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Antibacterial activity of Novel Prodrugs of Amoxicillin and Cephalexin

Samia Salah Abd Algane Al -Kurd

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Prepared by

Samia Salah Abd-Algane Al Kurd

B.Sc., Pharmacy, Al-Quds University, Palestine.

Supervisor

Prof. Dr. Rafik Karaman

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Prepared by: Samia Salah Abd Algane Al-Kurd

Registration No.: 21112814

Supervisor: Prof. Dr. Rafik Karaman

Master thesis Submitted and Accepted:, Date

The names and signatures of the examining members are as follows:

1.Head of committee:Dr. **Signature ……..**

2.Internal Examiner :Dr.Ahmad Amro **Signature ……..**

3.External Examiner :Dr .Hatem Saleem **Signature ……..**

Dedication

This thesis dedicated to my parents, husband, brothers, and sisters who have supported me all the way since the beginning of my studies

Declaration

I certify that the thesis submitted for the degree of master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not be submitted for a higher degree to any other university or institution

Signed: ……..

Samia Salah Abd Algane Al -Kurd

Acknowledgement

First, I would like to thank "Allah" who enabled me to complete my study successfully.

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Abstract

The two novel prodrugs of amoxicillin and cephalexin were synthesized to improve the stability and bitter taste of their parent drugs. The in vitro susceptibility for both prodrugs was determined against *Escherichia coli, staphylococcus epidermidis*, staphylococcus *aureus, Klebsiella pneumonia, streptococcus group A, streptococcus group B,* and compared to that of their parents.

The results revealed that both novel prodrugs have antibacterial activity on most bacterial strains with about the same potency as their parent drugs, In addition, *Klebsiella pneumonia, and staphylococcus epidermidis* showed resistance to both amoxicillin drug and its prodrug. Since klebsiella is gram negative bacteria and staphylococcus epidermidise is beta lactamase positive .

It is worth noting that those two novel prodrugs are among a small number of prodrugs that have activity themselves before undergoing conversion via enzymatic or chemical processes to their corresponding parent drugs. The novel prodrugs exhibit their antibacterial activity against different types of bacterial strains due to the presence of beta lactam ring in their structures.

In addition, it is expected that the novel two prodrugs will be much more stable in aqueous media than their corresponding parent drugs due to the fact that the sensitive amine group exists in the parent drugs was replaced with a more stable group, amide

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List of Abbreviations

AST	Antimicrobial susceptibility test
β-lactam	Beta-lactam
C_{max}	The maximum concentration
DFT	Density Functional Theory
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
6-APA	6-aminopenicillanic acid
7-ACA	7-aminocephalosporanic acid

Chapter One

1. Introduction

1.1.1 Historical Background

Infectious diseases are as old as lifetime itself. They account for a major proportion of overall diseases. Infectious diseases caused by microorganisms were plaguing mankind from ancient days until the half of 19^{th} century. Accordingly chemotherapy endeavor to cure these infectious diseases. In 1910, Ehrlich synthesized salvarsan for treatment of syphilis to become the first antimicrobial drug in the world. In 1929 Fleming observed that bacterial growth was inhibited in presence of *Penicillium notatum*. This observation makes penicillin the first broad antibiotic used in 1940s and led to its use during World War II. In 1935 Domagk developed sulfonamides [2], and the second synthetic antibiotics, which were discovered in 1962 were quinolones (e.g. Ciprofloxacin). Then it was followed in 1979 by the discovery of oxazolidinones [3].
Figure 1.1 shows the timeline discovery of antibiotics with natural and synthetic origins.

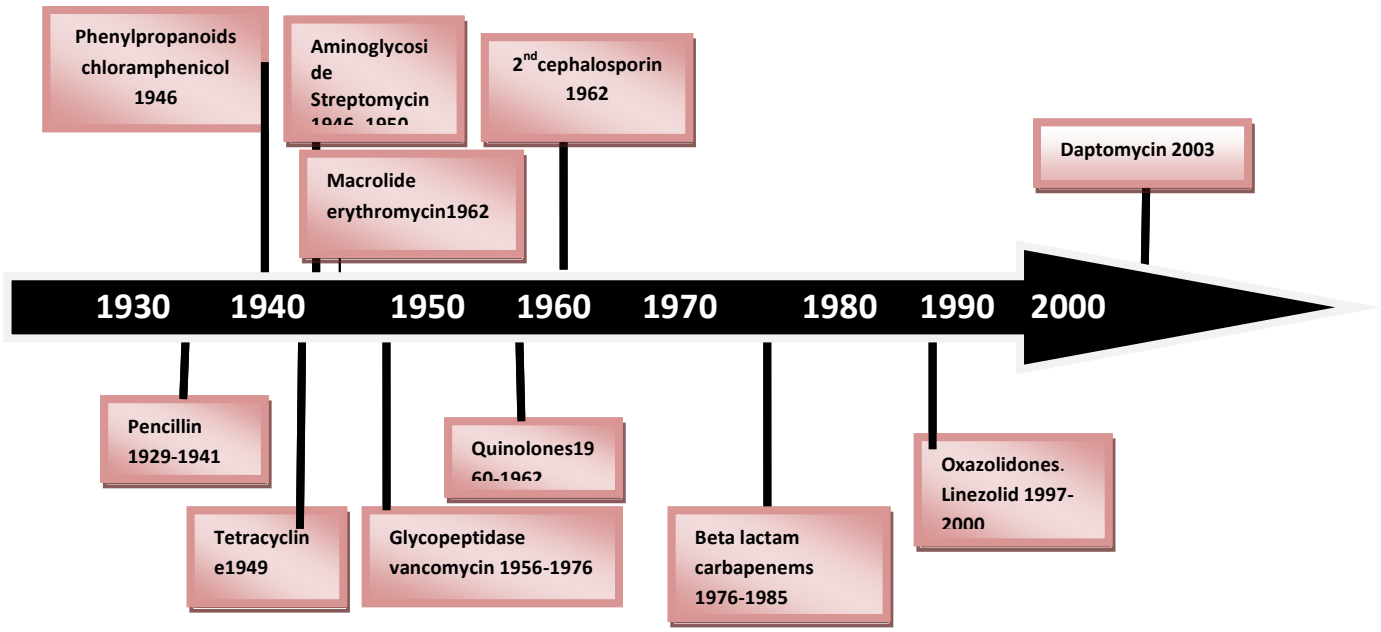


Figure (1.1): Timeline of discovery novel classes of antibiotics [3].

1.1.2 Discovery of Penicillin

John. Burdon Sanderson and William Robert independently noted that bacterial growth was prevented in the presence of fungi [4]. The same observation was noted by John Tyndall in 1876 upon surmising the antagonism of bacterial growth due to the low oxygen level, which presumably was consumed by fungi.

The first in vitro work was done by Cornil and Babes. Both scientists assessed the microbial inhibition and antagonism. They explained this observation as a substance produced by one microorganism that may serve as an antagonist for the growth of another [4, 5].

In 1887 Garre observed that the *staphylococcus pyogens* growth was inhibited in the presence of *Bacillus fluorescence*. Another notable observation which was noted by Ernest Duschesne in 1897 is the antagonism between *Penicillium* and *Escherichia* bacteria. In 1941, Selman Waksman named these observations as antibiosis [4]. The true story began in 1928 by Alexander Fleming, a Scottish bacteriologist, who became

Professor of bacteriology at St. Mary's hospital medical school in London. In the summer of 1928 before leaving London to his home in Suffolk he accumulated all his *staphylococcus aureus* culture plates on one edge of his laboratory board. When he returned he observed that there was a colony of mold growing on one side and the *Staphylococcus aureus* around this area disappeared. However, the *Staphylococcus aureus* away from the mold still had normal growth (Figure1.2).

Fleming was interested in this observation and he sub-cultured the mold and studied it. The culture of the mold was in nutrient broth and was for a period of eight days at room temperature. Fleming noticed that there was complete inhibition of growth of many bacteria. This fluid was first called mold juice; and later on the 7^{th} of march 1929, Fleming named the active substance penicillin [6, 7].

Fleming 1929's paper flagged the way to an important future research. First he pictured the mold and its growth temperatures, and observed that it has a rapid production at 20 ^{o}C. Then he studied other types of molds to check if their antibacterial activity and to confirm the unique nature of the strain he discovered.

To identify and test the antibacterial action of this strain of mold, Fleming made a cut in the agar plate where he saturated this cut with the mold's filtrate. Then when the ditch became solid, seven different microorganisms were applied to characterize the growth and inhibition mode for each. In addition, he tested the inhibition incidence in mm for different organisms and monitored the inhibitory changes as a result of dilutions of the filtrate. The procedures used by Fleming are equivalent to MIC and still in use up to these days.

Fleming had a background and expertise in immunological research at St. Mary's laboratory. This background helped him determine how to deal with foreign substance with host cell; he injected 20 mL of broth containing penicillin into rabbits and in mice which had no toxic effect as well [8]. Moreover, Fleming who already had experience in antiseptics, noted that his filtrate has an efficient effect when was applied on infected surfaces. During this time period Clutterbuck, Lovell and Raistrich extracted the active compound from the mold. They found that a pure compound could be separated by ether and watery acidic medium extractions. Upon evaporating the ether they recognized that

the activity of the compound was diminished which led them to conclude that the active ingredient (penicillin) is unstable compound in acidic aqueous medium [7].

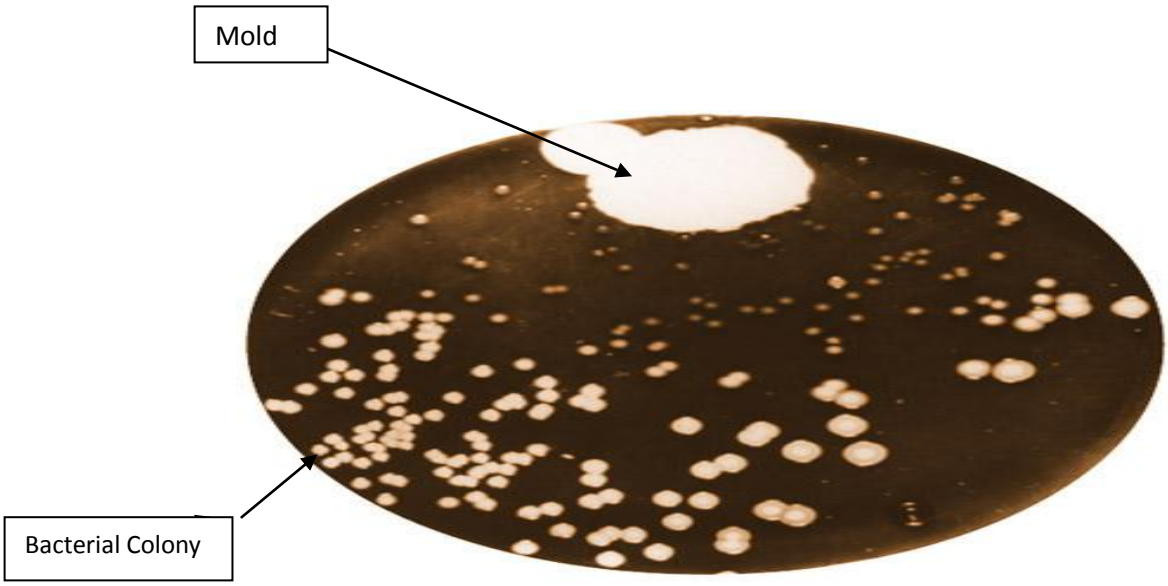


Figure (1.2): Fleming's original plate.

1.1.3 β- lactam Antibiotics Structure

Structurally, beta-lactam antibiotic molecules contain beta-lactam nucleus (6-amino pencillinc acid or 7-amino cephalosporinic acid), which provide the key for synthesis and modification (Figure1.3 a-b).

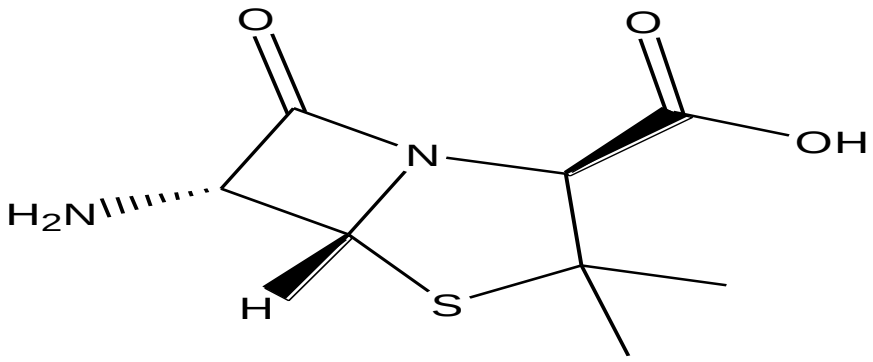


Figure (1.3a): 6-aminopenicillanic acid

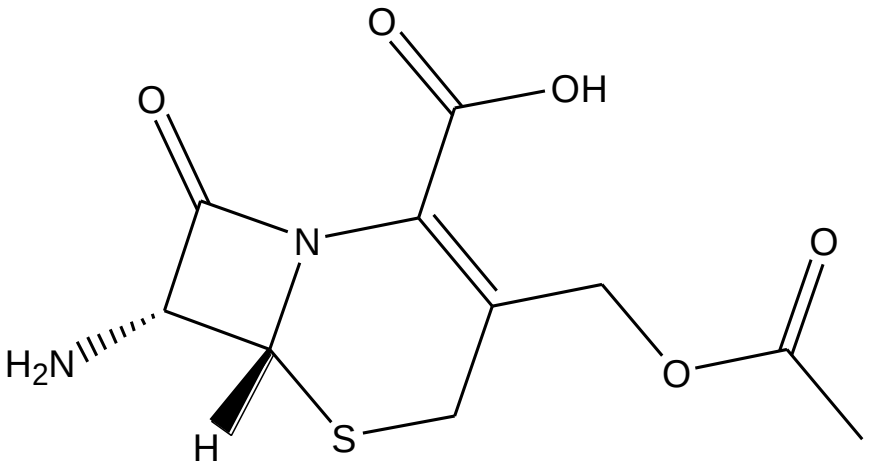


Figure (1.3b): 7-aminocephalosporanic acid

This important nucleus provides a key for novel beta-lactam agents to be synthesized by linking a unique side chain to 6-APA. Early work by J.C. Sheehan produced penicillin V by acylation of synthesized 6-APA. Thereafter, in 1960 methicillin was approved in the United States and became the first semisynthetic penicillin which is stable to enzymatic degradation, especially to penicillinase enzyme.

In addition, in 1967 carbenicillin was produced as semisynthetic compound by adding a carboxyl group instead of the amino group of ampicillin. Natural source did not stop here;

Abraham and Newton isolated a new family of beta-lactam antibiotics from *Cephalosporium acremonium* called cephalosporin C which contain 7-ACA nucleus instead of 6-APA in penicillin [5].

Chemical modification on beta-lactam antibiotics produced many semisynthetic compounds. For example, various salts or esters of penicillin such as procaine and bezathine were synthesized and used for intramuscular injection due to their poor solubility in water.

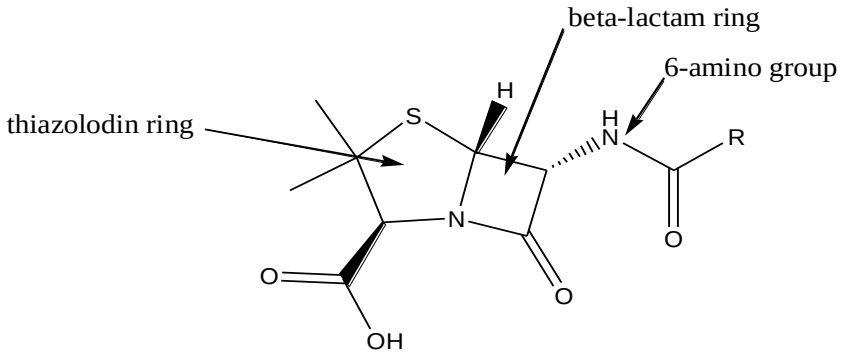
The reactive b-lactam ring containing in this group of antibiotics made them unstable and very labile. Therefore, a variety of modifications on the nucleus led to changes in their chemical properties such as increasing stability in acidic and basic media, lower degradation by enzymes and a broader spectrum of activity [9].

Pencillinc acid in (Figure 1.4a) shows the core structure of penicillin which upon conversion to its Na^+ or K^+ salts provides soluble compounds and upon substitution with benzathine gives insoluble agent as mentioned earlier.

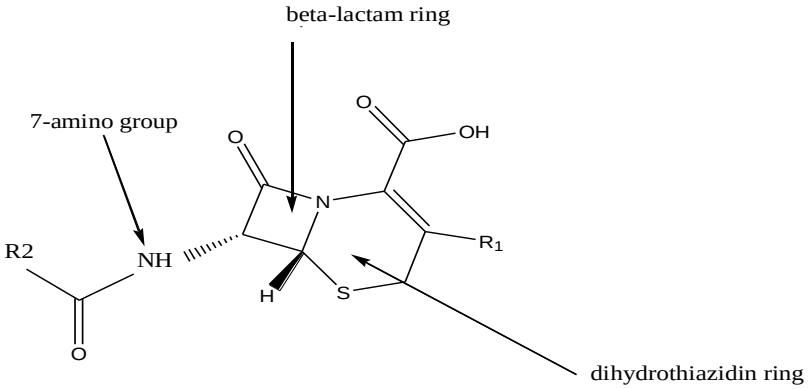
The more important modifications in this structure occur on the R group; because the β-lactam ring reactivity and stability depend on the side chain substitution. This is essential for the action of β-lactam antibiotics to act as anti-bacterial agents.

The first semisynthetic modification was changing the side chain R in penicillin G with other side chains. For example, phenoxyethyl, phenoxymethyl, where the β-lactam ring is less reactive to H^+ due to the change of the electron distribution and a creation of more stable entities.

Figure 1.4B shows the basic chemical structure of cephalosporin which has a basic structure as penicillin, but it has six-member dihydrothiazine ring instead of the thiazolidine ring in penicillin. Both R_1 and R_2 provide opportunity for essential side chains modifications which result in changes of many properties of the compounds.



(A) Penicillin



(B) Cephalosporin

Figure (1.4): Penicillin's and cephalosporin's core structure, where the R groups are the variable [1].

The main entity contained in both structures shown in Figure1. 4 essential for the antibacterial activity is the β-lactam ring (Figure. 1.5). This entity interacts with active sites in the bacteria and produces the desired antibacterial effect. This happens when C—N bond in the β-lactam opens and binds to a carbon atom in the bacteria's site of action by covalent bond, resulting in acylation of an important group needed for cell wall synthesis [1].

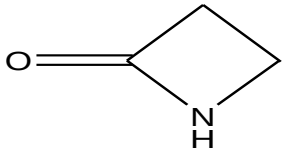


Figure (1.5): Beta-lactam ring

1.1.4 Mechanism of Action

Cell wall in bacteria is an important structure in both Gram positive and Gram negative bacteria because of stress bearing and shape maintaining function [10]. It is a complicated structure which is composed of multiple types of polymers, peptidoglycans, teichoice acid and lipopolysaccharides. However, the most important among these is peptidoglycan because it is essential for cells living under normal growth conditions.

Transpeptidase enzyme interacts with the peptide linkage contained in the pentapeptide chain of the uncrossed linked peptidoglycan (terminal D-alanine). This interaction results in D –alanine release and an acyl enzyme intermediate formation.

Penicillin behaves like terminal D-alanine in the pentapeptide chain. The CO-N bond in β-lactam structure crosses bond to the peptide bond during trans-peptidation. Thus new transition state is formed and peptide bond is cleaved; when the enzyme cleaves the β-lactam ring it forms a stable pencicilloyl-enzyme complex resulting in an inhibition of the transpeptidase enzyme (Figure1.6) [11] . Sequence analysis of the peptides derived from active site-labelled enzymes has established that both penicilloyl and an acyl moiety derived from substrate are covalently bound to the same site, as an ester of serine 36.[12]

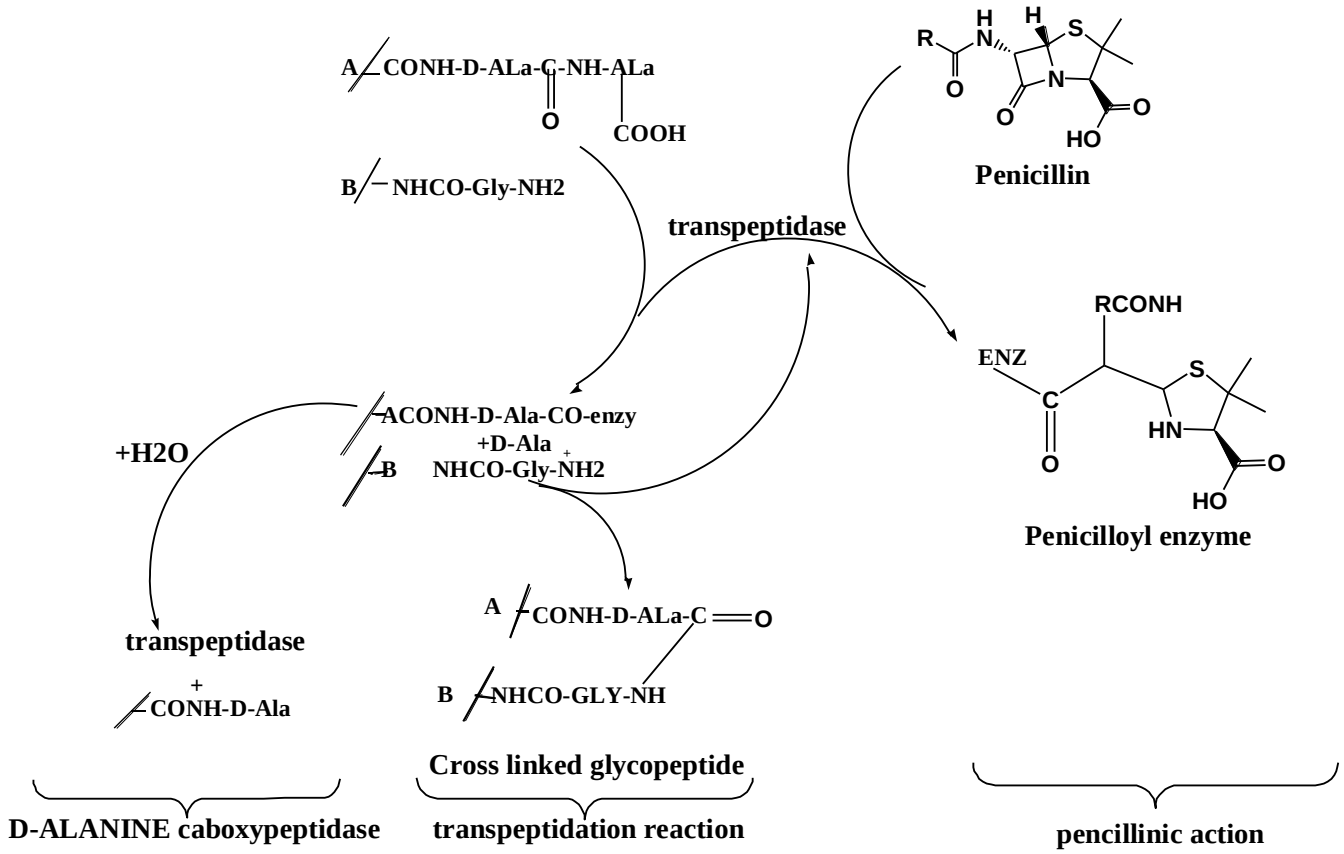


Figure (1.6): Proposed mechanism of transpeptidation

1.1.5 Development of Amoxicillin

In 1972 Amoxicillin was prepared in the UK, which has the same activity as ampicillin, but with higher bioavailability [13]. Later a combination of amoxicillin with clavulanic acid was developed to introduce oral bioavailability and broad spectrum activity against a variety of pathogens that produce β-lactamase enzyme [14].

As mentioned earlier, penicillin contains β-lactam ring which is important for activity and mechanism of action. On the other hand, it is sensitive and labile structure in presence of basic or acidic media and also can be inactivated by β-lactamases enzymes. However, an addition of an amide group to the 6-position in β-lactam ring can make the compound more stable in acidic conditions, due to the fact that the amide oxygen becomes less nucleophilic compared to that in amoxicillin [15].

In 1950, β-lactam group was limited for two drugs with low spectrum of activity, penicillin V and penicillin G. As a result, scientists in the field have had interest to introduce new compounds of penicillin by a fermentation process, however, this way gave limited diversity of compounds [13].

Later in 1957, 6-aminopenicillanic was used as a precursor to synthesize new beta lactams; and in 1960 stable methicillin was produced. Then the synthesis continued until the discovery of broader spectrum penicillin, ampicillin. In 1970 amoxicillin was made with better bioavailability than ampicillin [15].

1.1.5.1 pharmacology and Pharmacokinetics

As amoxicillin acts on cell wall of bacteria; it has bactericidal action against both gram positive and gram negative. Amoxicillin is used for many indications; treatment middle ear infection [15] laryngitis, bronchitis, pneumonia [16], and typhoid fever [15].
Amoxicillin is the most commonly prescribed antibiotic for children, it is well absorbed after oral administration, used for treatment in a variety of infections not only for broad spectrum also for outstanding advantage in comparison to other penicillin with higher bioavailability of 70-90%, and reaches C_{max} within 1-2hours [15]. Amoxicillin is widely distributed in the body and the apparent volume of distribution is 0.26 - 0.31ml/kg, it has half-life 1-1.5 hours[17]. Also it is excreted by the renal route and approximately 10-25% of the drug is bio-transformed into pencillinc acid [15].

1.1.6 Cephalosporin

Cephalosporins are related to penicillin β-lactam antibiotics; they act on cell wall of bacteria; interfering and lysing bacterial cell wall. This action is achieved by drug's crossing and binding to penicillin binding protein in the cell wall (site of action) [18].
Cephalosporin has no activity against enterococcus due to low affinity on penicillin binding protein. However, it has different activities against *Pseudomonas aeruginosa* and *Enterobacteriacea*, because of differences in binding on the active site located on the bacteria's cell wall [19].
Structure activity relationship and differences in side chain substitution at C7 position of the main core of cephalosporins led to a various pharmacokinetics properties, spectra of activity, and β-lactamase stability (Figure1. 4 b).
Alteration of the substituent on C7 by the addition of methoxy group (cephamycin) or replacing the sulfur in dihydrothiazine ring with oxygen (moxalactam) led to increase stability against enzymatic hydrolysis by β-lactamase [18].

1.2 Research problem

1.2.1 Stability

Conventional oral suspensions and solutions of antibiotics dispensed as powders need to be reconstituted with water at the time of use. Reconstitution process is formulation of choice for compounds with low stability; this reconstitution system allows acceptable but short life with storage in refrigerator [20]. The highly strain β–lactam ring that present in both penicillin and cephalosporin structures is unstable in solution; hydrolysis occur and as a result loss of activity.
So, when the side chain have been altered one can change the activity and physiochemical properties such as stability [9].

The degradation process is an irreversible chemical change in organic molecular structure [21]. The degradation of penicillin can occur in different conditions; acidic or alkaline, in presence of weak nucleophile as water and β-lactamase enzyme.

1.2.2 Bitter taste

The palatability of the active ingredient of a drug is a significant obstacle in developing a patient friendly dosage form. Organoleptic properties such as taste are an important factor when selecting a certain drug from the generic products available in the market that have the same active ingredient. It is a key issue for doctors and pharmacists administering the drugs particularly for pediatrics and geriatrics [22].

The problem of the bitter taste of drugs in pediatric and geriatric formulations still creates a challenge to pharmacists. Thus, different strategies should be developed in order to overcome this serious problem. The novel chemical approach to be discussed in this thesis involves the design of prodrugs for masking bitter taste of pharmaceuticals based on intramolecular processes using Density Functional Theory (DFT) methods [23]. No enzyme is needed to catalyze the intra-conversion of a prodrug to its corresponding drug. The rate of drug release is controlled by the nature of the linker bound to the drug. Bitter tasting molecules interact with taste receptors on the tongue to give bitter sensation. Altering the ability of the drug to interact with bitter taste receptors could reduce or eliminate its bitterness. This could be achieved by an appropriate modification of the structure and the size of the bitter compound [24].

Based on DFT calculations and experimental values obtained from intramolecular acid catalyzed hydrolysis in nine maleamic acid, Karaman's group designed and synthesized two pure prodrugs of amoxicillin and cephalexin by linking these two antibiotics with a maleic anhydride linker in order to: (1) improve the stability and aqueous solubility of the parent drug (2) provide drugs without bitter taste.[25]

The two synthesized prodrugs were designed such that the amine group in the parent drug converts to its corresponding amide. Thus, the prodrugs are expected to be more

stable than their corresponding amino parent drugs (Figure 1.7). The current thesis is devoted to study the antimicrobial activity of the two synthesized prodrugs.

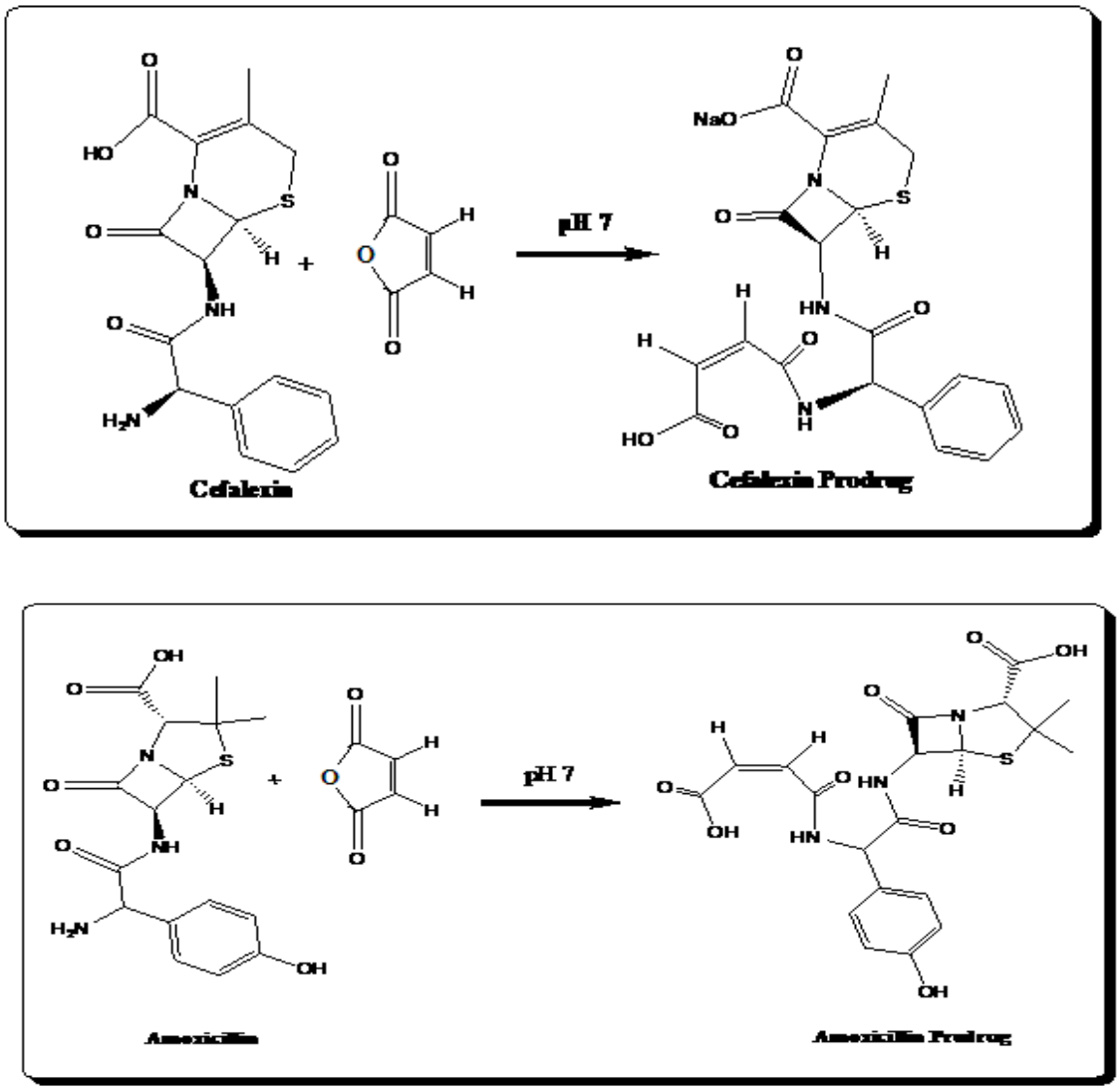


Figure (1.7): Chemical structures for the proposed prodrugs of Amoxicillin and Cephalexin[25]

1.3 Research objectives

1.3.1 General Objective

To assess the antibacterial spectrum of two novel prodrugs of amoxicillin, and cephalexin

1.3.2 Specific objectives

- To compare activity of prodrugs with their parent compounds.
- To determine the minimum inhibitory concentration (MIC) and (MBC) of the prodrugs.

1.4 Research Questions

1. Do the two synthesized prodrugs possess anti-bacterial activity?
2. What is the antibacterial activity of the prodrugs relative to their parent drugs?

Literature Review

Chapter Two
Literature Review

2.1 History of prodrug research

In 1958 Albert introduced the term prodrug in his article in *nature:* avail a pharmacological inactive moiety that can be used to change a drug's physiochemical properties. Later, Harper has built on this concept but the term he used is drug latentiation.

The use of the term generally applied when a drug is linked to chemical moiety by covalent bond and the cleavage to release the parent drug can be achieved by chemical or enzymatic mean.

Prodrug is designed to be utilized through a chemical approach, thus it is an alternative and short cut of redesigning drug molecules [26-29].

Prodrug approach can be utilized to:

1. Increase solubility and bioavailability of an active drug

2. Improve permeability and in turn increase drug’s absorption

3. Modify the drug’s distribution profile [30-33].

According to Albert s definition “a prodrug is inactive form of its parent drug” [29]. But there are few prodrugs that have activity before their conversion via enzymatic or chemical processes to their parent drugs [34].

2.1.1 Earlier Examples of Prodrugs

In 1899 aspirin was introduced as less irritating form of sodium salicylate [35], and methenamine which discovered by Shering to give its parent drug, formaldehyde, which acts as antibacterial agent for urinary tract infection [36].

Prontosil was discovered in 1935 as the first sulfa drug and a prodrug of sulfanilamide which ushered in the era of sulfonamide antibiotics. [37].

Acetanilide and phenacetin were originally not designed as prodrugs, however their nature as prodrugs was determined later; acetanilide exhibited its activity as a result of its metabolite acetaminophen, and phenacetin due to the fact that upon metabolism it undergoes o-dealkylation to acetaminophen. Therefore, the nature of these drugs being prodrugs was determined in hindsight. Other hindsight examples include phenylbutazone metabolized to oxymethylbutazone, primadone to phenobarbitone, codeine to morphine. In these examples both the prodrug and its parent drug have pharmacological activity [34].

Albert mentioned in his *"selective toxicity book"*, I apologize for having invented the term prodrug, now too widely used to alter, for literary purists tell me they would have preferred 'predrug' [33] .

Albert essentially affords the prodrug concept legitimacy as a tool in drug discovery and development.

A number of novel prodrugs that were designed based on enzyme models were studied by Karaman's group, among those are aza-nucleoside derivatives for the treatment of myelodysplastic syndrome, paracetamol as a pain killer, anti-malarial atovaquone, anti-Parkinson dopamine, anti-viral acyclovir, antihypertensive atenolol, antibacterial cefuroxime, anti-psoriasis monomethyl maleate, and phenylephrine as decongestant. In vitro kinetic studies in a wide pH range have shown promising results for obtaining novel prodrugs that may have enhanced dissolution, membrane penetration, and thus better bioavailability than their corresponding parent drugs. In conclusion, based on the

examples discussed, precise terminology can be drawn as follows: inactive prodrugs should be named predrugs and active prodrugs should be named drug-predrugs [34].

2.2.1 Antimicrobial Susceptibility Test (AST)

The importance to know whether a microorganism is responding to antibiotics therapy is old as chemotherapy itself [38].

With an increase in the use of traditional antimicrobial agents, bacterial resistance emerged, it has created a problem for clinicians to select suitable antimicrobial agent [39], so in vitro antimicrobial susceptibility test should be used for relevant bacterial pathogens [40].

The aim of in vitro (AST) is to give dependable predictor of how the pathogen responds to the chemotherapy in infected host, this important data provide the clinicians the tool to pick out the suitable antimicrobial therapy, helps in developing antimicrobial usage policy and supply information for epidemiological surveillance [38].

In 1920 Fleming made two important contributions on AST methods; in 1924 he introduced the use of the dish plate for estimating antimicrobial qualities of antiseptic solution [41]. Broth dilution method utilizing was Fleming's second contribution by which the use of turbidity as end point determination [42]. Later he developed this method by using pH as an indicator instead of turbidity [43] .

Diffusion technique of AST was introduced in 1940, absorbent paper containing antimicrobial agents introduced by Heatley [44], and Vincent introduce filter paper discs incorporating penicillin [45] .

Mohs utilized "radial streak disc method, where the disks has diameter 15 mm, this was the first procedure of a comparison of test organism in the same plate with test control [46].

In 1940, agar dilution AST method was developed by Schmitt Reymann. This method is done by incorporating antimicrobial agent into agar plates[42].

In 1950, everyone has realized that it is important to standardize AST measurement; consequently many organizations began addressing this important need. The world health organization set a report on standardization of AST methodology [47].

In 1966, a significant development happened with the introduction of standardization of disk method which was done after Bauer, Kirby and co-workers published their attempt to set the disk diffusion method as practical technique of testing with broad application to clinical laboratories [48]. This method became the basis of the national committee for clinical laboratory standard (NCCLS), disk diffusion standard [42].

2.2.2. Antimicrobial Susceptibility Testing Methods

2.2.2.1 Disk Diffusion Method

The disc diffusion method is simple, practical, qualification test, that is well standardized [49].The test is done by bacterial inoculums of 1-2 × 10^8 cfu/ml.to the mullar- Hinton agar plate, then paper antibiotic disks or strips with fixed concentration are placed on the inoculated agar surface. After plates incubation at 35°C for 16-18 hours the results are determined by reading and recording the zone of growth inhibition using the ruler. The diameter of the zone is related to the diffusion rate of the drug and to the susceptibility of organism [50] (Figure2.1).

The advantages of this method are the flexibility and test simplicity which does not require any special equipment. In addition, it is the least costly among susceptibility methods.

The disadvantages include that not all types of bacteria which has slow growth rate can be accurately tested using this method, and for some bacteria there is a need to use specialized media or incubation conditions for testing (e.g. *Neisseria.meningitidis, Hemophilas influenza*). In addition, no automation in this test is possible [49].

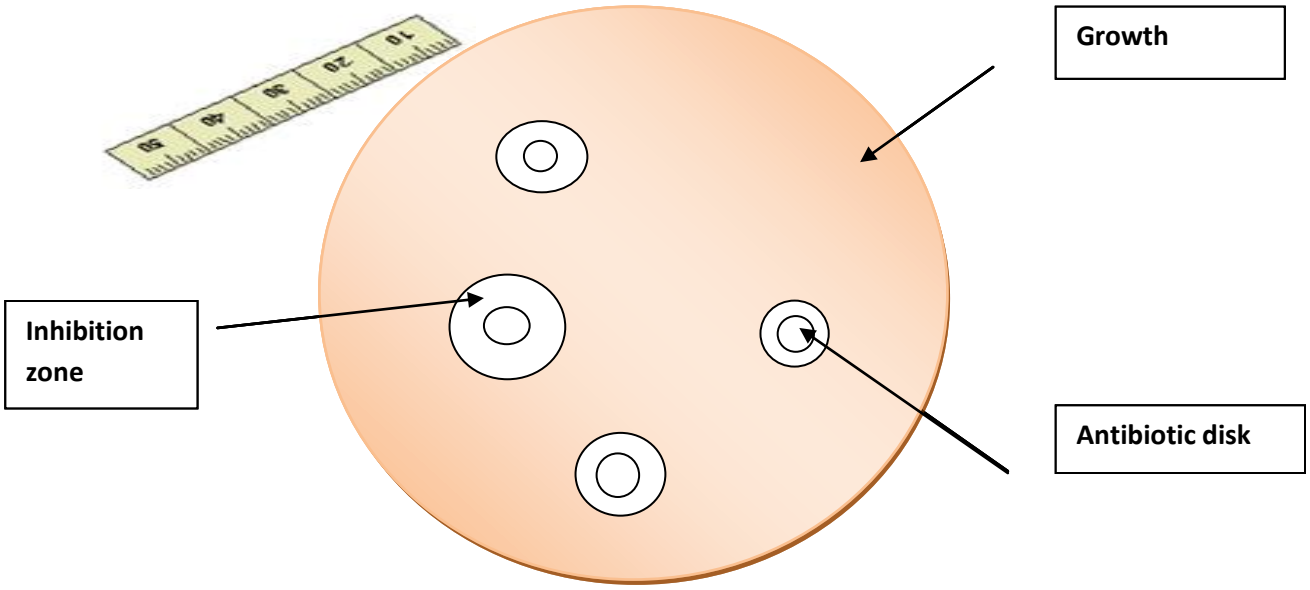


Figure (2.1): The antimicrobial susceptibility disk diffusion test: approximate disk placement and measurement of inhibition zone diameters.

2.2.2.2 Broth and Agar Dilution Method

The purpose of the broth and agar dilutions test is to set the lowest concentration of the assayed antimicrobial that inhibits the growth of tested bacterium and also to get quantitation result.

The MIC is a result of the lowest test concentration that inhibits the bacterium; however, it can be between that concentration and the next lower one. Hence, MIC does not always appear as an absolute estimate.[51]

2.2.2.2.1 Broth Dilution

The tube dilution method is one of the earliest antimicrobial susceptibility testing method; its technique performed by suspension of bacterium (which previously determined optimal concentration) is tested against different concentrations of an antimicrobial compound.

This method can be done either in tubes containing 2 ml as minimum volume (macro-dilution) or (micro-dilution) using micro-titration (Figure 2.2).

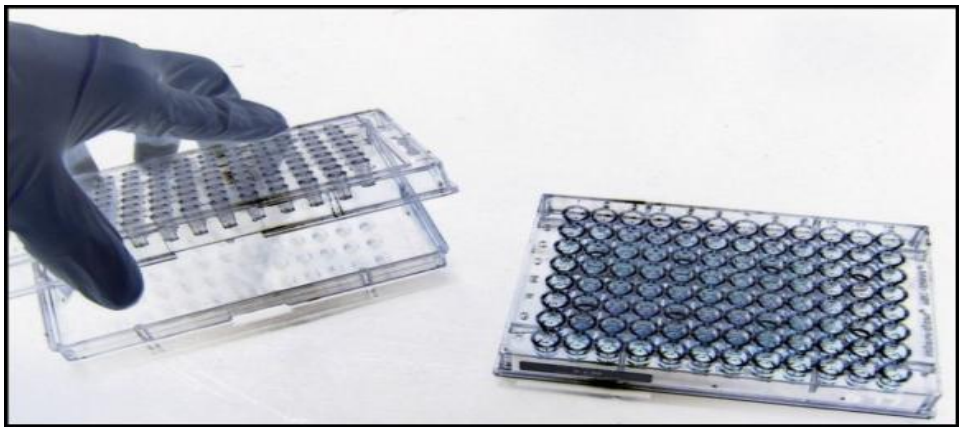


Figure (2.2): A broth micro dilution susceptibility panel containing 98 reagent wells and disposable tray inoculators.

The tubes containing antibiotics are inoculated with bacterial suspension and incubated at 35°C. After overnight incubation the tubes are tested to determine the MIC by visible bacterial growth estimated by turbidity. The lowest concentration that inhibits the growth of bacterial appears as minimum inhibitory concentration.

The advantages of this method is that it gives quantitative result [52]. In addition, the same tubes can be taken for minimum bactericidal concentration assay [51].

The disadvantages of this method; it is not simple since there is a need for preparing the antibiotic solutions for each test and errors may occur during preparation. Furthermore, it is costly and less flexible than the disk method.

2.2.2. 2.2 Agar Dilution

Agar dilution is performed by incorporation of different concentrations usually two folds of the antimicrobial agent into an agar plate; then the defined bacterial inoculum is

applied to the agar surface. Agar dilution test is recommended as standardized AST method for fastidious organisms [52].

2.2.2.3 E Test

The E_test is an in vitro procedure developed to set minimum inhibitory concentration of an antimicrobial compound on agar medium; the E_test avoids some disadvantages of the disk and broth methods. In addition, it simply gives quantitative MIC.
The E_ test consists of continuous concentration gradient of stabilized and dried drug that carried on a thin reagent strips; the E_test performed by swab the inoculated bacteria on an agar plate as the same principle in agar diffusion test; then one or more E_test strips of the antimicrobial compound to be tested are placed on the agar surface. Following an overnight incubation, the results appear as elliptical inhibitory zone (Figure 2.3) [53].

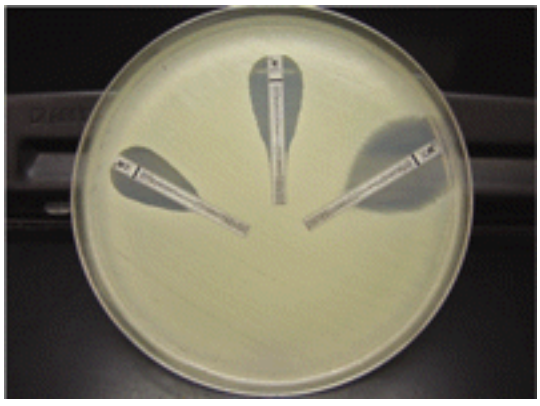


Figure (2.3): The minimum inhibitory concentration of each agent is determined by the intersection of the organism growth with the strip as measured using the scale inscribed on the strip [49].

2.2.2.4 Automated Instrument System

Use of instrument produces susceptibility test results in a short period and standardizing the reading of end points; its optical sensitive detection system which can detect changes in bacterial growth; the instrument incubates the trays over a period of time, testing them periodically with either fluorometric or photometer to examine a growth development [49].

2.3 Minimum Bactericidal Concentrations (MBC)

As mentioned previously the advantage of using the broth dilution method for MIC set is the ability to use the same tubes and continue working to determine MBC.[51]

2.4 Antimicrobial Activity of Amoxicillin and Cephalexin

2.4.1 Amoxicillin Activity

Penicillins have been divided into classes based on their spectrum of activity; the first agent that was used clinically to treat infections is the natural penicillin (penicillin G), but after the emergence of penicillinase in *staphylococci* penicillins became inefficient for these organisms. Therefore, development of penicillinase resistant-penicillins was initiated; this led to the development of three categories of penicillins: the aminopenicillins, carboxypencillins and ureidopencillin [54].

Aminopenicillins was the first class of penicillin antibiotic that has activity to both gram positive and gram negative bacteria; ampicillin compared to natural penicillin has more activity against *enterococci,* but somewhat less activity against *pyogens, streptococcus pneumonia, and Neisseria species.* On the other hand, it has some activity against *E.coli, proteus Mirabella, salmonella, shigella, listeria,* which are gram negative bacteria [54].

Amoxicillin has shown to be effective against a variety of infections, which are caused by gram positive and gram negative bacteria in humans and in animals [55]. Amoxicillin has a higher activity against gram positive than gram negative microorganisms [56]. In addition, it has greater efficacy relative to penicillinV and other antimicrobial such as ampicillin [15] and cefuroxime [57].

Different study reports showed that amoxicillin was effective at MIC ranges 0.06 μg/ml - 4 μg/ml against variety of microorganism, except *staphylococcus .epi* 64 μg/ml and *staphylococcus aureus* MIC up to 256 μg/ml [58].

In a study, amoxicillin and ampicillin showed that the kill rates for amoxicillin was higher than ampicillin for *E.coli,* and the rate of killing was the same for both agents for *Staphylococcus Aureus,* but amoxicillin showed longer bacteriostatic phase which was not observed with ampicillin [15].

In another study an investigation on the antibacterial activity of amoxicillin and ampicillin against 30 isolates of each *proteus mirabilis, Klebsiella, E.coli, Enterobacter* and *idol positive proteus* was carried out, and the results obtained are as follows:

89% of the strains of *E.coli* was inhibited by both drugs at 10 μg or less per ml, and at 5. μg or less *Proteus.mirabilas* was inhibited by both drugs.

On other hand, high response of resistance to amoxicillin and ampicillin was seen among strains of *klebselia, enterobacter and idol positive* species [59].

In addition, other studies showed that amoxicillin was especially active against *group A hemolytic streptococci, penicillin G susceptible staphylococcus aureus* and *pneumococci* only 28% of *S. aureus* isolates which were resistant to 50 μg of penicillin G per ml were susceptible at 50 μg/ml or less to amoxicillin, 76% of *p.mirabilas* isolates were susceptible to amoxicillin at 1.56 μg/ml or less and 20% showed resistant to 12.5 μg/ml or more, 75% of *E.coli* isolates were susceptible to 6.65 μg/ml or less and most of the remaining isolates were resistant to 50 μg/ml or more [60].

2.4.2 Cephalexin Activity

First generation cephalosporins are cefazolin, cephapirin and cephalothin for intravenous use and cephalexin, cephradine, and cefadroxil which are used orally. All of these cephalosporins are similar in spectrum of activity. They have high activity against gram-positive cocci. They have low activity against gram negative bacteria. In addition, most strains of *Escherichia coli*, *Klebsiella* species, and *Proteus mirabilis* are sensitive to this class of drugs. They have no activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-*resistant Staphylococcus epidermidis* (MRSE), as well as *enterococci*, *Listeria monocytogenes*, *Bacteroides fragilis*, *Citrobacter*, *Enterobacter*, *Proteus* (other than *mirabilis*), *Providencia*, *Pseudomonas*, and *Serratia* organisms. Gram-positive anaerobes like *Peptostreptococcus* and non-penicillinase producing *Bacteroides* species are usually sensitive [18].

Cephalexin is used for the treatment of the upper and lower respiratory tract infections, genitourinary system, skin, soft tissue, bones, joints and many other infections due to susceptible organisms [61].

Experimental Part

Chapter Three

Experimental Part

3.1. Media Preparation

Brain heart infusion agar, Muller Hinton agar (Becton ,Disckinsonand company sparks USA)and nutrient broth(hemedia laboratories pvt.ltd) were prepared in concentrations of 52 gm/L, 38 gm/L and 13 gm/L, respectively.

3.2. Preparation of the Buffer Solution

Buffer solution (pH=7.4) was prepared by dissolving 0.68 gm of potassium dihydrogen phosphate in100 ml water, then NaOH was added and the solution was stirred.(pH meter model HM-30G: TOA electronics™ was used to measure the pH value for all buffers and reaction media involved in this study.)

3.3 Test Microorganisms

Reference strains obtained from American Type Culture Collection (ATCC) were used *(Staphylococcus aureus (ATCC 25923), Staphylococcus epidermidis (ATCC 12228), Escherichia coli (ATCC 25922), Klebsiella pneumonia (ATCC 700603), Streptococcuspyogens (ATCC 19615),* and *Streptococcus group B* obtained from microbiological labs (Al-Quds University).

3.3.1Preparation of Inocula

Part of an isolated bacterial colony was inoculated in 5 ml nutrient broth & incubated for 24 hours at 37^{o}C, the growth turbidity in nutrient broth was adjusted by further incubation or dilution with sterile physiological saline; after comparison with that of a McFarland nephlometer tube no. 0.5 (10^{8}cfu/ml) using spectrophotometer at 625 nm (optical density of 0.08-0.1)

3.4 Antimicrobial Activity Screening Methods

3.4.1 Disk Diffusion Method

With a sterile cotton applicator 10^8cfu/ml of each bacterial strain was swabbed on Muller Hinton agar (for *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Klebsiella pneumonia)* while brain heart infusion agar was used for *Streptococci spp*. in the following manner

1. The cotton applicator was dipped into the bacterial suspension, rotated several times and pressed against the inside wall of the tube to remove excess inoculum.
2. The agar plate was then streaked in three different directions and around the agar margin to ensure even distribution of the inoculum.
3. The plates were left to dry for 3-5 minutes.
4. Using sterile forceps the disks, which contain prodrugs, drugs, negative control were then distributed evenly on the surface of the agar plates.
5. The plates were incubated upside-down at 37 ^{o}C.
6. The inhibition zone around each disk was measured using a transparent ruler.

3.4.2 Broth Dilution Method

3.4.2.1 Preparation of Media

For each strain 13 tubes, each contains 9.9 ml Muller Hinton broth were prepared and autoclaved.

3.4.2.2 Preparation of the Active Ingredient Dilutions

Two main prodrugs of amoxicillin and cephalexin along with their parent drugs (Pure standards (>99%) of amoxicillin and cephalexin were available commercially from Sigma Aldrich & cephalexin), as positive control were used. 500 mg of each drug and prodrug were dissolved in 10 ml of buffer solution pH 7.4, that has no effect on tested microorganisms and the prodrugs have maximum stability[25], to give a final concentration of 50 mg/ml. Then several dilutions of stock solution were prepared as shown in Table 3.1.[62]

Table 3.1: Dilutions of active ingredients

Tube no.	Stock solution 50 mg/ml	Buffer (mL)	Final concentration mg/ml	Volume of broth	Final volume added to each tube
1	1 ml	0	50	9.7 ml	0.3 ml
2	1 ml	0	50	9.75 ml	0.25 ml
3	1 ml	0	50	9.8 ml	0.2 ml
4	1 ml	0	50	9.9 ml	0.1 ml
5	0.9 ml	0.1	45	9.9 ml	0.1 ml
6	0.8 ml	0.2	40	9.9 ml	0.1 ml
7	0.7 ml	0.3	35	9.9 ml	0.1 ml
8	0.6 ml	0.4	30	9.9 ml	0.1 ml
9	0.5 ml	0.5	25	9.9 ml	0.1 ml
10	0.4 ml	0.6	20	9.9 ml	0.1 ml
11	0.3 ml	0.7	15	9.9 ml	0.1 ml
12	0.2 ml	0.8	10	9.9 ml	0.1 ml
13	0 ml	1	0	9.9 ml	0.1 ml

The experiment was repeated with *Klebsiella*, which required lower concentrations to find MIC and MBC. Broth tubes containing the active ingredients with different concentrationswere prepared as shown in the Table 3.2.

Table 3.2: Dilutions of active ingredients used for klebselia

Tube no.	Stock solution 50mg/ml	Buffer (mL)	Final concentration mg/ml	Final concentration mg/0.1ml
1	1	0	50	5
2	0.9	0.1	45	4.5
3	0.8	0.2	40	4
4	0.7	0.3	35	3.5
5	0.6	0.4	30	3
6	0.5	0.5	25	2.5
7	0.4	0.6	20	2
8	0.3	0.7	15	1.5
9	0.2	0.8	10	1
10	0.1	0.9	5	0.5
11	0.05	0.95	2.5	0.25
12	0.025	0.975	1.25	0.125
13	0	1	0	0

3.4.2.3 Incorporation of Active Ingredients into Media

For the incorporation of the active ingredients into media, 13 broth tubes each contains broth volume as shown in Table 3.1 in to broth tube 1, 300 microliter (0.3 ml) of stock solution was added into broth tube 2, 250 microliter was added and 200 microliter into broth tube 3.

The procedure was repeated for the remaining dilutions by adding 100 microliter (0.1 ml) for each tube, the final concentrations of the active ingredients in broth are shown in Tables 3.3 and 3.4.

Table (3.3): Final concentration of the active ingredients,amoxicillin ,cephalexin(drug and prodrug) in the medium. Against *staphylococcus aureus, Staphylococcus epidermidis Escherichia coli , Streptococcus pyogens group A,* and *Streptococcus group B*

Tube no.	1	2	3	4	5	6	7	8	9	10	11	12
Concentration microgram/ml	150	125	100	50	45	40	35	30	25	20	15	10

Table (3.4): Final concentration of the active ingredients amoxicillin ,cephalexin(drug and prodrug) used for Klebsiella

Tube no.	1	2	3	4	5	6	7	8	9	10	11	12
Concentration microgram/ml	50	45	40	35	30	25	20	15	10	5	2.5	1.25

3.4.2.4 Determination of Minimum Inhibitory Concentration (MIC)

All tubes were inoculated with 10 µl of the tested bacterial suspension; the tubes were then incubated for 24 hours at 37^{o} C.

After incubation, the tubes were examined for turbidity, indicating a growth of microorganisms; the organism will grow in the negative control tube (tube no.13) that does not contain antimicrobial agent to inhibit growth. The lowest concentration of the prodrug that inhibits a growth of the organism, as detected by a lack of visual turbidity is designated as the MIC (Figure 3.1.)

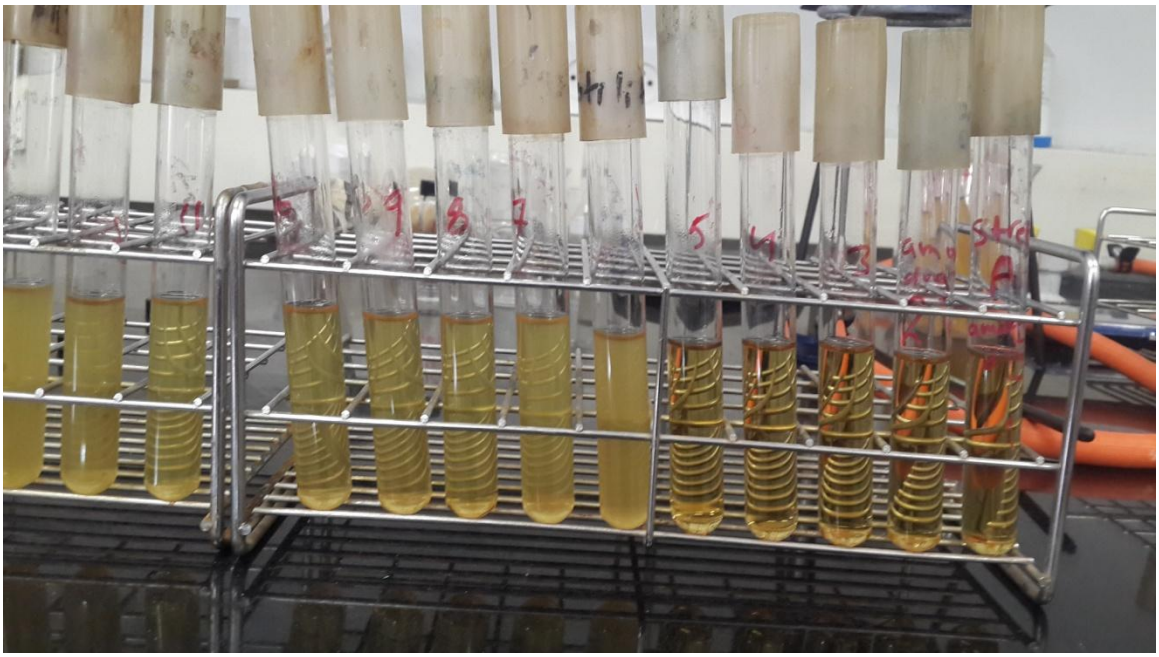


Figure (3.1): Broth dilution susceptibility test; the tube number 5 lacks of visual turbidity.

MBC is interpreted to be at a tube that shows no growth on the agar plate for example Figure 3.1 shows that MIC for the test illustrated in the figure in tube no. 5 is 45 microgram per ml; while MBC is checked by testing viable colonies in tubes 1-5.

Therefore, after reading the results of MIC by recording the lowest concentration that inhibits the organism growth, the following procedure is followed:

1. Sub-culturing of all tubes which have no visible growth by spreading loop full over quarter of the agar plate.
2. incubation at 37 °C and After overnight incubation read the result and record as follow:

- Bacteriostatic if similar number of colonies are present
- Partial bactericidal if reduced number of colonies are found
- No growth indicates that the whole inoculums have been kille[51]

Results and Discussion

Chapter Four

Results and Discussion part

4.1 Screening of drug an prodrug inhibition of bacteria showing zone of inhibition diameter in mm (Table 4.1 and Figures 4.1-4.2)

Table 4.1: drug and prodrug inhibition of bacteria showing zone of inhibition diameter in (mm)

Drugs and prodrugs	Staph. Epidermidis (G+)	Staph. aureus (G+)	Streptoc occi. B (G+)	Streptoco cci. A (G+)	klebselia (G-ve)	E. coli (G-ve)
Amoxicillin	no inhibition zone	44 mm	30 mm	40 mm	no inhibition zone	33 mm
Amoxicillin prodrug	no inhibition zone	30 mm	30 mm	26 mm	no inhibition zone	31mm
Cephalexin	25 mm	44 mm	33 mm	37 mm	26 mm	29 mm
Cephalexin Prodrug	20 mm	40 mm	32 mm	30 mm	21 mm	24 mm

Amoxicillin drug and prodrug(P value =.141)
Cephalexin drug and prodrug(p value=.003)

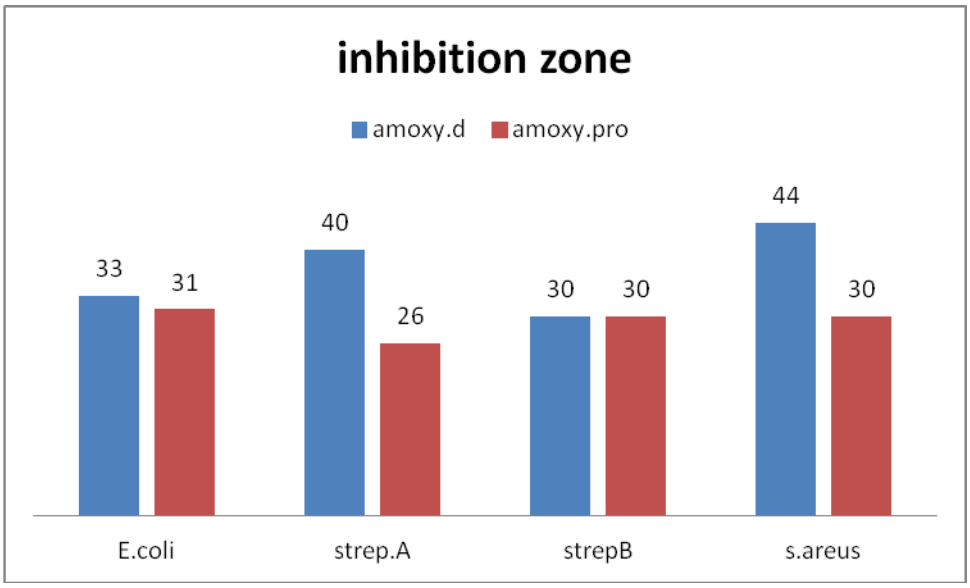


Figure (4.1): antibacterial activity of drug and prodrug of amoxicillin against bacterial strain.

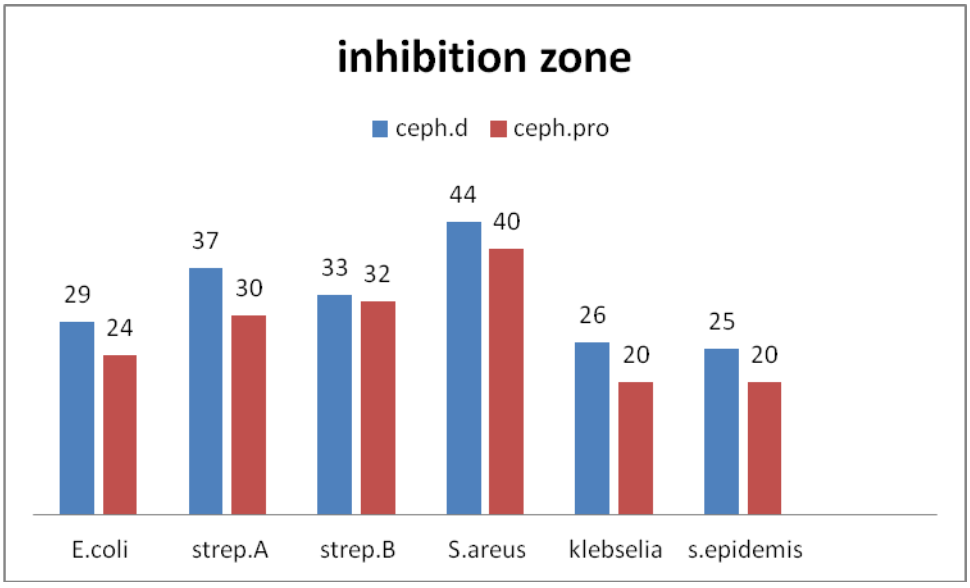


Figure (4.2): antibacterial activity of drug and prodrug of cephalexin against bacterial strain.

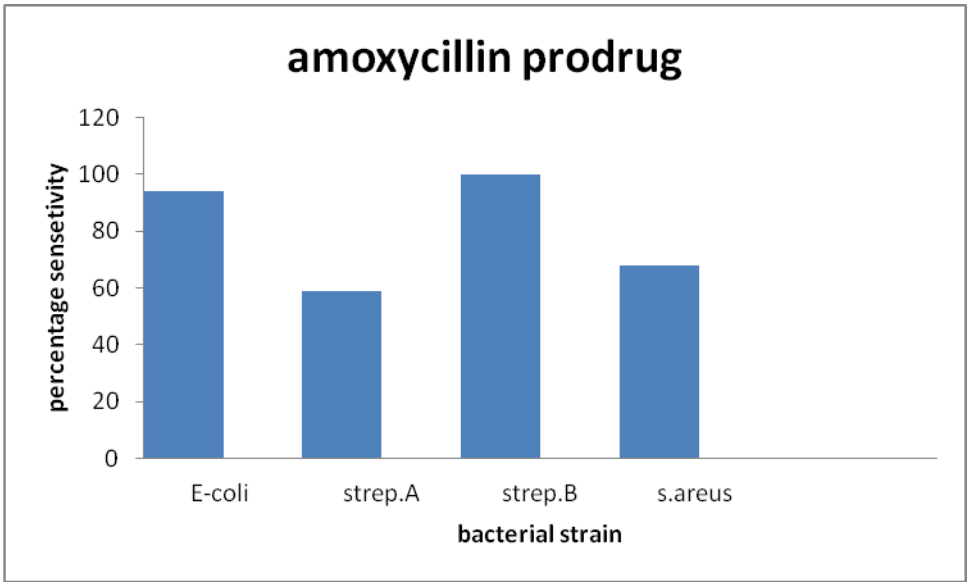


Figure (4.3). Percentage of prodrug to drug of amoxicillin against bacteria.

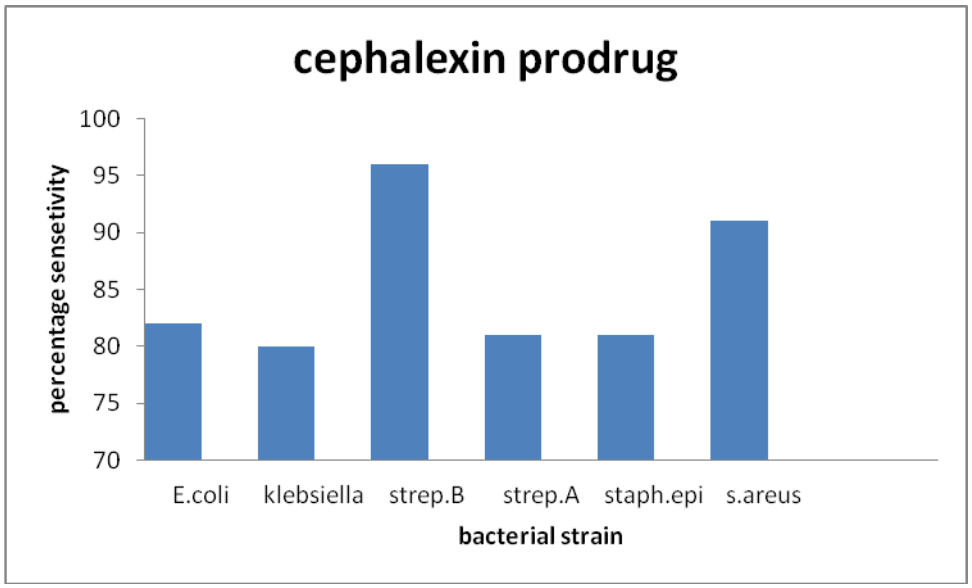


Figure (4.4): Percentage of prodrug to drug of cephalexin against bacteria.

Chemical modification on the reactive β–lactam ring can change the chemical properties and spectrum of activity of a certain antibacterial agent. Novel amoxicillin and

cephalexin prodrugs were designed and synthesized by karaman's group. The prodrugs design was accomplished using DFT calculations.

The goal of the present work was to study the antibacterial spectrum of these two new prodrugs since the β-lactam ring still present, which is needed for the antibacterial activity.

The results shown in Table 4.1 revealed that the novel two prodrugs have antibacterial activity on most bacterial strains studied with about the same potency as their parent drugs for amoxicillin. And its not significantly difference (p value =.141)

Klebsiella showed resistance to amoxicillin drug and its prodrug since *klesiella*is is a gram negative bacteria, which exhibits resistance to amoxicillin drug [59], and there is a need of clavulanic acid to overcome its resistance.

Staphylococcus epidermidis also showed resistance to amoxicillin drug and its prodrug since it is β -lactamase positive. Cephalexin drug and its prodrug showed inhibition against all bacterial strains used including *Klebsiella pneumonia* and *E.coli*, since cephalexin drug has broader spectrum than amoxicillin (Table 4.1)

In addition, the results showed the percentage sensitivity of prodrug to drug against bacterial strains used in this work. For amoxicillin, 94% against *E.coli*, 59% against strep. A, 100% against strep. B and 68% against *staph. arues.* (Figure 4.3). For cephalexin the percentage of prodrug to drug is 82% against E.coli, 80% klebselia, 96% *streptococcus group. B*, 81% strep. A, 81% *staphylococcus. Epidermidiss* and 91% for *staphylococcus. areus* (Figure 4.4).

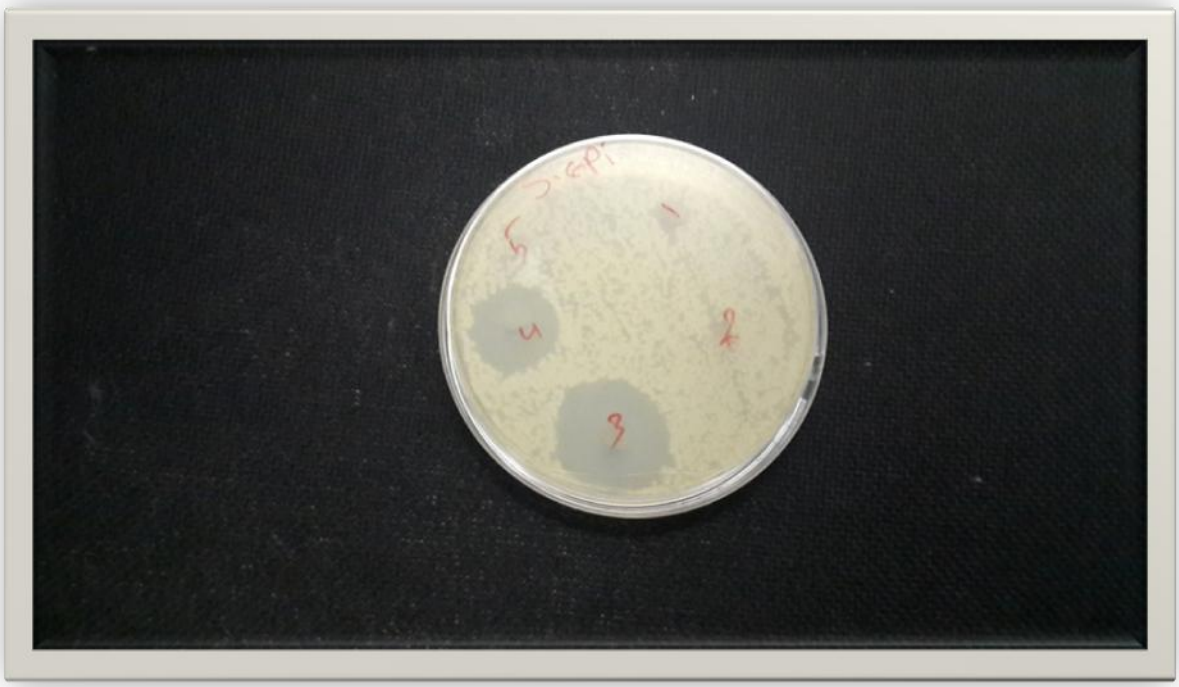


Figure (4.5): Zone of inhibition in mm determined for each agent against *staphylococcus epidermidis*: (1) amoxicillin drug, (2) amoxicillin prodrug, (3) cephalexin drug, (4) cephalexin prodrug and (5) negative control (buffer)

Figure (4.6): Zone of inhibition in mm determined for each agent against *E .coli*: (1) amoxicillin drug, (2) amoxicillin prodrug, (3) cephalexin drug, (4) cephalexin prodrug and (5) negative control (buffer)

Figure (4.7): Zone of inhibition in mm determined for each agents against *staphylococcus aureus:* (1) amoxicillin drug, (2) amoxicillin prodrug, (3) cephalexin drug, (4) cephalexin prodrug and (5) negative control (buffer)

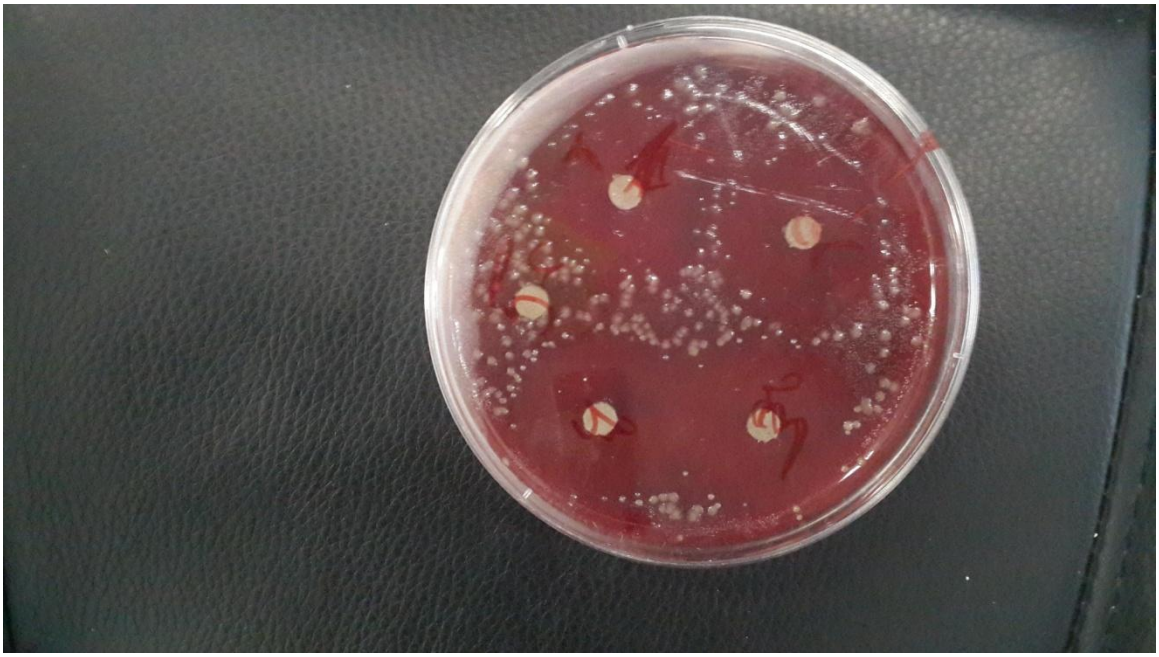


Figure (4.8): Zone of inhibition in mm determined for each agent against *streptococcus group B*: (1) amoxicillin drug, (2) amoxicillin prodrug, (3) cephalexin drug, (4) cephalexin prodrug and (5) negative control (buffer)

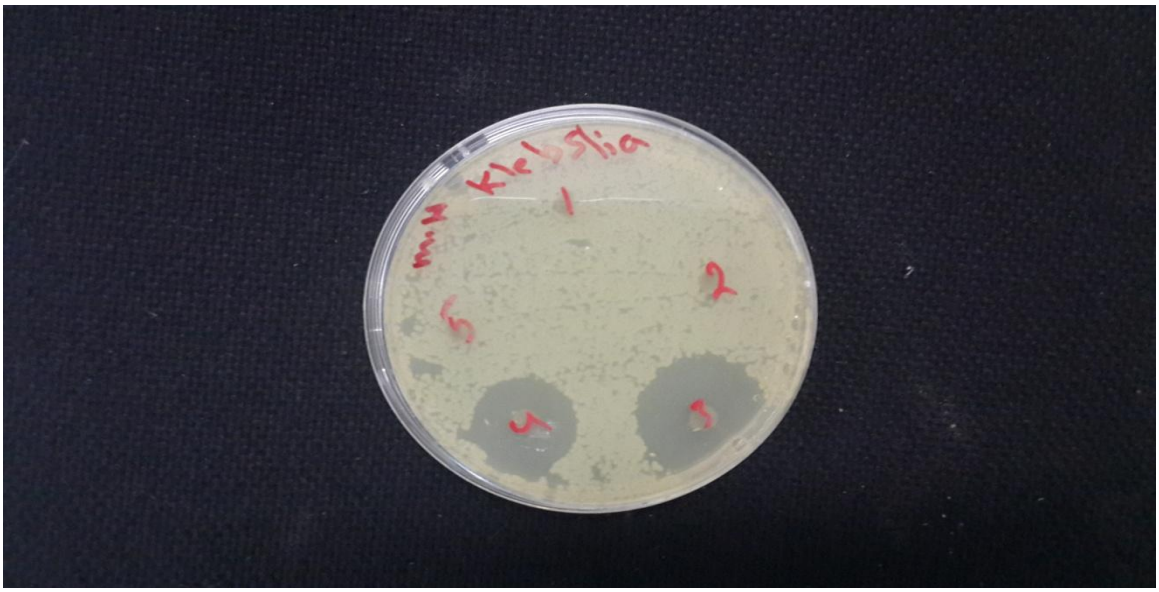


Figure (4.9): Zone of inhibition in mm determined for each agent against *Klebsiella*: (1) amoxicillin drug, (2) amoxicillin prodrug, (3) cephalexin drug, (4) cephalexin prodrug and (5) negative control (buffer)

Figure (4.10): Zone of inhibition determined for each agent against *streptococcus group A*: (1) amoxicillin drug, (2) amoxicillin prodrug, (3) cephalexin drug, (4) cephalexin prodrug and (5) negative control (buffer).

4.2 MIC and MBC result in microgram/ml

Table (4.2): MIC, MBC of (amoxicillin, cephalexin) drug and prodrug

Atcc bacteria	Cephalexin drug		Cephalexin prodrug	
	MIC	**MBC**	**MIC**	**MBC**
Streptococcus. Group B	45	50	50	100
E-coli	10	10	15	20
Staphylococcus. areus	35	40	35	40
Staphylococcus .epidermidis	45	125	50	150
Klebsiella	5	5	10	15
Streptococcus.Group A	45	100	50	100
Atcc bacteria	**Amoxicillin drug**		**Amoxicillin prodrug**	
	MIC	**MBC**	**MIC**	**MBC**
Streptococcus. Group B	100	100	>150	>150
E-coli	10	10	10	10
Staphylococcus. areus	50	100	>150	>150
Staphylococcus. Epidemidis	---	----	----	----
klebseilla	-----	----	----	----
Streptococcus . group A	45	100	>150	>150

cphalexin drug and prodrug MIC(p value =.004) ,cphalexin drug and prodrugMBC(pvalue =.098)

In this study the MIC and MBC values for both amoxicillin and cephalexin prodrugs were determined and compared with the values of their parent drugs.

Amoxicillin parent drug against *strep. B*, staph *arues*, *strep. A* was more potent than its prodrug since lower concentrations of drug are needed to inhibit and kill bacteria.

Amoxicillin parent drug has the same MIC value as the prodrug against *E.coli* 10ug/ml this finding is similar to previous studies.[59, 63] This means that they are equal in potency.as they have the same MIC and MBC results.

Amoxicillin drug has different MIC value than the prodrug against sstaphylococcus. areues for the parent drug the MIC value was 50 ug/ml which is similar to that reported in previous studies.[60]

All results that are above 150 ug/ml need higher concentration to determine the exact value since 150 microgram is the maximum concentration used in this work (Table 4.3).

Cephalexin parent drug was slightly more potent than the prodrug, because the MIC and MBC values of the parent drug were not significantly (p value = 0.98) lower than that of the prodrug. Table 4.4 indicates that the prodrug of cephalexin was more potent than the prodrug of amoxicillin; since all MIC and MBC measured values for cephalexin prodrug were less or equal to 150 ug/ml.

Conclusions and Future directions

Conclusion and Future directions

Chapter five

5.1 Conclusion

The two synthesized prodrugs of amoxicillin and cephalexin were designed such that the amine group in the parent drug is replaced with its corresponding amide. Thus, both prodrugs are expected to be more stable than their corresponding parent drugs, amoxicillin and cephalexin.

It is worth noting that those two novel prodrugs are among a small number of prodrugs that have activity themselves before undergoing conversion via enzymatic or chemical processes to their corresponding parent drugs. The novel prodrugs exhibit their antibacterial activity against different types of bacterial strains due to the presence of beta lactam ring in their structures.

5.2 Future direction

The steps to be taken in the future will include the followings:

(1) A determination of the exact MIC, MBC for those results having values above 150 ug/ml.

(2) An assessment of the antibacterial activity on other types of bacteria in addition to those used in this work.

(3) In vivo testing of the novel prodrugs in the absence and presence of clavulanic acid.

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الملخص

دراسة الفعالية المضادة للكائنات الدقيقه لدوائيْن أوليّيْن جديديْن مبتكريْن من مضادات البكتيريا؛ الأموكسيلين والسيفالكسين

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لقد تم تصميم وتركيب دوائيْن أوليّيْن جديديْن مبتكريْن من مضادات البكتيريا؛ الأموكسيلين والسيفالكسين(prodrugs) لتحسين المذاق المرّ وقلة الثبات .

لقد تم دراسة الفعالية المضادة للكائنات الدقيقه ضد

Escherichia coli, staphylococcus epidermidis, staphylococcus *aureus*

Klebsiella pneumonia, streptococcus group A, streptococcus group B,

وقد تم مقارنة الفعاليه مع الأموكسيلين والسيفالكسين (drugs) بالاضافه تم تحديد اقل تركيز من الماده الفعاله يمكن ان يمنع نمو الكائنات الدقيقه او يقضي عليها كليا (MIC,MBC), اظهرت الدراسه ان الدوائين(prodrugs) لهم فعاليه مقاربه لفعاليه الأموكسيلين والسيفالكسين الاصليين بلنسبه لأنواع البكتيريا المستخدمه ,فقد كانت (*Klebsiella pneumonia, staphylococcus epidermidis*) مقاومه للاموكسيلين (drug and prodrug). *(*ا *Escherichia* coli) فقد كان اقل تركيز لتثبيطها لكلا الدوائين اموكسيلين (drug and prodrug) 10ug/ml.

بلنسبه الى السيفالكسين لم يكن هناك فرق كبير في قيم(MIC,MBC) لكلا الدوائين (drug and prodrug)