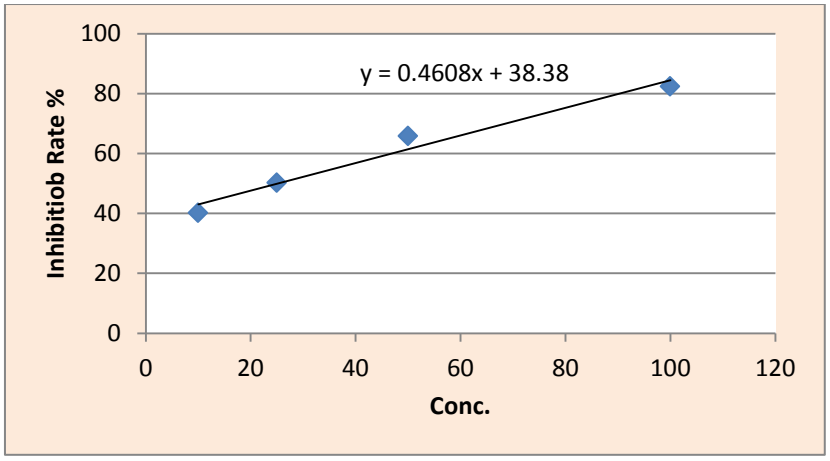


Figure (3-42) Linear relation between inhibition rate and concentration of Compound (B1)

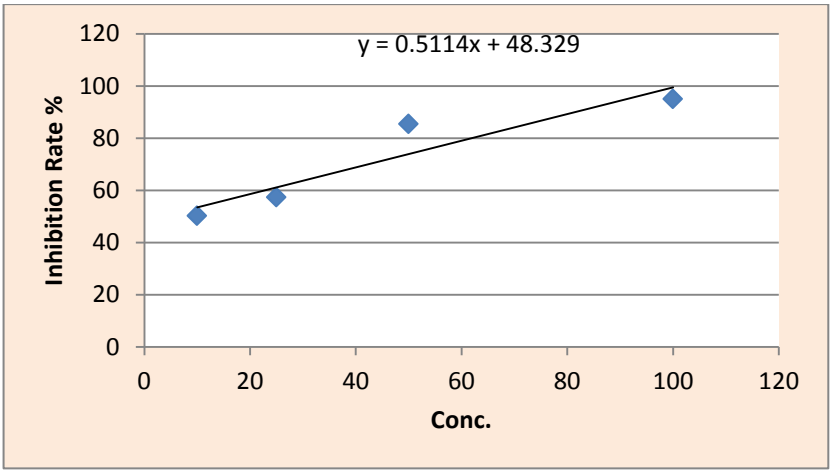


Figure (3-43) Linear relation between inhibition rate and concentration of Compound (B2)

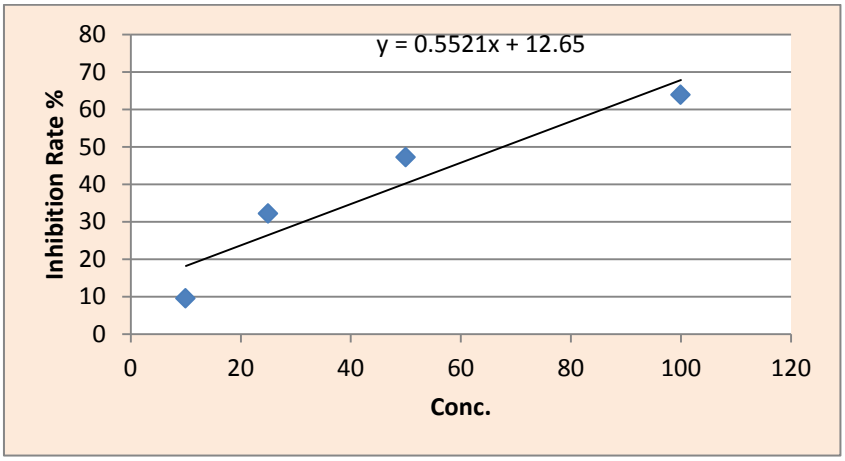


Figure (3-44) Linear relation between inhibition rate and concentration of Compound (C)

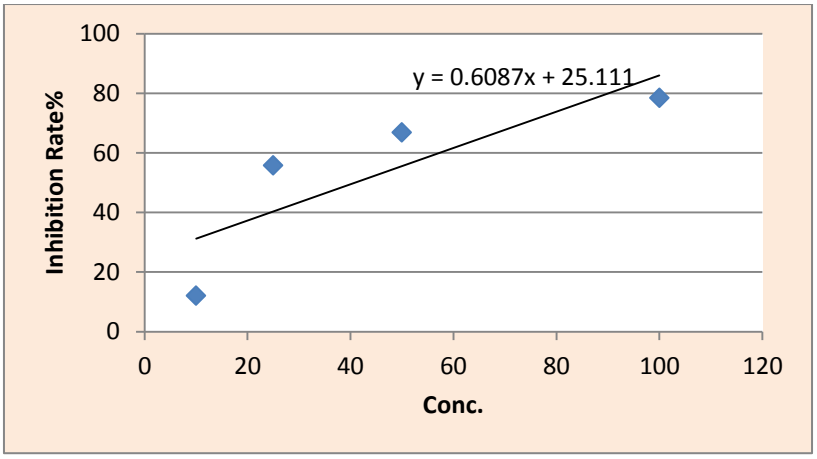


Figure (3-45) Linear relation between inhibition rate and concentration of Compound (D)

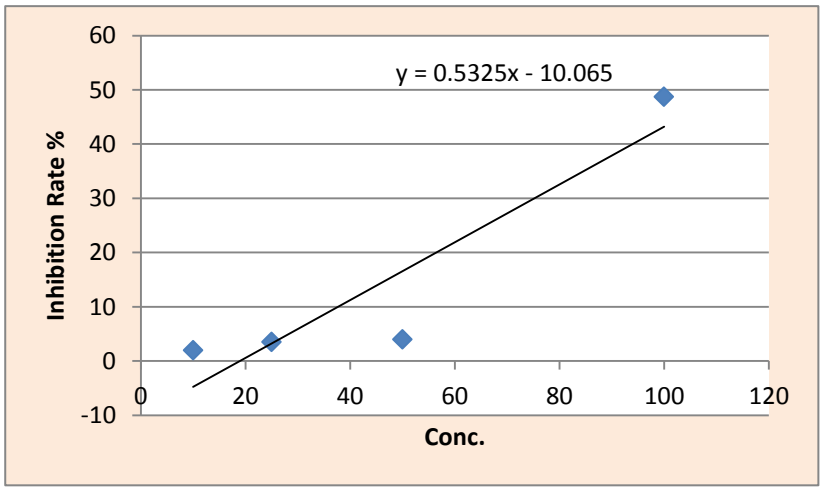


Figure (3-46) Linear relation between inhibition rate and concentration of Compound (E)

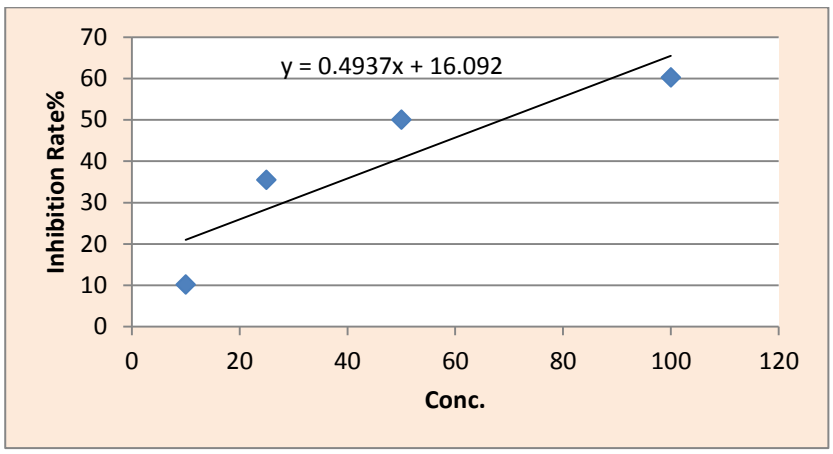


Figure (3-47) Linear relation between inhibition rate and concentration of Tetracycline

The prepared compounds showed inhibition activity in a concentration of 100 µg/ml where the compounds (B2, A2, B1, A1, D, B and C) have highest inhibition ability than tetracycline where the inhibition zone diameter ranged between (19-36)mm with IC_{50} (3.33, 20.9, 25.26, 32.54, 40.9, 41.8 and 67.6)µM respectively .

The compound (A and E) have inhibition ability less than tetracycline where the inhibition zone diameter (16-17)mm with IC_{50}(108.78 and 112.7) µM respectively.

The antimicrobial studies suggested that the Schiff bases were found to be biologically active and their metal complexes showed significantly enhanced antibacterial activity against microbial strains in comparison to the free ligands . The Overtone's[160,161] concept and Tweedy's[162] chelation theory can be used to explain the enhancement in antimicrobial activity of the metal complexes. According to the Overtone's concept of cell permeability, the lipid membrane surrounding the cell favors the passage of only lipid-soluble materials; therefore, liposolubility is an important factor which controls the antimicrobial activity. On chelation, polarity of the metal ion is reduced to a greater extent due the overlapping of the ligand orbital and partial sharing of the positive charge of the metal ion with donor groups. Moreover, delocalization of the π-electrons over the whole chelate ring is increased and lipophilicity of the complexes is enhanced. The increased lipophilicity enhances the penetration of the complexes into the lipid membranes and blocks the metal binding sites in the enzymes of microorganisms. These complexes also disturb the respiration process of the cell and thus block the synthesis of proteins, which restricts further growth of the organism. In general, metal complexes are more active than ligands as they may serve as principal cytotoxic species[163].

A large variety of biological activities such as antibacterial, anti-inflammatory and antifungal are shown by the complexes containing N, S and O

atoms in the chelating ligands which are bonded to the metal ion in many ways[164].

The compounds (B, C and D) are considered as Schiff bases show high inhibitory activities due to the fact that strong donating groups are found in their structures and the donating groups are (OH , O-CH_3 and N(CH_3)2) and because (C=N) group is found in their structures which is peculiar to the Schiff bases. The compound (E) in addition to the presence of (Cl) which is a withdrawing group has less inhibition activity than the activity of these compounds against bacteria[165].

In the whole series, the MIC of chemical compounds ranged between 25 and 100 µg/mL against Proteus mirabilis bacteria. Compounds A1, A2, B1 and B2 were found to be best, as they exhibit the lowest MIC of 10 µg/mL against Proteus mirabilis (Table 3-5).

3.2.2.2 Urease Inhibitors

This study was performed to test the effects of eight chemical compounds on urease enzyme activity.

The results of the inhibition study showed that all prepared compounds have inhibition effects on urease activity and they differ in their inhibition degree of urease activity. The calculated values of Ki and i % for each inhibitor and specified the kind of inhibition whether competitive or non-competitive by estimation of Km and Vmax for each enzyme and inhibitors. To compare between the inhibition strength for each inhibitor, a constant concentration for all inhibitors; both competitive and non-compotitive to deduce the Ki which were measured in molarity as shown in the calculations paragraph in chapter two. The results of this study showed that the compound A has inhibitor strength value Ki =0.17 M and (Vmax value=909 µmole/min/ml) and (Kmapp

value =2.545 mM), and this inhibitor was competitive type as Vmax value for inhibitor is the same for the Vmax for enzyme as shown in figure (3-48).

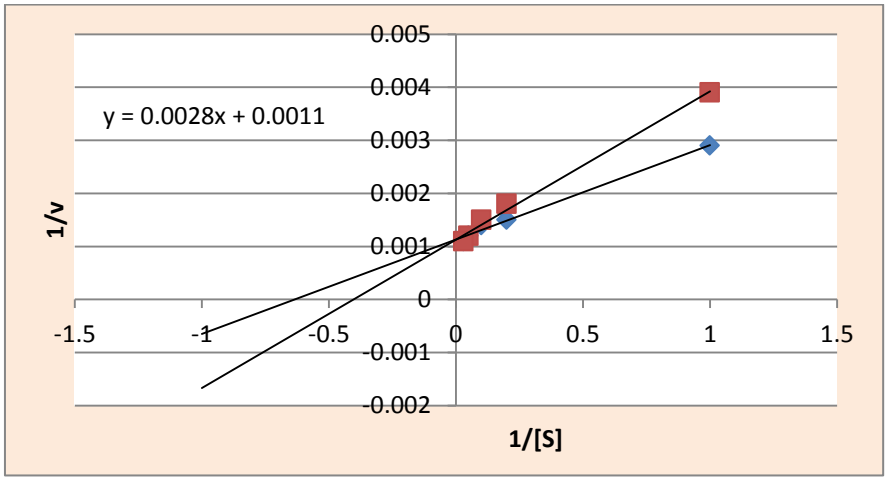


Figure (3-48) The relationship between concentration of urea and the activity of urease drawn by linear weaver Burk equation with the presence and absence of compound (A)

Compound A
Enzyme

Compound A1 is Complex with inhibitory strength (Ki=0.052 M) which is of competitive type because V_{max} is the same for the enzyme and the inhibitor (Vmax value = 909 µmole/min/ml) whereas the value of Km app. was equal to 4.726 Mm as shown in Figure (3-49).

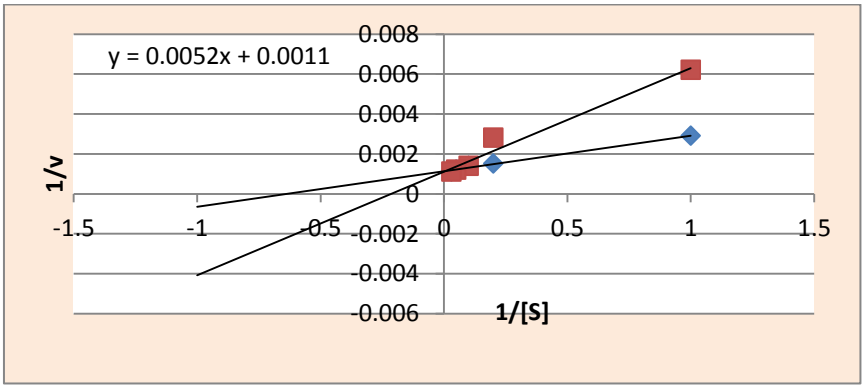


Figure (3-49) The relationship between concentration of urea and the activity of urease drawn by linear weaver Burk equation with the presence and absence of compound (A1)

Compound A1
Enzyme

Compound A2 is complex, where inhibitory strength (Ki=0.018 M) and this is considered as competitive inhibitor where Vmax for enzyme and inhibitor is the same (Vmax =909 µmole/min/ml) while Michaelis constant (Km app. = 10.326 mM) as shown in Figure (3-50).

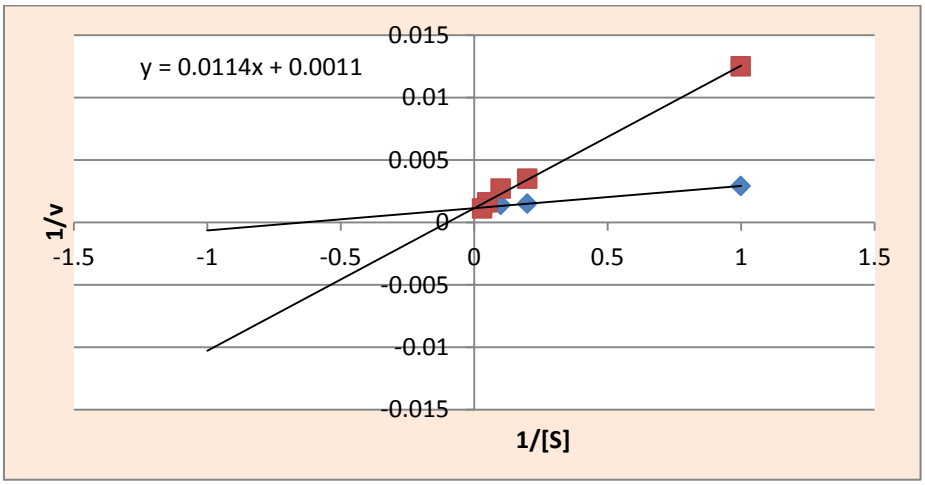


Figure (3-50) The relationship between concentration of urea and the activity of urease drawn by linear weaver Burk equation with the presence and absence of compound (A2)

Compound A2
Enzyme

Compound B is Schiff base, with inhibitory strength (Ki=0.1 M) which is of competitive type because V_{max} is the same for the enzyme and the inhibitor (Vmax value = 909 µmole/min/ml) whereas the value of Km app. was equal to 4.09 Mm as shown in Figure (3-51).

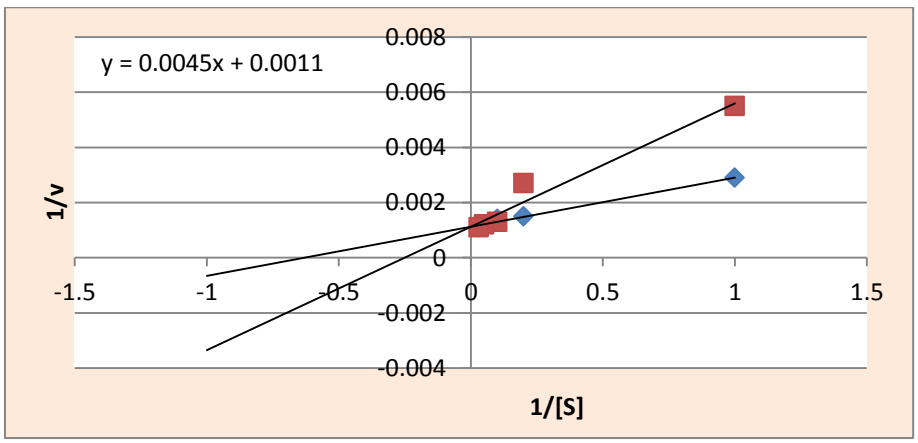


Figure (3-51) The relationship between concentration of urea and the activity of urease drawn by linear weaver Burk equation with the presence and absence of compound (B)

Compound B
Enzyme

Compound B1 is Complex, where inhibitory strength (Ki=0.036 M) and this is considered as competitive inhibitor where Vmax for enzyme and inhibitor is the same (Vmax =909 µmole/min/ml) while Michaelis constant (Km app. = 6.1812 mM) as shown in Figure (3-52).

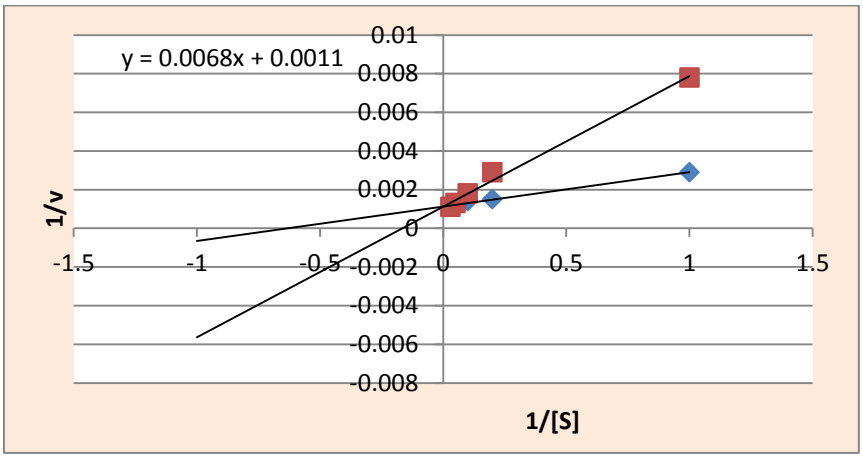


Figure (3-52) The relationship between concentration of urea and the activity of urease drawn by linear weaver Burk equation with the presence and absence of compound (B1)

Compound B1
Enzyme

Compound B2 is Complex, where inhibitory strength (Ki=0.017 M) and this is considered as competitive inhibitor where Vmax for enzyme and inhibitor is the same (Vmax =909 µmole/min/ml) while Michaelis constant (Km app. = 10.908 mM) as shown in Figure (3-53).

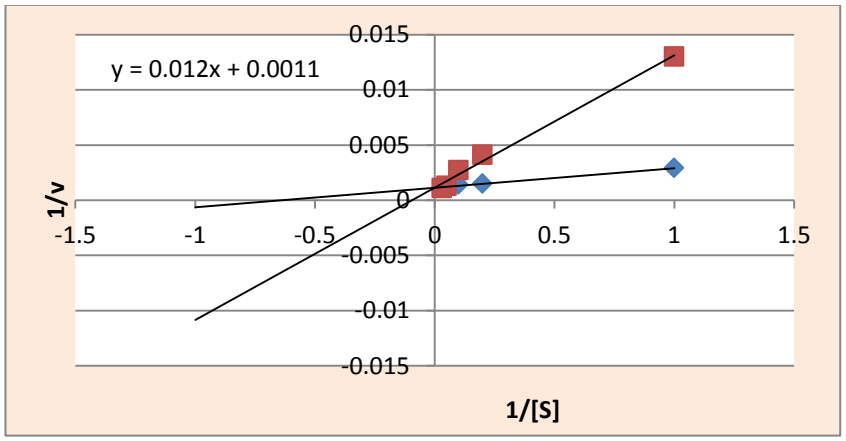


Figure (3-53) The relationship between concentration of urea and the activity of urease drawn by linear weaver Burk equation with the presence and absence of compound (B2)

Compound B2
Enzyme

Compound C is Schiff base, where inhibitory strength (Ki=0.12 M) and this is considered as non-competitive type as Vmax value for enzyme is different from Vmax value for inhibitor while Km value was equal for both enzyme and inhibitor, therefor this kind of inhibitor is considered as non-competitive inhibitor shown in Figure (3-54).

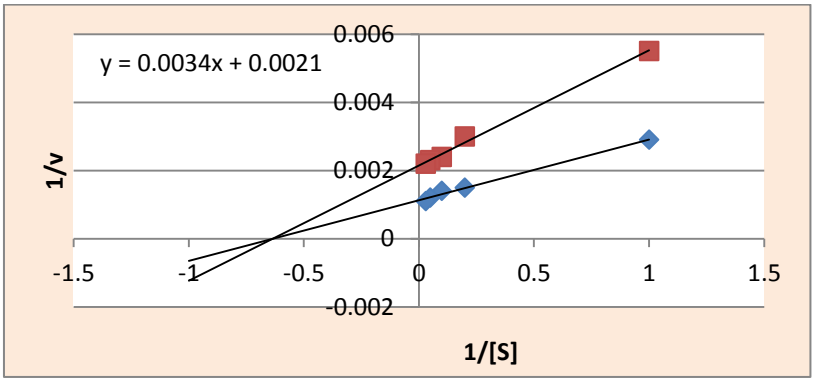


Figure (3-54) The relationship between concentration of urea and the activity of urease drawn by linear weaver Burk equation with the presence and absence of compound (C)

Compound C
Enzyme

Compound D is Schiff base, where inhibitory strength (Ki=0.059 M) and this is considered as competitive inhibitor where Vmax for enzyme and inhibitor is the same (Vmax =909 µmole/min/ml) while Michaelis constant (Km app. = 4.363 mM) as shown in Figure (3-55).

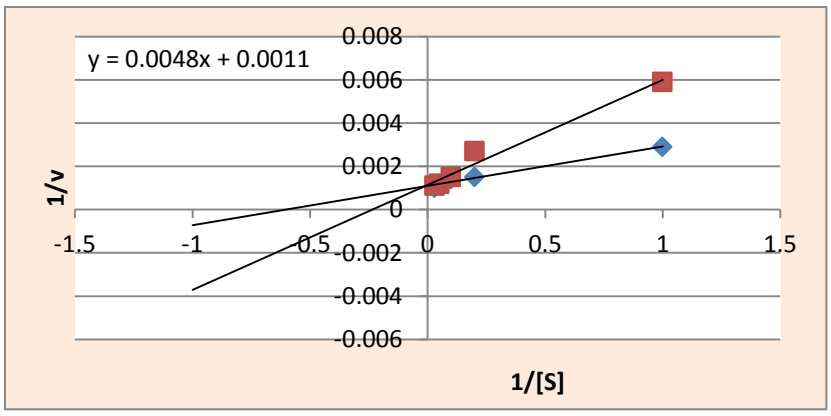


Figure (3-55) The relationship between concentration of urea and the activity of urease drawn by linear weaver Burk equation with the presence and absence of compound (D)

Compound D
Enzyme

Compound E is Schiff base, where inhibitory strength (Ki=0.25 M) and this is considered as competitive inhibitor where Vmax for enzyme and inhibitor is the same (Vmax =909 µmole/min/ml) while Michaelis constant (Km = 2.272 mM) as shown in Figure (3-56).

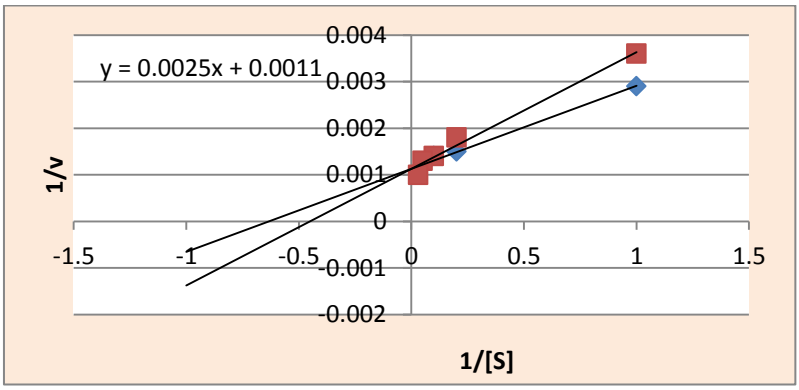


Figure (3-56) The relationship between concentration of urea and the activity of urease drawn by linear weaver Burk equation with the presence and absence of compound (E)

Compound E
Enzyme

From equation (2-1) , Vmax and Km were calculated both for enzyme and the inhibitor. From these results, it was found that Km value is inversely proportional with inhibitory strength, i.e., so when Ki value is high, need high concentration of the inhibitor is needed to have the same effect.

It is noticed from the results that the complex of compound B, is considered as the strongest inhibitor used in this study as it's Ki value 0.017 M. Results indicated that Cu complexes exhibit very good activities against urease activity .

The presence of (C=N) bond which is characteristic of Schiff base has high inhibition activity on enzyme activity and the presence of functional group on the phenyl ring of aldehyde (hydroxy) acts as a donating group that form a strong inhibitor in comparison with compound E which is the weakest inhibitor where Ki value is high in comparison with other inhibitors which is (0.03M).

This is because the functional group on the phenyl ring of aldehyde is considered as a withdrawing group (Cl).

It is clear from the results of the present study the metal complex and donating group that is present in the chemical structure of the compounds increase the inhibitory ability. The following Table (3-6) shows Vmax, Km and Ki for these compounds.

Table (3-6) Estimation of Vmax, Km and Ki for urease inhibitors and the type of inhibition

Compound No.	V_{max} (mM)	K_m (mM)	K_i (mM)	Inhibition type
Enzyme only	909	1.6	-	-
A	909	2.54	0.17	Competitive
A1	909	4.72	0.052	Competitive
A2	909	10.36	0.018	Competitive
B	909	4.09	0.1	Competitive
B1	909	6.18	0.036	Competitive
B2	909	10.90	0.017	Competitive
C	476.19	1.6	0.11	Non-Competitive
D	909	4.36	0.059	Competitive
E	909	2.27	0.25	Competitive

The percentage of inhibition (i %) was calculated for each inhibitor as shown in chapter two .

The concentration of inhibitor and substrate were constant to compare the inhibition percentage for each inhibitor. It is obvious from calculation of the

inhibition percentage, shown in chapter two, that the highest percentage of inhibition was (97.6%) when the:

Compound B2 was used and compare with other inhibitors using the same concentration of the compounds. which it considered as the complex with high activity and this may be due to the presence of metal (Cu) characteristic of complex. The least inhibition percentage was (50%) by Compound E. The following Table (3- 7) shows the percentage of the inhibition:

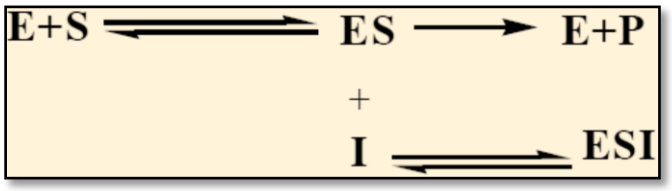
Table (3-7) Estimation of Vmax, Km and Ki for urease inhibitors and the type of inhibition

Compound No.	Vi µmole/min/l	Vo µmole/ min/l	a (relative activity)	%i (percentage of inhibition)
A	6.78	16.42	0.41	59.3
A1	2.004	16.42	0.12	87.7
A2	0.43	16.42	0.02	97.3
B	3.36	16.42	0.20	79.5
B1	1.16	16.42	0.07	92.7
B2	0.39	16.42	0.02	97.6
C	4.61	16.42	0.28	71.9
D	2.35	16.42	0.14	85.6
E	8.60	16.42	0.52	50

From the results of the study, it has been found that all the prepared compounds could be inhibitors of urease. the compounds B2 and A2 have the best activity among these inhibitors. It was also noticed those compounds (A,A1, B, B1, D and E) were competitive inhibitors of urease, except the compound (C) was non-competitive inhibitors of urease.

The Vmax remains constant despite the increase in Km and this is an evidence that it's of competitive type. They compete with substrate (urea) to bind with active site of urease and when urea concentration is increased the % inhibitor effect is reduced increasing the chance of substrate binding with enzyme, and the enzyme regains its activity which has been lost [166] .

Competitive inhibition increases Km and substrate concentration to reach Vmax but the Vmax is constant and unchanged. Therefore in the presence of competitive inhibitor a much greater substrate concentration is required to attain any fraction of Vmax. Km is inversely proportional with affinity. This formula shows competitive inhibition[166] .



It is clear From the results of the present study the increase in urea (substrate) concentration in the media did not affect the activity of urease; its addition doesn't return to the Vmax to its original value before addition of inhibitors, as a result Vmax remaine low, and Km constant. The resulte of decrease Vmax while Km value remain constant is evidence its of non-competitive type[167]. In this inhibition type there will be binding of the substrate and inhibitor at different site of the enzyme, they bind independently. this lead to the formation of complex (ESI), i.e., the substrate and inhibitor binds with enzyme at the same time. The appearance of (ESI) is due to the presence of any amount of inhibitor in reaction media and its formation does not to formation of product and the quantity of this enzyme remains unchanged.

From enzymatic and biological studies noticed that complex compounds are strong inhibitors of the urease enzyme where the inhibition force of the compound B2 was (Ki=0.017 M) which is the highest inhibition force for the prepared compounds(%i= 97.6 %). The biological activity for this compound was tested on Proteus mirabilis and was noticed to have the highest inhibition at (100µg/ml) where the inhibition zone diameter was 36 mm. In addition to the other complex compounds, which have a high inhibition ability where compound A2 had Ki (0.018M) and also the compound B1 had inhibitory force (Ki=0.036M) results of the biological activity of these compounds were high on Proteus mirabilis with (100µg/ml) with inhibition zone diameter between(21-35) mm this result may be due to the present of heavy metal ion (Cu^{+2} and Ni^{+2}).

Inhibition of urease by heavy metal ions is important. Heavy metal ions inhibit both plant and bacterial ureases at the following order of effectiveness :

$Hg^{+2} \approx Ag^{+} > Cu^{+2} >> Ni^{+2} > Cd^{+2} > Zn^{+2} > Co^{+2} > Fe^{+3} > Pb^{+2} > Mn^{+2}$

Hg^{+2}, Ag^{+} and Cu^{2+} ions nearly always listed as the strongest inhibitors [168].

The inhibition of urease by heavy metal ions is result from the reaction of these ions with a sulfhydryl group in the active center of the enzyme by a reaction analogous to the formation of metal sulfides .The formation of sulfides with the active center was confirmed experimentally by the correlation between the inhibitory efficiency of metal ions and the solubility products of their sulfides[168,169].

The inhibition force for the compounds contain (C=N) group characteristic for the Schiff base and strong donating group(OH,CH_3-N-CH_3 and O-CH_3) have a high enzyme activity with high inhibition percentage like(B, C

and D). The compound E was weak inhibitor in comparison with other compounds studied in this study. The inhibition force for this compound was weak (Ki=0.25M) where it is highest Ki value for the studied compounds and had the least inhibition force.

From this comparison of these results, found a relationship between the biological and enzyme activities.

The study of urease inhibition has medical, environmental, agricultural importance. Urease is considered a virulent factor for pathogenic bacteria lead to many infections like urolithiasis, UTI, pyelonephritis, gastric ulcers….etc . The development in discovery and the importance of urease inhibition leads to reduction in pollution and help to increase the uptake of nitrogen by plants and treatment of the infection caused by urea splitting microorganisms[170,171]. Urease inhibitors are considered as effective treatment because they inhibit urolithiasis caused by microorganisms that produce urease when infecting the urinary tracts .

3.3.Conclusion

1. From the results of this study, it is found that the prepared urease inhibitors compounds (A, A1, A2, B, B1, B2, D and E) are competitive inhibitors for urease which compete the substrate for binding to the active site of urease. While compound (C) are non-competitive types of inhibitor that does not compete with substrate for the active site of enzyme.

2. Compound B2 has the highest inhibitory effect against urease. Ki=0.017 M; i=97.6%; Vmax= 909 unit/ml; Km=10.90 mM and it showed competitive type of inhibition. While compound E has the lowest inhibitory effect with (Ki=0.25 M, Vmax 909 unit/ml, Km 2.27 mM, i %=50 %).

3. All the prepared compounds have biological activity against *Proteus mirabilis* bacteria , compound B2 gave the highest biological activity .
4. These compounds behave successfully as inhibitors of urease enzyme. The inhibitors take the following order according to their increasing in i% and Zone inhibition in mm.

$$\boldsymbol{Cu(B2)_2 > Cu(A2)_2 > Ni(B1)_2 > Ni(A1)_2 > D > B > C > A > E}$$

5. The metal complexes are more active than other compounds due to the presence of heavy metal that has a role in their activity.
6. The Copper complexes were found to be the more efficient in inhibition process as compare to Ni complexes. This support the use of Copper complexes as commercial inhibitor of urease enzyme.

3.4.Suggestion of the future work

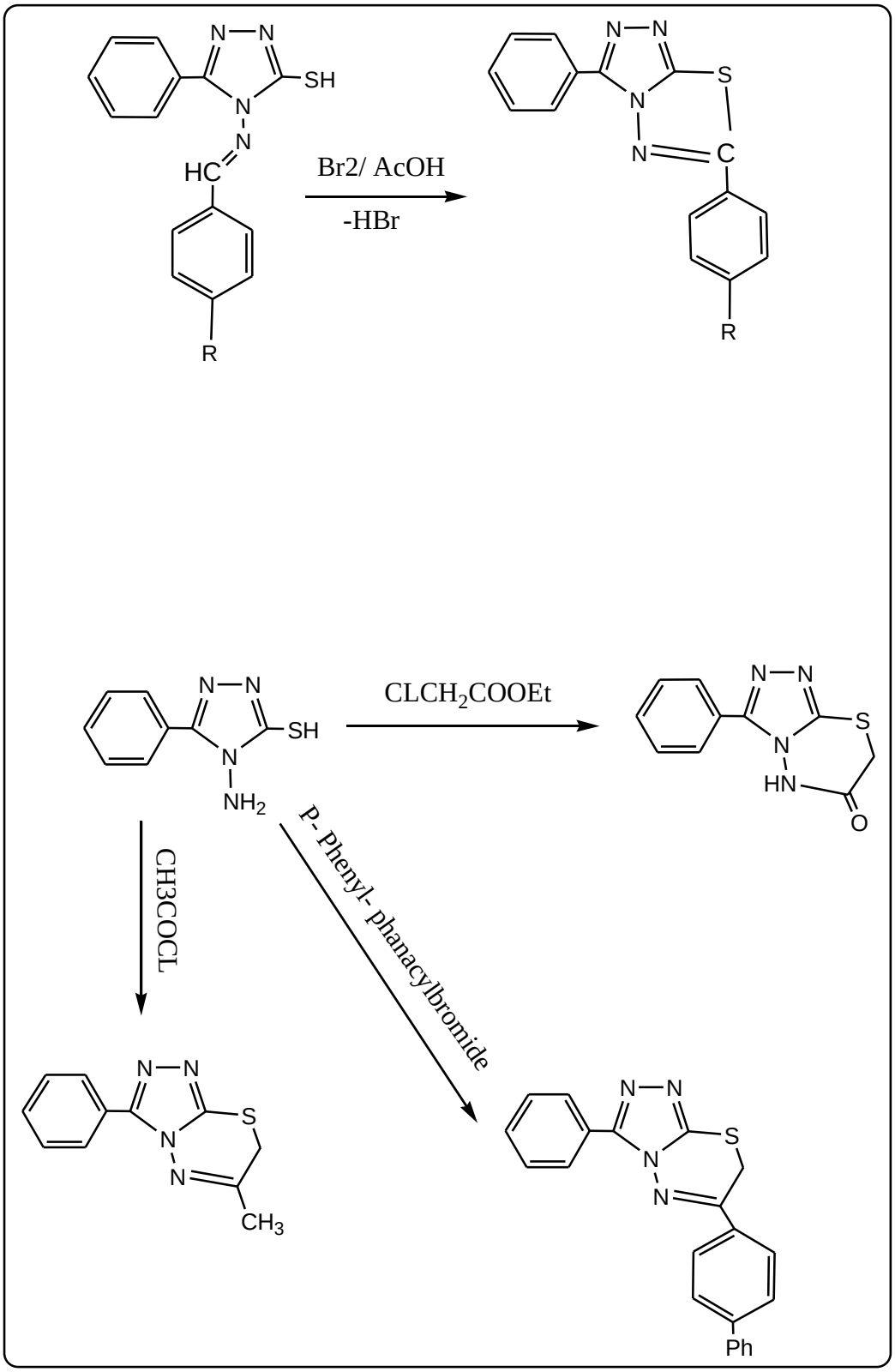
1. Preparation of new 1,2,4-triazol Schiff base derivatives with different aldehyde such as vanillin and furfuraldehyde also preparation of new complexes with different heavy metals such as Hg^{+2}, Ag^{+} and study their effects on biological and enzymatic activities.

2. Study the mechanism of action of these compounds on the enzyme through X-ray study.

3. Carry out experiments of enzyme inhibition *in vivo* on experimental animals with histological and physiological studies.

4. Evaluation of the cytotoxic activity of the synthesized compounds in vitro.

5. Cyclization between the two functional groups (SH and NH_2) of Triazol or between (SH and N=CH) of Schiff base by using different cyclization reagents to form various heterocyclic rings. As shown


Br2/ AcOH
-HBr
CLCH2COOEt
CH3COCL
P- Phenyl- phanacylbromide

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الخلاصـــــــــة

تشمل الدراسة الحالية تحضير عدد من مشتقات Triazol-١،٢،٤ من تفاعل ٤ الأمينية-٥-فينيل-٢،4-H-1٤-4،2-الترايازول-٣-ثيول (A) مع الألدهيدات العطرية المختلفة لتحضير قواعد شيف (B,C,D,E)، بالإضافة إلى تحضير المعقدات المختلفة (A1, A2, B1, B2) التي حضرت من تفاعل المركبين (A, B) مع النيكل (II) والنحاس (II) المعدنية. جميع المركبات ومعقداتها المحضرة شخصت باستخدام: مطيافية الأشعة تحت الحمراء (FTIR)، والأشعة فوق البنفسجية والمرئية الطيفية (UV-Vis.) ، طيف الرنين النووي للهيدروجين (^{1}HNMR) وقياسات التوصيلية الكهربائية. وفقاً للبيانات الطيفية للمعقدات وجد أن معقدات النيكل (II) تأخذ شكل رباعي السطوح عدا معقدات النحاس(II) حيث تأخذ شكل مربع مستو.

قُدرت الأنشطه المضادة للبكتريا للمركبات اعلاه ضد الـ(*Proteus mirabilis*) بإستخدام طريقة انتشار الابار الصغيره (Well-Diffusion).

تمت دراسة تأثيرالتثبيط المركبات المحضرة على نشاط إنزيم اليوريز. وأظهرت النتائج تأثير جيد في تثبيط إنزيم اليوريز. تم حساب السرعة القصوى (V_{max})، ثابت قوة التثبيط (K_i)، ثابت ميكايل (K_m) ونسبة التثبيط (i%) للإنزيم باستخدام معادلة خط ويفر بيرك (Line Weaver Burk Equation). وأظهرت جميع المركبات المحضرة إستعداد تثبيط تنافسي حيث إن قيمة (V_{max}) نفسها مع زيادة (K_m) باستثناء مركب (C) أظهر تثبيط غير تنافسي حيث إن قيمة (V_{max}) إنخفضت مع نفس الزيادة لـ(K_m).

بين جميع ألمركبات ألتي درست في هذا العمل، كانت المعقدات هي مثبطات قوية لإنزيم اليوريز حيث إن المعقد (B2) له قوة تثبيط (K_i = 0.017 M) و نسبة التثبيط (i% = 97.6)

جمـــــــــــــــــهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعـــــــــــــــة النهريـــــــن
كليـــــــــــــــة العـــــــــلوم
قـــــــــــــــسم الكيميـــــــاء

تحضير مركبات ١و٢و٤- ترايزول وتطبيقاتها على البكيريا المتقلبة الرائعة المنتجة لليوريز

رسالة
مقدمة الى كلية العلوم- جامعة النهرين
وهي جزء من متطلبات نيل درجة الماجستير في علوم الكيمياء

من قبل
زهراء عبد العزيز تركي الطائي
بـكـالـوريـوس ٢٠١١

المشرف
الاستاذ المساعد الدكتور: سلمان علي احمد

١٤٣٥هـ ٢٠١٤م