An-Najah National University

Faculty of Graduates Studies

Design, Synthesis and Biological Activities of Curcumin Based Alkaloids

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Dedication

To my father

To my mother

To my brothers

To my husband

To all friends and colleagues

To my professors

To everyone who works in this field

To all of them, I literally dedicate this work

Acknowledgment

First of all, praise is to Allah for helping me in making this research possible.

Then, I would like to express my great thanks and sincere gratitude to my supervisors Dr. Othman Hamed and Dr. Shehde Jodeh for their guidance, suggestions and assistance and encouragement during the preparation of this thesis.

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And special mention goes to my parents, brothers, husband and friends for their continuous support at all times.

الإقرار

أنا الموقع أدناه مقدم الرسالة التي تحمل عنوان:

Design, Synthesis and Biological Activities of Curcumin Based Alkaloids

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Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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Date: **التاريخ: 28/10/2018**

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List of Abbreviations

Abbreviation	Full name
Da	Dalton
TB	Tuberculosis
HIV/AIDS	Human Immunodeficiency Virus infection and Acquired Immune Deficiency Syndrome
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
NCE	New Chemical Entity
FDA	Food and Drug Administration
FT-IR	Fourier -Transform Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
DMSO	Dimethyl Sulfoxide
EtOAc	Ethyl Acetate
mmol	millimole
NB	Nutrient Broth
MHA	Mueller-Hinton agar
EMB	Eosin methylene blue agar
S. *aureus*	*Staphylococcus aureus*
S. *epidermidis*	*Staphylococcus epidermidis*
E. coli	*Escherichia coli*
CFU	colony-forming units
CLSI	Clinical and Laboratory Standard Institute
ATM	Aztreonam
DA	Clindamycin
CIP	Ciprofloxacin
NA	Nalidixic acid
TE	Tetracycline
SXT	Cotrimoxazole
K	Kanamycin
FOX	Cefoxitin
NX	Norfloxacin
LEV	Levofloxacin
MIC	Minimum Inhibitory concentration
MBC	Minimum Bactericidal Concentration
ERIC	enterobacterial repetitive intergenic consensus
rpm	round per minute
UV	Ultra Violate
bp	Base pair

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Abstract

A new series of curcumin based alkaloids were synthesized. The synthesized alkaloids belong to two classes, the first class contains an alky amine chain and the other class of alkaloids contains a heterocyclic ring such as isoxazole, diazepine and pyrazole. The prepared compounds were characterized by various spectroscopic techniques such as FT-IR, ^{13}C and $^{1}HNMR$ spectroscopy.

The compounds were synthesized using two new methods, and the yields in some cases as shown in the experimental part were quantitative. In the first method, curcumin was reacted with the diamine compounds in ethanol in presence of catalytic amount of sulfuric acid. The second method involved mixing the reactants, curcumin and diamine compounds, heating them gradually to melt, then heating them at 160^{o}C for 30min.

Investigation of antimicrobial activity of the prepared alkaloids against four types of bacteria, demonstrated the ability to inhibit gram-positive microorganisms with MIC ranging between 1.5 and 6.25µg/mL for *S. aureus*. Among all tested derivatives, isoxazole **23**, diazepine **26**, pyrazole **27**, amines with methyl **28** and mostly piperazine **25** exhibited remarkable potency against gram-positive bacteria, S. aureus. Also, it was found that

the other gram-positve bacteria used, *S. epidermidis*, isoxazole **23** and piperazine **25** were the most effective.

Gram- negative bacteria were found to be much more resistant than gram-positive, in which E. coli was susceptible to the piperazine **25** only.

Genotoxic study showed that compound **25** with piperazine group damaged the DNA of E. coli strains. Based on this result, it is recommended to make *in vivo* genotoxicity studies using animal models and human cell lines for proper assessment of the safety of using compound **25** for therapeutic purposes.

An extensive study is underway to optimize the effectiveness of piperazine type of compounds and to determine their mode of action.

1 INTRODUCTION

Since the old time, humans have suffered from illnesses and infections caused by bacteria. In the 20th century, researchers have found a way to stop this suffering through antibacterial agents. Since then, a diversity of antibacterial agents have been developed and used. However, due to their overuse, resistance to the antibacterial agents was quickly observed.[1] Therefore, there is a great need to develop novel antibacterial agents with high efficiency and low drug resistance.

In this study, natural curcumin based derivatives were prepared and their antibacterial activities and genotoxicities were examined.

1.1 Antibacterial agent's overview

1.1.1 Background

Antibacterial agents are drugs used to prevent the bacterial infections by either killing or inhibiting the bacterial growth.[2]Most of these agents are relatively small molecules with a molecular weight less than 1000 Da.

The old classical definition of an antibiotic is a compound produced by a microorganism which inhibits or stops the growth of another microorganism. But over the years, this definition has been extended to include synthetic and semi-synthetic products.

The first antibiotic discovered was of a natural origin which is Penicillin (Fig 1.1). It was discovered by Alexander Flaming in 1928 while he was working on a culture of bacteria, he observed a green mold, Penicillium chrysogenum, in one of his culture plates with no presence of bacteria,

so he postulated that the mold must excreted an antibacterial agent.[3]Since then, many other antibacterial agents were developed by synthesis, like the sulfa drugs, or by chemical modification of natural compounds.

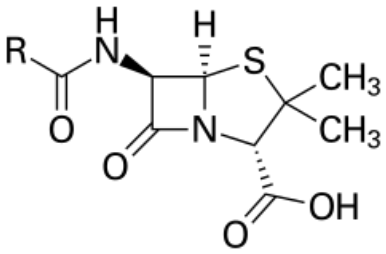


Figure 1.1: Penicillin chemical structure

The process of developing compounds against bacterial infections is often believed to be more difficult than for other therapeutic areas for several reasons, including bad penetration into bacterial cells, innate resistance mechanisms such as drug-inactivating enzymes, and the need of relatively high concentrations that would cause severe side effect.[4]The side effects could be ranging from mild to very severe according to the type of: antibiotic drug used, the microbes targeted, and the individual patient. The side effects could include fever, diarrhea, nausea and major allergic reactions, including photodermatitis and anaphylaxis.

1.1.2 The importance of antibacterial agents

The introduction of synthetic antibiotics into clinical practice has led to a numerous advantages including the ability to cure effectively and to treat both acute bacterial infections and chronic infectious diseases like TB.

The discovery of the antibiotics has lowered the suffering and loss of human lives by infectious diseases and changed medical practice in various

ways. For example, a lot of surgical interventions nowadays like joint replacements or organ transplants, antibiotics are given to the patient before surgery to reduce the risk of infection. Also, antibiotics protect prematurely born babies and cancer patients in addition to patients who are suffering from chronic diseases, including asthma, diabetes, rheumatoid arthritis and HIV/AIDS.

On the other hand, these therapeutic interventions and surgical procedures will be of a high risk if the resistance levels to the current antibiotics continue to rise without developing new anti-infective agents to substitute them. If nothing is done, then the chance of returning to the pre-antibiotic era could be very high.[5]

Infectious diseases are still the main cause of death in the world, as 14 million deaths in 2011 were caused by infection diseases. The risk of fast spreading of the infections through the world is becoming easier than any time before, which could be related to the easiness of intercontinental travel, the displacement of populations caused by poverty and starvation. Thus, drug resistance is arising in considerable levels. In addition, new diseases appear due to environmental and demographic change. [6]

1.1.3 Classification of antibacterial agents

Antibiotics can be classified according to their chemical structure, mechanism of action, and speed of action.[7]

According to the mechanism of action, antibacterial compounds are divided to three types: those that target the bacterial cell wall like penicillin, the cell

membrane like polymyxins, or interfere with essential bacterial enzymes like quinolones and sulfonamides(Fig. 1.2).[8]

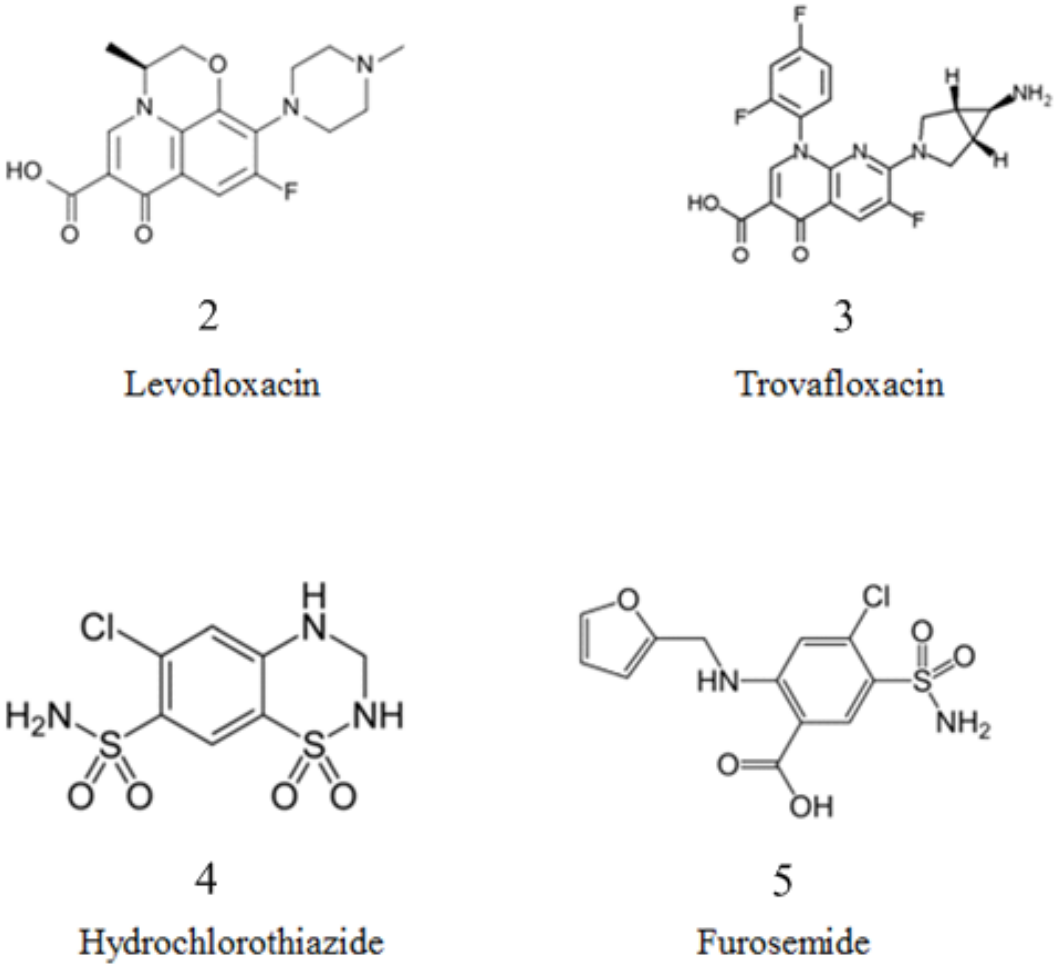


Figure 1.2: Quinolones and sulfonamides antimicrobial agents

Antibacterial agents according to their speed of action and residue production, are classified into two groups: **non-residue-producing,** which is a fast acting antibacterial agents, that quickly kill bacteria, and disappear very fast afterwards via evaporation or breakdown so they don't leave active residue behind, like for examples peroxides, alcohols and chlorine.

The second group is **residue-producing or long lasting antibacterial agents**, this type leaves long-acting residues on the surface to be antiseptic so that they have a longer action. Examples on this type are triclosan (compound 6, Fig.1.3), triclocarban (compound 7, Fig.1.3), and benzalkonium chloride (compound 8, Fig.1.3).

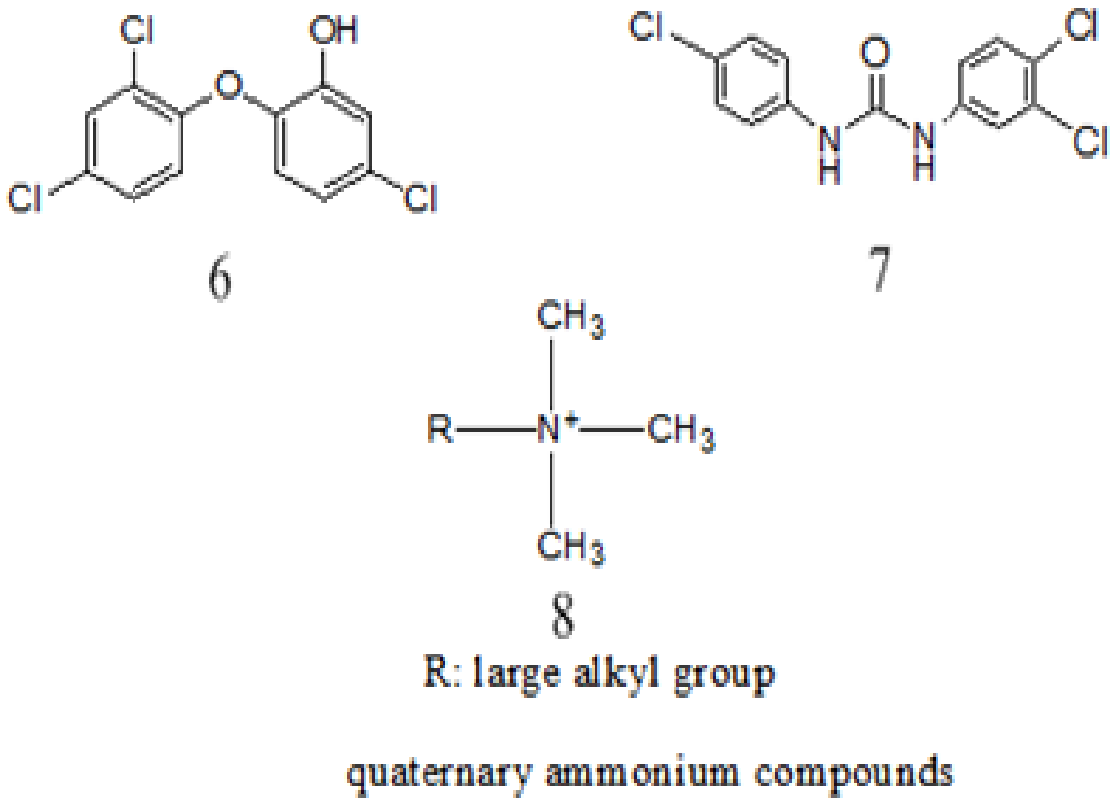
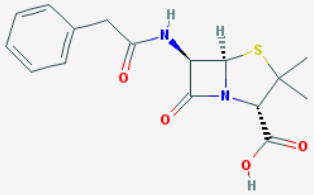
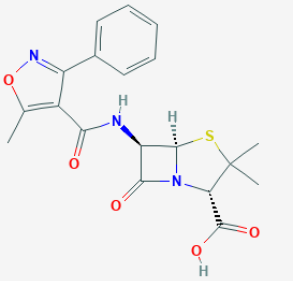
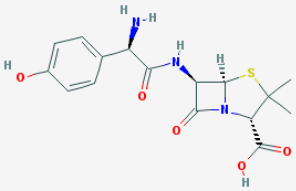
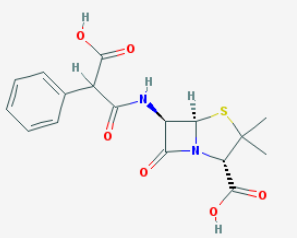
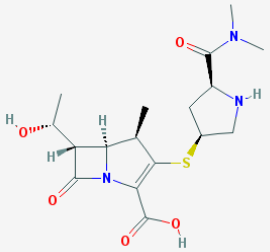
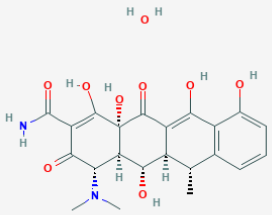
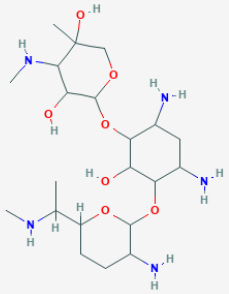
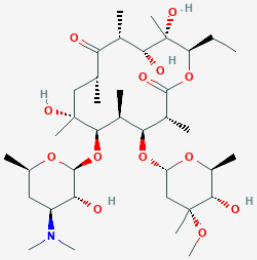
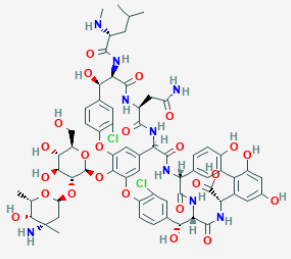
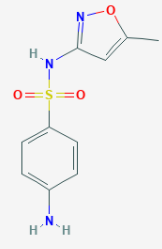


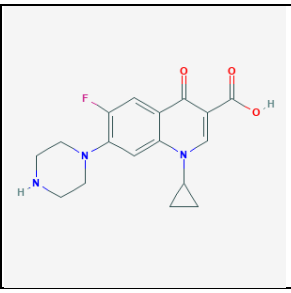
Figure 1.3: Examples of long lasting antibacterial agents

Antibiotics can also be divided into different groups according to their chemical structures as shown in Table1.1, e.g. ß-lactams, quinolones, tetracyclines, macrolides, sulphonamides and others.[9]

Table 1.1: Antibiotic compounds classified according to their chemical structures

Class/group	Subgroup/Example	Chemical structure
ß-lactams/Penicillins /Cephalosporins	Benzyl-penicillins/Phenoxypenicillin	
	Isoxazolylpenicillins/ Oxacillin	
	Aminopenicillins/Amoxicillin	
	Carboxypenicillins/Carbenicillin	

	/Meropenem	
Tetracyclines	/Doxycycline	
Aminoglycosides	Gentamicin	
Macrolides	Erythromycin A	
Glycopeptides	Vancomycin	
Sulfonamides	Sulfamethoxazole	

Quinolones	Ciprofloxacin	

1.1.4 Cellular targets of antibacterial agents

Antibacterial agents may cause cellular damage to bacteria in three different ways: bacterial cell wall biosynthesis, bacterial proteins, and DNA synthesis, replication or repair.

Bacterial cell walls are very important for the existence of bacterium, since they preserve cell contents and a high osmotic pressure needed for cellular function. Therefore, inhibition of bacterial cell wall biosynthesis is an effective way for killing bacteria. An example of antibacterial agents that act on the cell wall of bacteria is the β-lactam amoxicillin shown in Fig.1.4.[10]

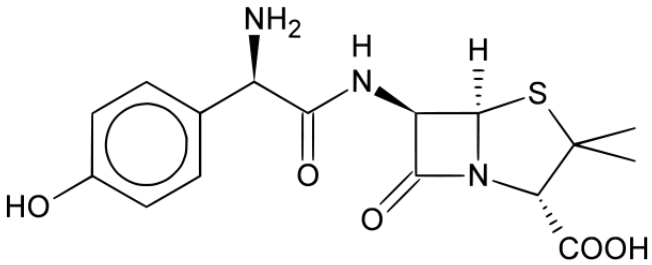


Figure 1.4: Chemical structure of β-lactam amoxicillin

The processes of DNA synthesis, replication and repair are substantial for the existence of bacterial cells, so a compound that can interfere with these processes can be very effective in killing bacteria. Common examples of

these compounds are: quinolones, such as ciprofloxacin (molecule 10, Fig.1.5), that targets the replication process. Sulfonamides, like the dual drug Bactrim® which has a 1:5 ratio of trimethoprim (molecule 11, Fig1.5) and sulfamethoxazole (molecule 12, Fig.1.5), that inhibits nucleic acid synthesis.

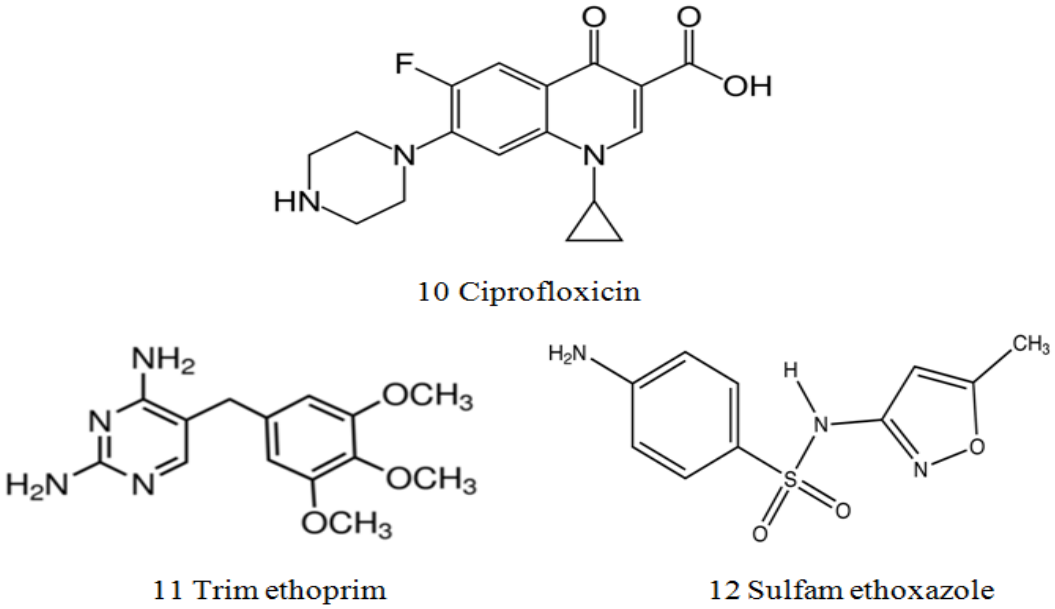


Figure 1.5: Examples of quinolones and Sulfonamides

Other clinically used antibacterial agents may target both DNA and RNA of a bacterium such as oxazolidinone linezolid 13, (Fig.1.6).[11]

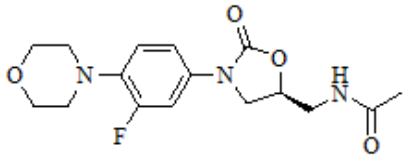


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Figure 1.6: Chemical structure of oxazolidinone linezolid

1.1.5 Effects of pH on antibacterial activities

Antibacterial molecules possess various functionalities within the same molecule. Thus, under various pH values, antibiotics can be neutral, cationic, anionic, or zwitterionic. Different functionalities within a single molecule cause its physico-chemical and biological properties, sorption behavior, photo reactivity and antibiotic activity and toxicity to be pH dependent. [12]

Ciprofloxacin antibiotic 10 for instance, has a chemical structure (Fig1.7a) that contains both acidic and basic functionalities. The acid constant, pKa, of the carboxyl group is 6.09 and for the secondary amino group is 8.74. At a pH value of 7.4, the iso-electric point of ciprofloxacin, the molecule is neutral as it carries both negative and positive charges (Fig.1.7b). Solubility, hydrophobictiy and hydrophilicity, and so the distribution coefficient log K_D, are all depending on pH. [13]

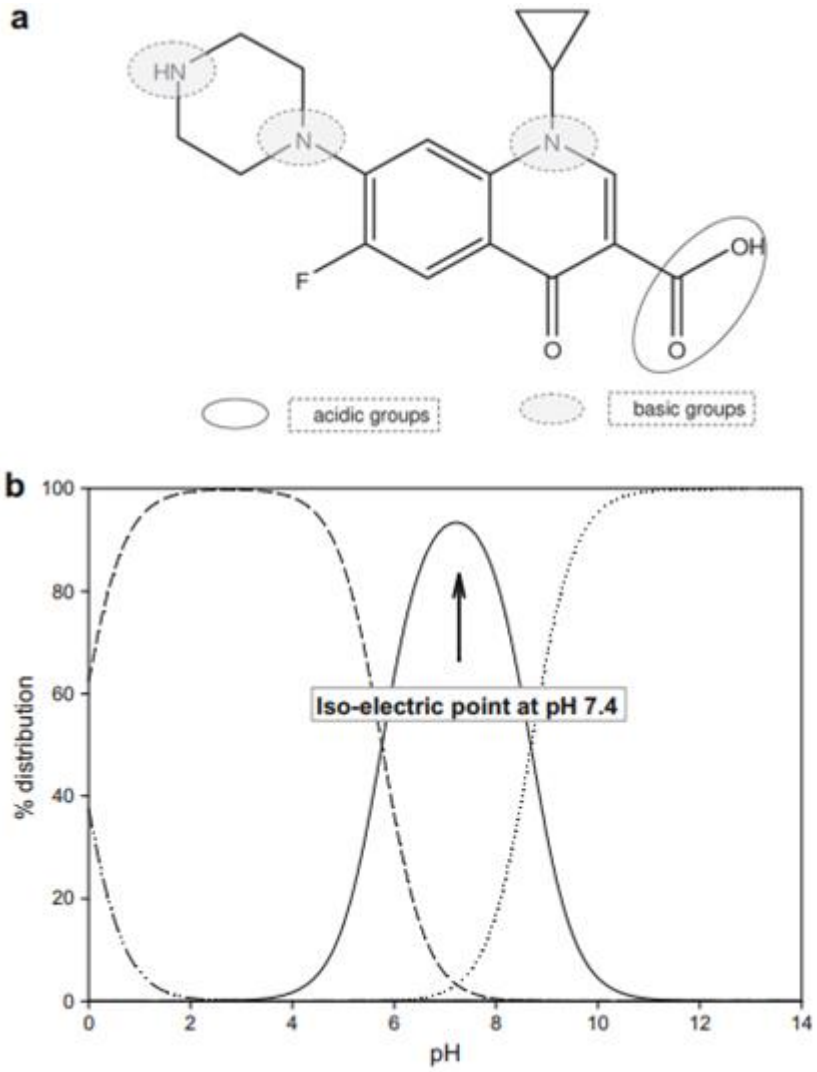


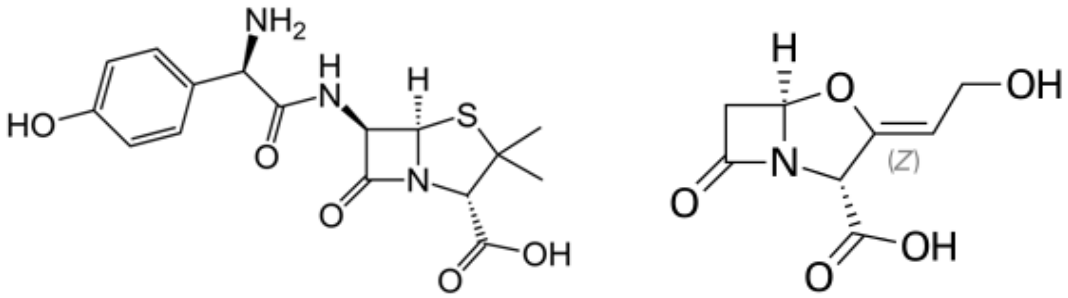
Figure 1.7: (a) Chemical structure of ciprofloxacin, (b) ciprofloxacin carries diverse electrical charges at different pH, so different chemical species are present

1.1.6 Resistance of bacteria to antibacterial agents

The over use of synthesized and natural antibacterial agents has caused the bacteria to develop ways to resist these drugs. There are three major mechanisms bacteria can use to resist antibacterial compounds: antibiotic inactivation, target alteration and decreased intracellular drug concentration.[14, 15]

An effective method for treatment of the resistance issue is to use two reagents with dual biological functions. These “dual action” drugs are categorized in the literature as: dual drugs and cross-linked dual action drugs.

An example of the dual drugs is Augmentin®, which has a combination of a penicillin antibiotic, (amoxicillin, Fig.1.8), and the β-lactamase inhibitor (clavulanic acid, Fig.1.8). In this dual action drug, clavulanic acid inhibits the β-lactamase enzyme, which maintain the integrity of amoxicillin leading it to exert its own antibacterial activity. Augmentin® is used against infections of Augmentin®-sensitive, β-lactamase producing bacterial strains, like skin infections caused by S. aureus bacteria.[16]

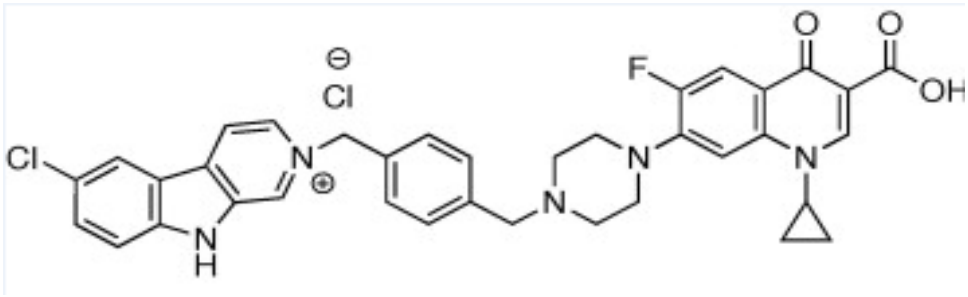


14 Amoxicillin 15 Clavulanic acid

Figure 1.8: chemical structures of (14) amoxicillin and (15) clavulanic acid

The second category of dual action drugs is chemically cross-linked by a non-cleavable linkage, or by a cleavable linkage. An example of the cross-linked by a non-cleavable linkage is the incorporation cross-linking of the algicidal natural product nostocarboline (16, Fig.1.9) with ciprofloxacin (17, Fig.1.9) through 1,4 dimethylbenzene linker. The cross-linked hybrid

showed a MIC value of about two orders of magnitude larger than those of ciprofloxacin alone.[17]

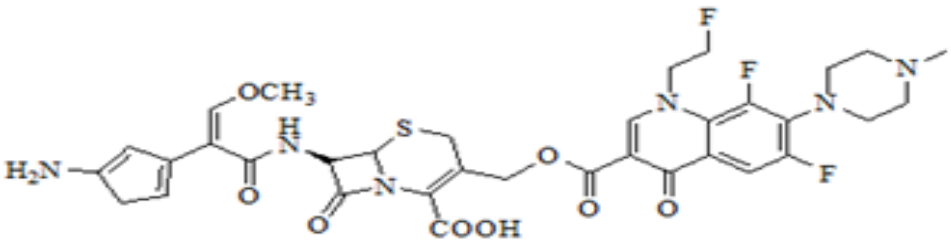


16 17

Nostocarboline Ciprofloxacin

Figure 1.9: Cross-linking of (16) nostocarboline with (17) ciprofloxacin

An example of the cross-linked via a cleavable linkage is the ester-linked derivative (Fig.1.10) which combine cephalosporin (desacetylcefotaxime) linked with a quinolone (fleroxacin). It showed a high activity against different gram positive bacteria (MIC of 1.0µg/mL) against *S. aureus*, and gram negative like (MIC of 0.125µg/Ml) against E. coli strains.[18, 19]



18

Figure 1.10: Structure of ester-linked derivative

It is obvious that novel medications are required to overcome the dangerous resistance issue of bacteria.

1.1.7 The current state of antibacterial research

Most antibacterial agents used to treat infections were obtained from a limited number of sources, based on natural products, and these were discovered over 40 years ago. Since then, only two new broad-spectrum chemical entities (NCEs) have been developed and approved for human use: the oxazolidinones represented by linezolid and the lipopeptide antibiotic, daptomycin.[20]

However, few NCEs for specific diseases like TB have been found. The constant decreasing number of new drug registrations (Fig.1.11) lead to widen the innovation gap as more pharmaceutical companies leave this sector and financial funds become less than before.[21]

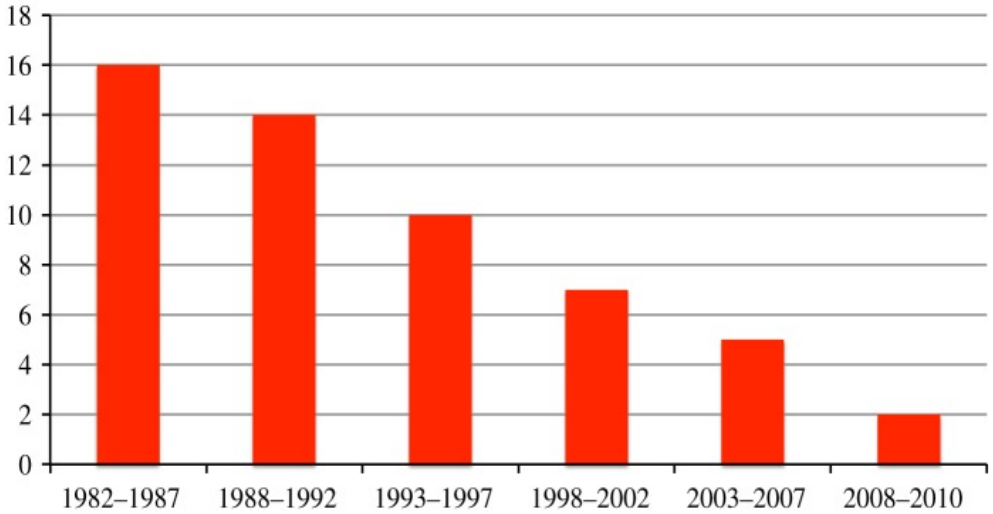


Figure 1.11: Number of new antibacterial agents approved for human use in the past 30 years

1.2 Aims of this study

This study is a continuation of a previous work carried out in our laboratories for developing novel curcumin antibacterial agents, and determine their mechanism of action. The overall objectives of the study are three folds:

1. Design, and synthesize a new class of natural product derivatives from curcumin.
2. Characterize the synthesized compounds by various spectroscopic methods (IR, NMR).
3. Evaluate the prepared compounds for antibacterial activities and genotoxicity.

1.3 Curcumin

Curcumin (1E, 6E)-(1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione)is a natural polyphenolic compound extracted from turmeric roots (*Curcuma longa*) in tropical and subtropical areas around the world. It is originating from India, Southeast Asia and Indonesia.[22] It exists in the form of an orange–yellow crystalline powder that is insoluble in water and ether but soluble in ethanol, dimethylsulfoxide, and acetone. [23]

Chemically, it is a bis-α, β-unsaturated β-diketone that shows keto-enol tautomerism. The first isolation of curcumin was done in 1815 by Vogel and Pelletier, and it was synthesized for the first time in 1910.[24]Curcumin is considered one of the linear diarylheptanoid class of natural products in which two oxy-substituted aryl moieties are connected by a seven member

carbon chain. The C7 chain of linear diarylheptanoids has unsaturation, oxo functions, enone moiety, and 1,3-diketone group. The C7 chain is generally unsubstituted except for the oxo and hydroxyl groups. The unsaturation in the linker unit has an E-configuration (trans C=C bonds).

Besides curcumin, turmeric consists of other chemical constituents known as the curcuminoids (Fig.1.12).[25]

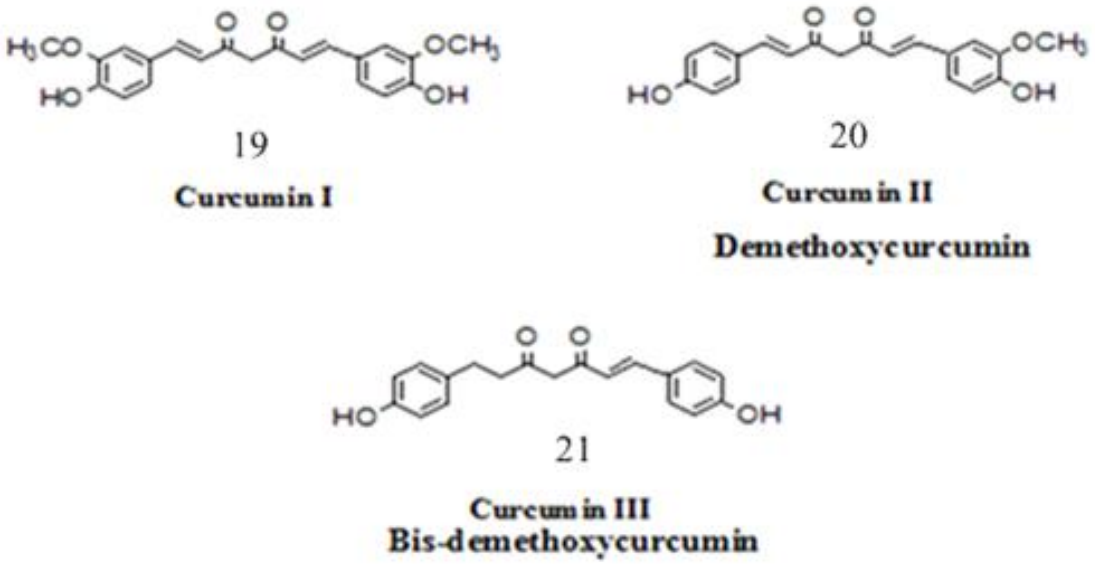


Figure 1.12: Chemical structures of curcuminoids

The curcuminoids are responsible of the yellow color of turmeric. The main curcuminoids in turmeric are curcumin (molecule 19, Fig.1.12), demethoxycurcumin (molecule 20, Fig.1.12), bisdemethoxycurcumin (molecule 21, Fig.1.12) and the recently identified cyclocurcumin. Commercial curcumin contains about 77% of curcumin **[**(E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], 17% of demethoxycurcumin, and 3% of bisdemethoxycurcumin.[26]

Curcuminoids and turmeric products have been classified as safe by the Food and Drug Administration (FDA) in USA.[27]in which a healthy dose up to 12 g/day of curcumin is considered safe for human consumption without emerging any side effect.[28]

1.3.1Biological activity of Curcumin

Curcumin exhibits remarkable biological activities including anti-inflammatory, antioxidant, anticarcinogenic, antiviral and antimicrobial activity. In addition, curcumin has many therapeutic properties like antineoplastic, antiapoptotic, antiangiogenic, cytotoxic, immunomodulatory and antithrombotic, wound healing, antidiabetogenic, antistressor and antilithogenic actions, which leads to the large interest of this material.[29]

One of the most important factors that are responsible for curcumin's therapeutic effects is its ability to scavenge reactive oxygen and nitrogen free radicals. The two O-methoxy substitutions on the curcumin's aromatic rings are responsible for these effects, as many reports had found that curcumin has better radical scavenging and antioxidant ability than the other two curcuminoids. The hydrogen bonding between the phenolic OH and the O-methoxy groups in curcumin markedly affects the O–H bond energy and H-atom elimination by free radicals, so making it a better free radical scavenger.[30]

The antioxidant ability of curcumin in the presence of metals like Cu(II), Fe(II), or Pb(II) is due to its chelating power.[31]Despite that transition metal chelation by curcumin can occur by either the diketone moiety or the

O-methoxy phenol moiety, chelation is often observed only through the diketo group.

The causes and the actual mechanism of the curcumin antitumor activities are still not understood. Hydrogenation of the heptadiene part in curcumin to produce tetrahydrogenated curcumin had significantly increased the antioxidant activity but decreased the antitumor and antiinflammatory effects.[32]

On the other hand, its poor bioavailability due to low serum levels, limited tissue distribution, rapid metabolism, and poor solubility limits the effective clinical application of curcumin.[33]

In order to overcome these disadvantages and improve curcumin bioavailability, many researchers used different strategies, among them are adjuvants such as piperine,[34]delivery systems based on nanoparticles,[35]Liposomes,[36]or micelles.[37]

Another method to enhance the compound's biological activity is to modify the curcumin skeleton structure. For example, the introduction of different groups instead of phenolic hydroxyl groups can prevent protons elimination and subsequent degradation of curcumin under basic conditions.[38]

During the last few decades, a number of compounds with an adjusted curcumin structure were synthesized and were found to give better biological activities.[39]

The important antineoplastic activity of curcumin, besides its low molecular weight and lack of toxicity, makes this compound a good natural product of likely chemotherapeutic derivatives or analogues.[40]

Subsequently, a wide variety of curcumin analogues have been prepared. One commonly used structural modification amputates the central conjugated beta diketone in curcumin to the monocarbonyldienone compounds which exhibit cytotoxicities against different types of cancer.[41]

A variety of metal ions can be complexed with the diketo moiety of curcumin, where these metal complexes often show a higher stability than the readily degraded curcumin itself. The first complexes of curcumin that were prepared with medically valuable transition metals, such as Pd, Pt, Rh, and In, were published in 1997. In most cases the resulting complexes exhibited favorable biological activities compared with the parent ligands for a number of molecular targets.[42]

The biological activity of metal complexes of curcumin and its derivatives relies on many factors, like: the selected metal, curcumin skeleton and its modifications, ancillary ligand(s), the shape of the resulting complex, exchange capability of the ligands, charge, and others.[43, 44]

Thus, Curcumin is a promising candidate to preserve the required cis-coordination of platinum-based anticancer agents together while improving their selectivity.

1.3.2 Antibacterial activity of curcumin

It was found that curcumin has a powerful activity against various microbes. It suppress the growth of a plethora of bacteria like S. epidermis, Klebsiella pneumoniae, E.coli, Bacillus subtilis, Staphylococcus aureus.[45-47]

In addition to pathogenic bacteria’s found in shrimp, mice and chicken, such as Vibrio, Bacillus, Salmonella, Staphylococcus and Helicobacter pylori specie.[48]The bactericidal influence of curcumin is because it causes a leakage of bacterial membrane.[49]

Because of the increasing resistance of Gram-positive and Gram-negative bacteria, there is a high demand to identify alternative antimicrobials, including those from natural materials with low human cytotoxicity.

2 MATERIALS AND METHODS

2.1 General experimental

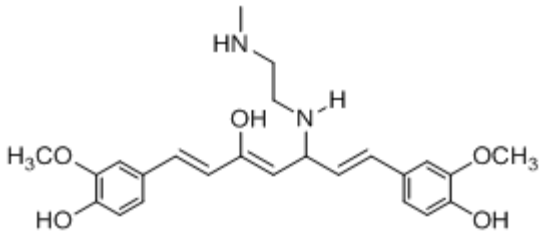
All reagents were purchased from Aldrich Chemical Company, and used as received except otherwise specified. All prepared compounds were characterized by IR spectroscopy and NMR Spectroscopy, ^{1}HNMR and ^{13}CNMR.

All ^{1}HNMR experiments are reported in δ units, parts per million (ppm) downfield from tetramethylsilane (internal standard) and were measured relative to the signal for DMSO-d6 (2.5ppm). Nuclear Magnetic Resonance spectra were recorded on a Varian VXR S400 NMR spectrometer with a proton resonance frequency of 400 MHz

Infrared (IR) spectra were recorded by using FTIR Spectrum 820 PC FT-IR, Shimadzu.

2.2 Preparation of curcumin-based amine derivatives

2.2.14,4'-((1E,3Z,6E)-3-hydroxy-5-((2 (methylamino)ethyl)amino) hepta-1,3,6-triene-1,7-diyl)bis(2-methoxyphenol)



22

Figure 2.1: Chemical structure of compound **22**

To a large test tube fitted with a magnet stir, curcumin (0.50g, 1.4mmol) was added, followed with a N-methylethylenediammine (0.104g, 1.4mmol). The test tube and its contents was placed in an oil bath. The mixture was heated with stirring to 160ºC for 30 min, and kept at this temperature for another 30 min. Then it was cooled to room temperature, producing a brown solid that was crushed into powder and washed several times with water and then isopropyl alcohol. The insoluble residue was recrystallized from hexane/EtOAc (2:1 by volume) to give 0.31g (yield 52%). The chemical structure of the product is shown in Figure 2.1.

Spectroscopic data of compound **22** is shown in Figure 2.2. Melting point is 148-154ºC. IR (neat): ν_{max} cm^{-1}: 3350 (-C-OH, and R_2N-H), 3022 (=C-H), 2910 (C-H, aliphatic), 1605 (C=C, aliphatic), 1584 (C=C, aromatic), 1218 (C-N), 1186 (C-O ether) and 1081 (C-O alcohol).

^{1}HNMR (400 MHz, DMSO-d6) δ: 2.6 (s, 3H, CH_3); 2.6 (d, 2H, N-CH_2); 2.6 (d, 2H, N-CH_2), 3.68 (s, 6H, OCH_3), 5.44 (s, 2H, OH), 6.94 (s, 1H); 6.23 (d, 1H, *J = 15.3 Hz*); 6.56 (d, 1H, *J = 15.3 Hz*); 6.83-7.21 (m, 8H).^{13}CNMR (400 MHz, DMSO-d6) δ: 35.2, 47.5, 52.3, 56.3, 92.2, 112.1, 118.9, 122.2, 132.3, 134.9, 148.1, 149.3, 148.6, 182.1.

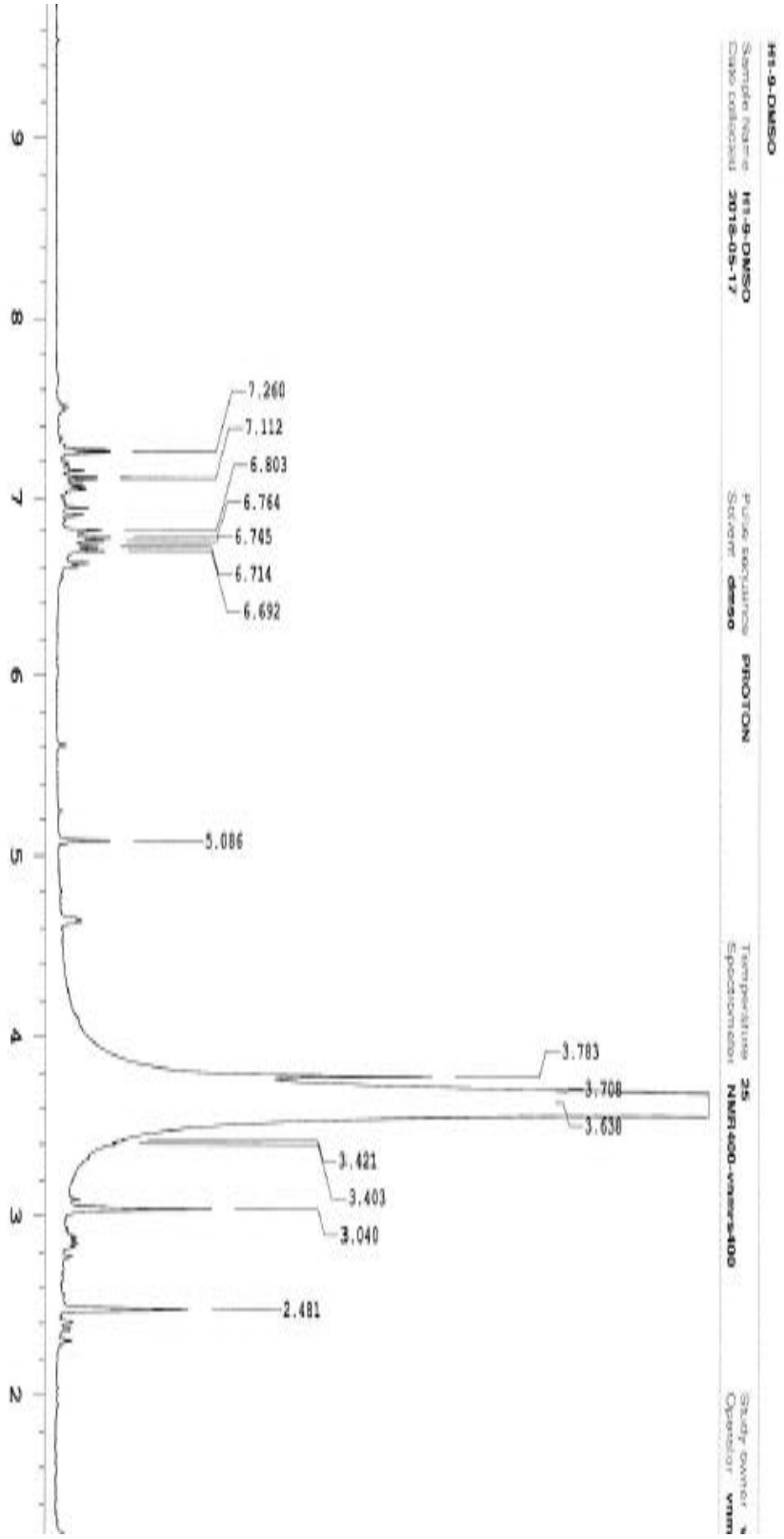
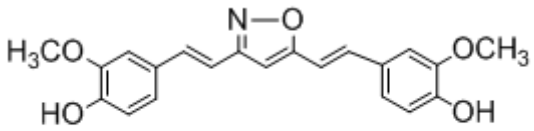


Figure 2.2: [1]HNMR spectrum of compound **22**

2.2.2 4,4'-((1E,1'E)-isoxazole-3,5-diylbis(ethene-2,1-diyl))bis(2-methoxyphenol)



23

Figure 2.3: Chemical structure of compound **23**

To a 100mL round bottom flask fitted with a magnet stir and a condenser, curcumin (1.0 g, 2.7mmol) was added followed with 50mL of ethanol. The mixture was stirred until a clear solution was obtained. Hydroxyl amine hydrochloride (0.42g, 6mmol) was added to the solution followed with 5 drops of concentrated H_2SO_4. The reaction mixture was refluxed for 2 h. The solvent was evaporated under vacuum, then the residue was washed several times with 10% Na_2CO_3, water and dried. The residue was recrystallized from hexane/EtOAc (2:1 by volume). The chemical structure of the product is shown in Figure 2.3.

Spectroscopic data of compound **23** is shown in Figure 2.4. Melting point is 167-172ºC. IR (neat): ν_{max} cm^{-1}: 3400 (-C-OH),1643, 1620 (-C=N), 1514, 1276, 1213, 1031.^{1}HNMR (400 MHz, DMSO-d6): δ 3.85 (s, 6H, 2OCH3), 6.25 (s, 2H, OH), 6.71 (s, 1H, C4-H), 6.84-7.01 (m, 3H), 7.04-7.15 (m, 4H), 7.26 (m, 3H). ^{13}CNMR (300 MHz, $CDCl_3$) δ ppm: 56.1, 56.1, 98.3,

110.6, 110.8, 113.1, 113.4, 116.0, 116.1, 116.2, 122.0, 122.2, 127.5, 127.8, 129.2, 135.3, 136.0, 148.2, 148.4, 162.7, 168.8.

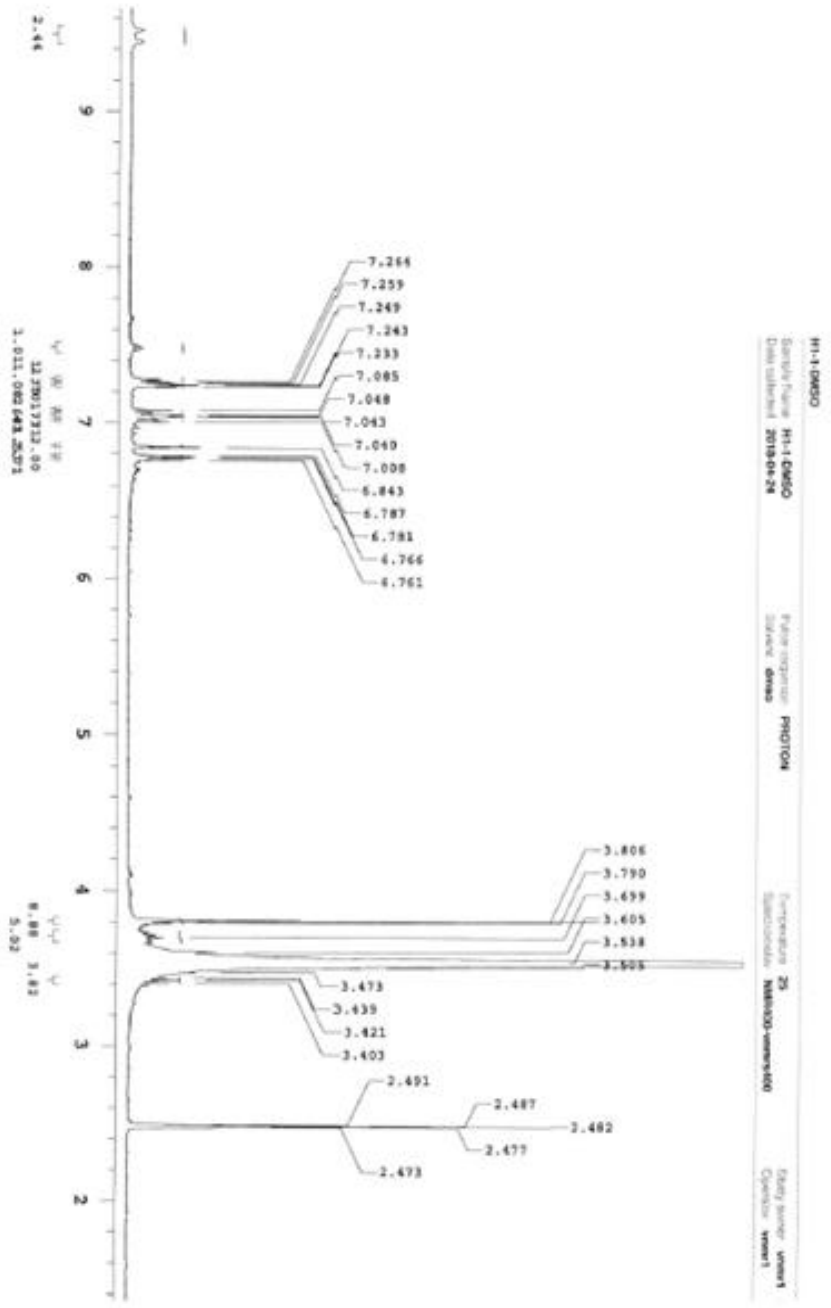
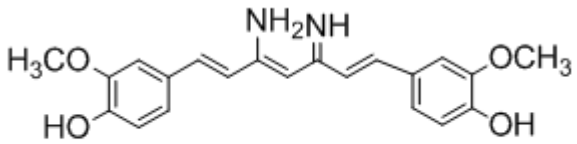


Figure 2.4: [1]HNMR spectrum of compound **23**

2.2.3 4,4'-((1E,3Z,6E)-3-amino-5-iminohepta-1,3,6-triene-1,7-diyl)bis(2-methoxyphenol)



24

Figure 2.5: Chemical structure of compound **24**

To a large test tube fitted with a magnet stir, curcumin (01.0g, 2.7mmol) was added, followed with ammonium acetate (0.46g, 6mmol). The test tube and its contents was placed in an oil bath. The mixture was heated with stirring to 160ºC for 30 min, and kept at this temperature for another 30 min. Then it was cooled to room temperature, producing a brown solid that was crushed into powder and washed several times with water and then isopropyl alcohol. The insoluble residue was recrystallized from hexane/EtOAc (2:1 by volume) to give 0.84g (yield 57.5%). The chemical structure of the product is shown in Figure 2.5.

Spectroscopic data of compound **24** is shown in Figure 2.6. Melting point is 146-151ºC. IR (neat): ν_{max} cm^{-1}: 3215 (-C-OH), 1628 (C=N), 1554 (C=C), 1508, 1455.^{1}HNMR (400 MHz, DMSO-d6) δ ppm: 3.83 (s, 6H, OCH_3), 5.55 (s, 2H, OH), 6.68-7.87 (m, 7H), 6.98-7.21 (m, 4H), 8.63 (bs,

2H), 9.21 (1H, bs). ^{13}CNMR (300 MHz, $CDCl_3$) δ ppm: 56.2, 94.3, 112.1, 116.6, 116.7, 122.8, 127.4, 128.1, 135.9, 137.3, 147.9, 148.1, 149.2, 164.6.

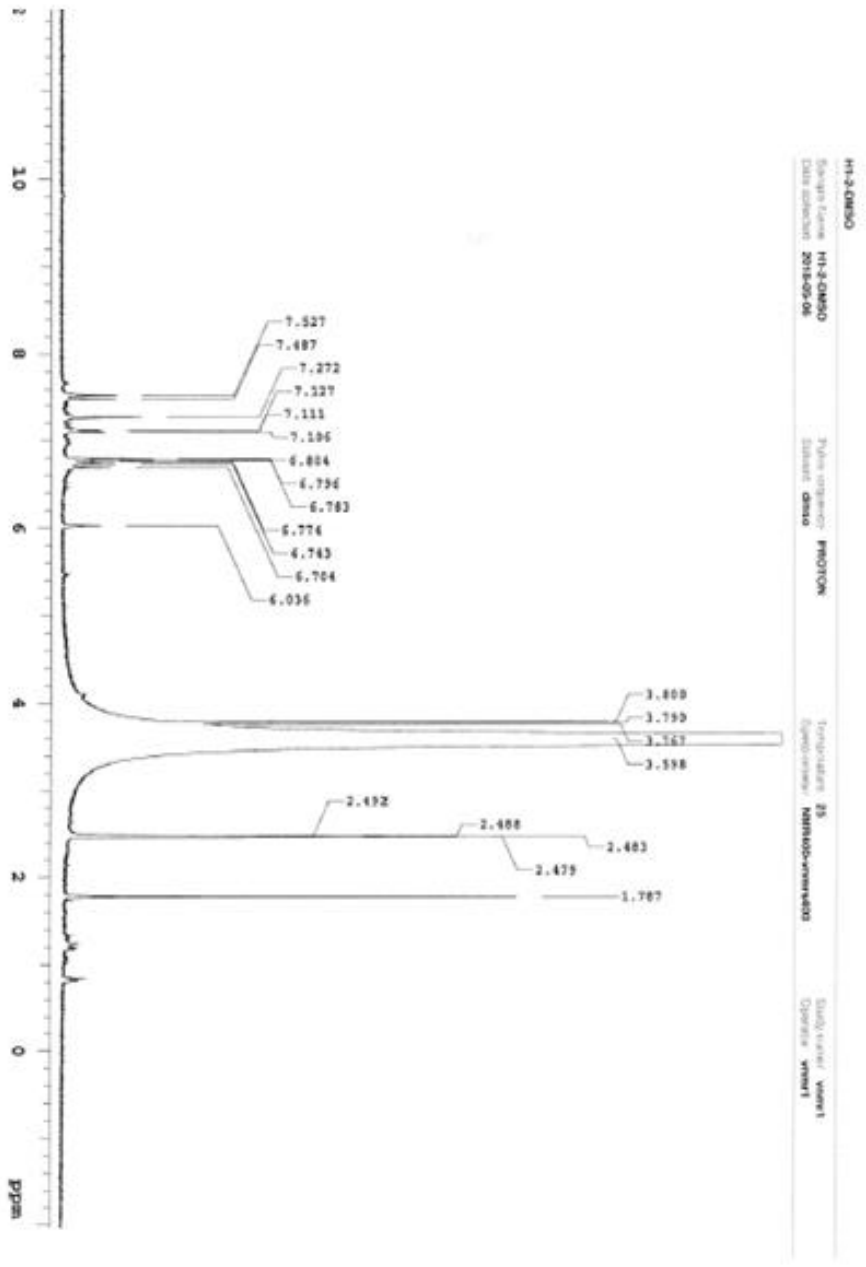
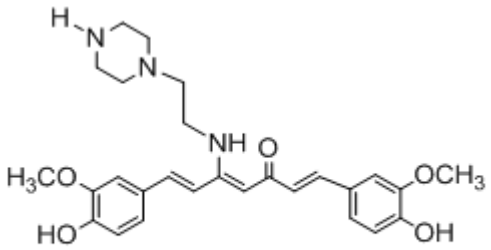


Figure 2.6: ^{1}HNMR spectrum of compound **24**

2.2.4 (1E,4Z,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-5-((2-(piperazin-1-yl)ethyl)amino)hepta-1,4,6-trien-3-one



25

Figure 2.7: Chemical structure of compound **25**

To a large test tube fitted with a magnet stir, curcumin (0.5g, 1.4mmol) was added, followed with a 1-(2-aminoethyl) piperazine (0.18g, 1.4mmol). The test tube with its contents was placed in an oil bath. The mixture was heated with stirring to 160ºC for 30 min, and kept at this temperature for another 30 min. Then it was cooled to room temperature, producing a brown solid that was crushed into powder and washed several times with water and then isopropyl alcohol. The residue was recrystallized form water/ethanol mixture. The chemical structure of the product is shown in Figure 2.7.

Spectroscopic data of compound **25** is shown in Figure 2.8. Melting point is 142-148 ºC.IR (neat): ν_{max} cm^{-1}3345 (O-H and N-H), 3050 (=C-H), 2948 (-C-H), 1670 (C=O), 1610 (C=N). ^{1}HNMR (400 MHz, DMSO-d6)δ ppm: 2.39 (t, 2H), 2.49 (t,2H), 3.79 (s, 6H, OCH_3), 5.48 (s, 2H, OH), 6.8-7.87

(m, 6H), 6.98-7.21 (m, 4H), 8.63 (bs, 1H), 9.21 (1H, bs). ^{13}CNMR (300 MHz, $CDCl_3$) δ ppm: 43.7,46.5, 56.1, 57.5, 54.4, 105.8, 111.3, 116.8, 122.8, 123.5, 124.1, 127.2, 135.3, 142.6, 172.5, 188.1.

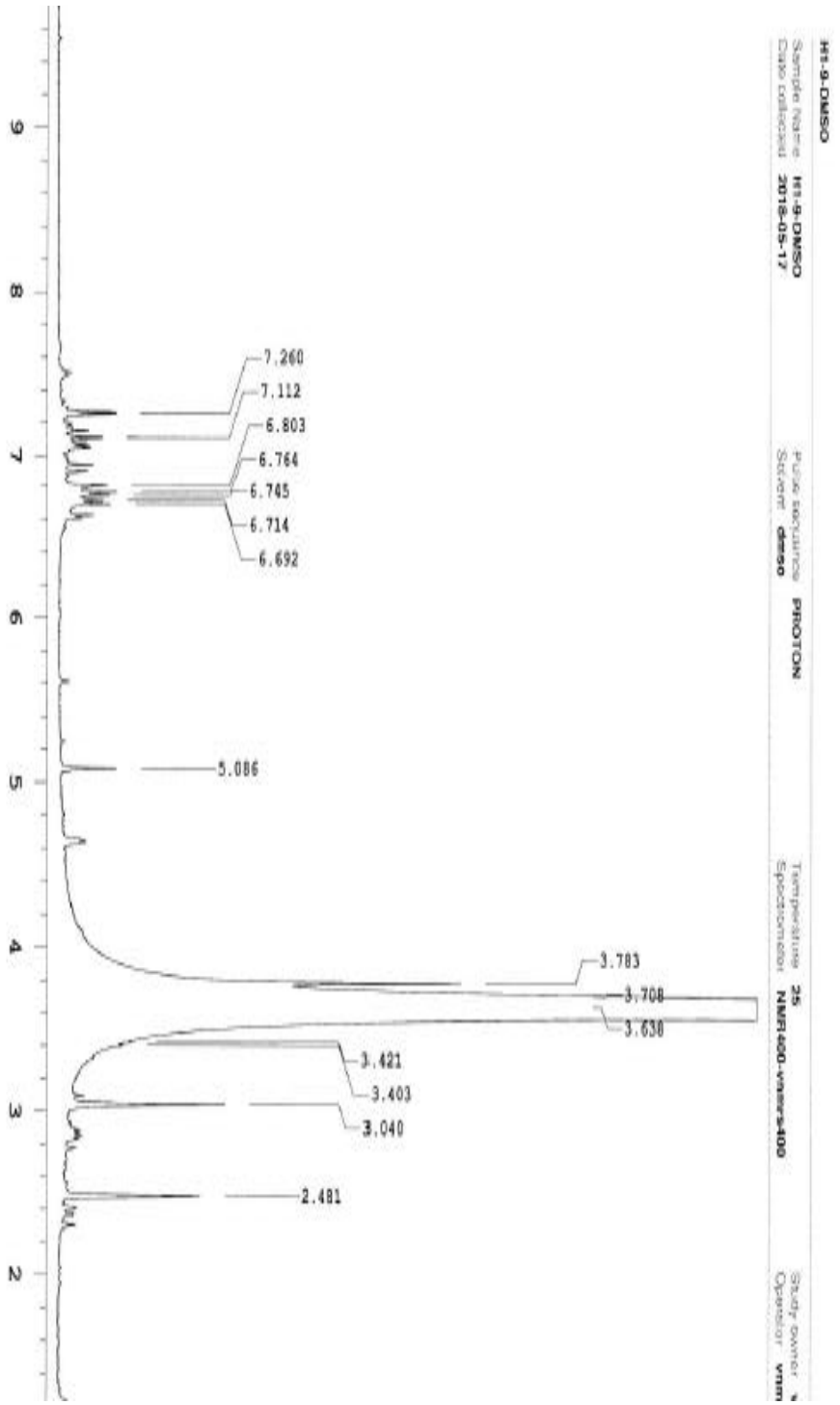
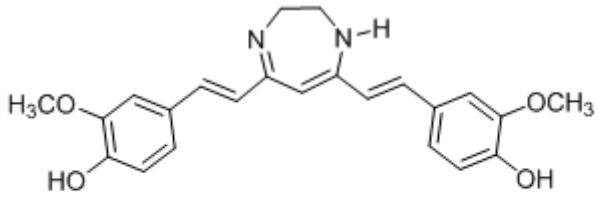


Figure 2.8: ^{1}HNMR of compound **25**

2.2.5 4,4'-((1E,1'E)-(2,3-dihydro-1H-1,4-diazepine-5,7-diyl)bis(ethene-2,1-diyl))bis(2-methoxyphenol)



26

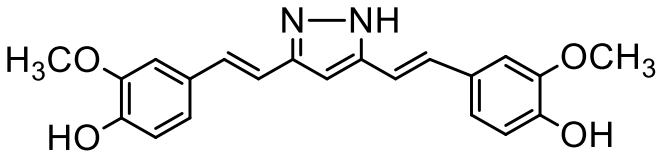
Figure 2.9: Chemical structure of compound **26**

To a large test tube fitted with a magnet stir, curcumin (0.5g, 1.4mmol) was added, followed with ethylenediamine (0.1mL, 1.5mmol). The test tube and its contents was placed in an oil bath. The mixture was heated with stirring to 160ºC for 30 min, and kept at this temperature for another 30 min. Then it was cooled to room temperature, producing a brown solid that was crushed into powder and washed several times with water and then isopropyl alcohol. The residue was recrystallized from water ethanol mixture. The product structure shown in Figure 2.9.

Yield is 86.3% (0.51g), melting point is 65-68°C (hydrated) and the IR(neat): v_{max} cm^{-1}: 3605 (-C-OH), 3020, 1640 (-C=N), 1600, 1080 (C-O ether). ^{1}HNMR (400 MHz, DMSO-d6) δ: 3.16 (s, 2H, CH_2), 3.764 (s, 4H, CH_2CH_2); 3.78 (s, 6H, OCH_3), 5.72 (s, 2H, OH), 6.85 (m, 2H), 6.91 (d, 2H, J = 15.2 Hz), 7.05 (d, 2H, J = 12.1 Hz), 7.20 (s, 2H), 7.40- 7.60 (m, 2H).

^{13}CNMR (400 MHz, DMSO-d6) δ: 24.5, 48.3, 56.4, 112.9, 116.3, 120.6, 122.4, 127.8, 129.3, 148.2, 149.3, 165.8.

2.2.6 4,4'-((1H-pyrazole-3,5-diyl)bis(ethane-2,1-diyl))bis(2-methoxyphenol)



27

Figure 2.10: Chemical structure of compound **27**

To a 100mL round bottom flask fitted with a magnet stir and a condenser, curcumin (1.0 g, 2.7mmol) was added followed with 50mL of ethanol. The mixture was stirred until a clear solution was obtained. Hydrazine hydrate solution (80%, 1.2g, 30mmol) was added to the solution followed by 5 drops of concentrated H_2SO_4. The reaction mixture was refluxed for 2 h. The solvent was evaporated under vacuum, the residue was washed several times with 10% Na_2CO_3, water and dried. The residue was recrystallized from hexane/EtOAc (2:1 by volume). The product structure is shown in Figure 2.10.

Melting point is 167-172ºC. Spectroscopic data for compound 27 is shown in Figure 2.11. IR (neat): ν_{max} cm^{-1}: 3400 (-C-OH), 3197, 2932, 1600, 1516, 1453, 1433,12741643, 1620 (-C=N), 1514, 1276, 1213, 1031.

^{1}HNMR (400 MHz, DMSO-d6): *δ:* 3.84 (s, 6H, 2OCH_3), 6.23 (s, 2H, OH), 6.67 (s, 1H, C4-H), 6.78-7.05 (m, 3H), 7.08-7.21 (m, 4H), 7.31 (m, 3H). ^{13}CNMR (300 MHz, $CDCl_3$) *δ* ppm: 56.2, 56.2, 98.1, 110.3, 110.9, 113.2, 113.3, 116.0, 116.2, 116.1, 121.6, 122.2, 127.5, 127.9, 129.3, 135.3, 136.0, 148.3, 148.2, 162.7, 168.8.

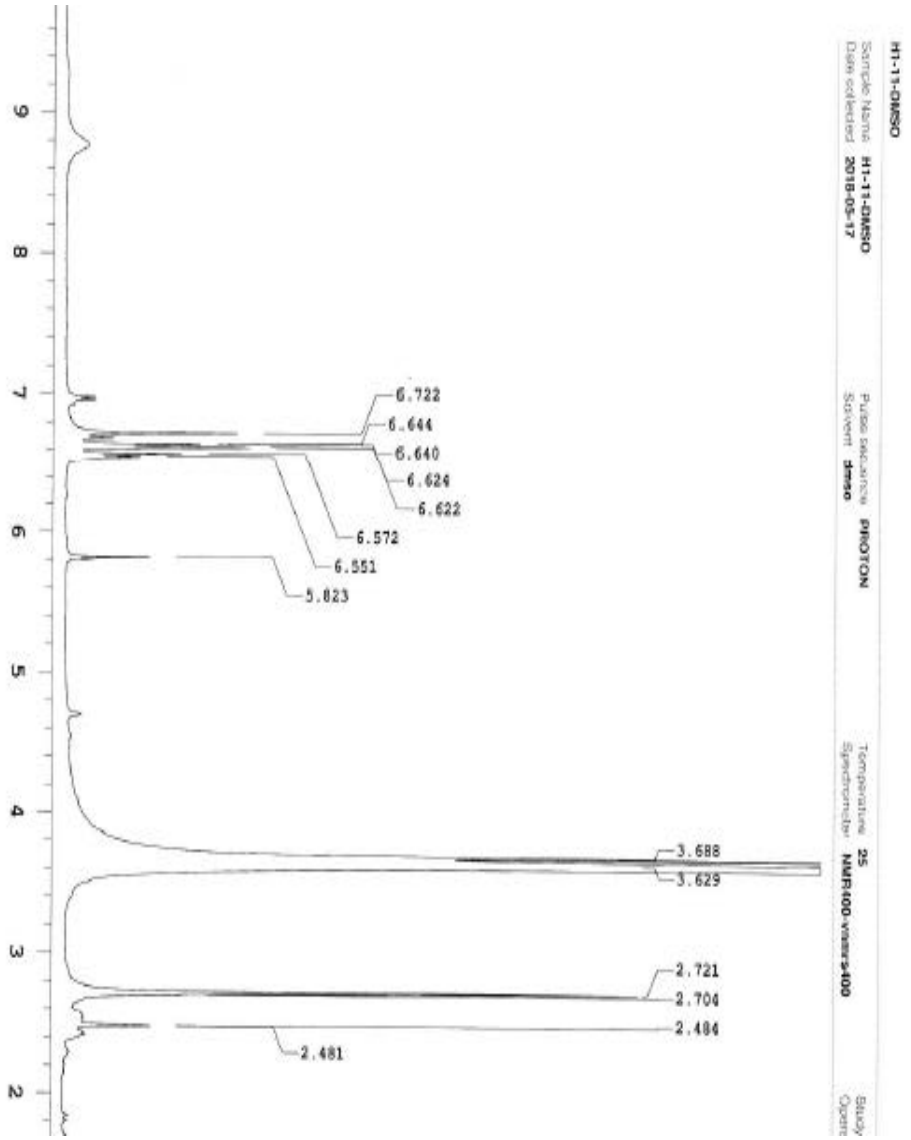
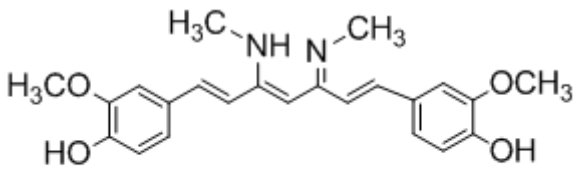


Figure 2.11: ^{1}HNMR spectrum of compound **27**

2.2.7 4,4'-((1E,3Z,5E,6E)-3-(methylamino)-5-(methylimino)hepta-1,3,6-triene-1,7-diyl)bis(2-methoxyphenol)



28

Figure 2.12: Chemical structure of compound **28**

To a 100mL round bottom flask fitted with a magnet stir and a condenser, curcumin (1.0g, 2.7mmol) was added followed with 50mL of ethanol. The mixture was stirred until a clear solution was obtained. Methylamine solution 40% (0.465g, 6mmol) was added to the solution followed with 5 drops of concentrated H_2SO_4. The reaction mixture was refluxed for 2 h. The solvent was evaporated under vacuum, the residue was washed several times with 10% Na_2CO_3, water and dried. The residue was recrystallized from ethanol/water solution (1:2 by volume). The structure of the product is shown in Figure 2.12.

Melting point is 171-177ºC. Spectroscopic data is shown in Figure 2.13. IR: ν_{max} cm^{-1} 3602 (-C-OH), 3050 (=C-H), 2943 (C-H), 1630 (C=N), 1610 (-C=C), 1514, 1276, 1213, 1031. ^{1}HNMR (400 MHz, DMSO-d6): δ 2.86 (s, 6H, 2 NCH_3), 3.83 (s, 6H, 2OCH_3), 6.27 (s, 2H, OH), 6.69 (s, 1H, C4-H), 6.79-7.05 (m, 3H), 7.06-7.19 (m, 4H), 7.28 (m, 3H). ^{13}CNMR (300

MHz, $CDCl_3$) δ ppm: 31.8, 39.6, 56.2, 56.6, 98.4, 110.7, 110.9, 113.2, 113.3, 116.0, 116.3, 116.3, 121.9, 122.6, 127.5, 127.9, 129.2, 135.2, 136.1, 148.3, 148.4, 162.8, 168.8.

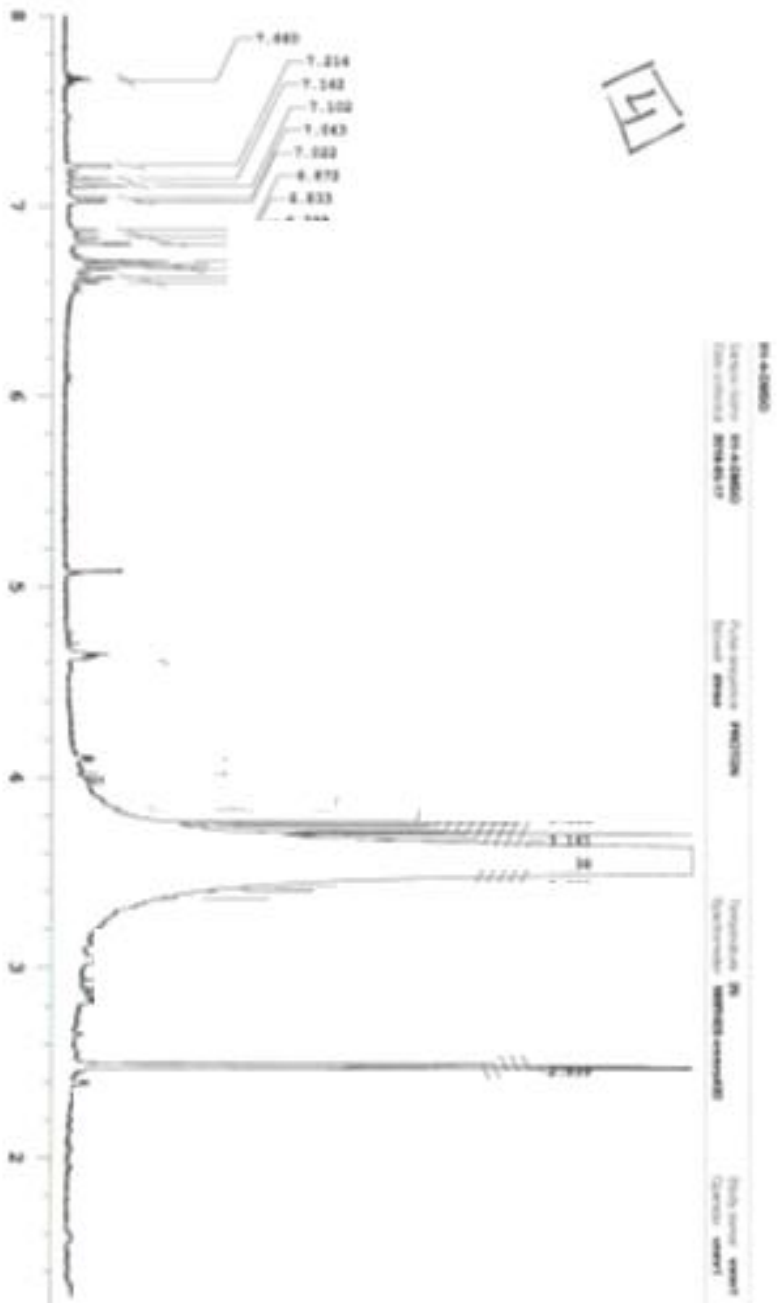
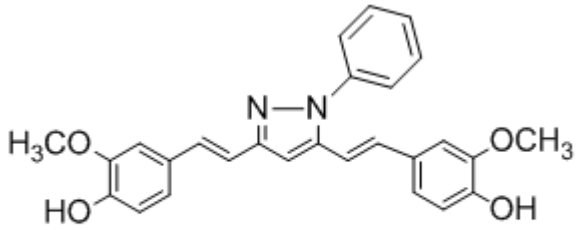


Figure 2.13: ^{1}HNMR of compound **28**

2.2.8 4,4'-((1E,1'E)-(1-phenyl-1H-pyrazole-3,5-diyl)bis(ethene-2,1-diyl))bis(2-methoxyphenol)



29

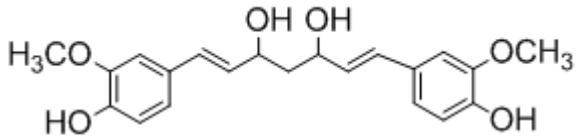
Figure 2.14: Chemical structure of compound **29**

To a large test tube fitted with a magnet stir, curcumin (0.5g, 1.4mmol) was added, followed with phenylhydrazine (0.3g, 2.7mmol). The test tube and its contents was placed in an oil bath. The mixture was heated with stirring to 160ºC for 30 min, and kept at this temperature for another 30 min. Then it was cooled to room temperature, producing a brown solid that was crushed into powder and washed several times with petroleum ether then with diethyl ether. The produced solid was recrystallized from hexane/EtOAc (2:1 by volume). The product structure is shown in Figure 2.14.

Yield is 60% (0.48g), melting point is 98-104°C, IR (neat): v_{max} cm^{-1}: 3602 (-C-OH), 3355 (–C–NH), 3350, 1618 (-C=N), 1602 (C=C|), and 1082 (C-O ether) of (–O-CH_3). ^{1}HNMR (300 MHz, $CDCl_3$) δ ppm: 3.85 (s, 6H, OCH_3), 6.01 (s, 2H, OH), 6.78 (s, 1H, C4-H), 7.01 (d, 2H, J = 14.7 Hz, C2-

H and, C6-H), 7.12 (d, 2H, J = 14.7 Hz, C1-H and C7-H), 7.15–7.32 (m, 8H, Ar-H), 7.47 (m, 2H, Ar-H), 7.69 (m, 1H, Ar-H). ^{13}CNMR (300 MHz, CDCl3) δ ppm: (300 MHz, CDCl3): 56.0, 56.2, 101.3, 110.1, 111.0, 112.0, 115.9, 116.2, 117.5, 120.8, 125.3, 128.3, 128.3, 128.8, 129.1, 129.0, 129.1, 131.3, 133.3, 139.8, 142.9, 147.2, 147.9, 148.3, 148.1, 151.4, 161.1.

2.2.9 Preparation of (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-diol



30

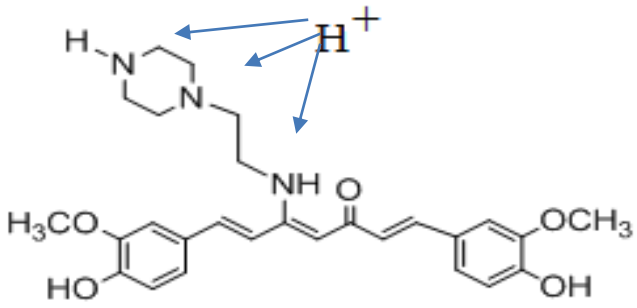
Figure 2.15: Chemical structure of compound **30**

In a round bottom flask fitted with a magnetic stir bar, curcumin (2.0g, 5.4mmol) was dissolved in 10mL of methanol. The $NaBH_4$ (0.2g, 5.3mmol) was frequently added to the flask. The flask was then placed in a 50ºC water bath with continuous stirring until deep yellow color of curcumin turned to pale yellow (it took about 1 h). The reaction was cooled to room temperature and water (10mL) was added dropwise to destroy excess $NaBH_4$ and to precipitate the product. The product was collected by suction filtration and washed several times with water and dried. The product weight was 1.93g (yield 88%). The chemical structure of the product is shown in Figure 2.15.

Melting point is 121-126 ºC. IR (neat): ν_{max} cm^{-1} 3654 (O-H), 3045 (=C-H), 1620 (C=C), 1582 (C=C aromatic), 1513, 1455, 1430, 1374, 1272.

2.2.10Protonation of alkaloid 25

In order to study the effect of converting the amine group to ammonium, 0.15g of compound **25** was placed in a small beaker, and then1.00mL of 1.0% HCl was added. This mixture was stirred for one hour, then it was filtered and dried. Possible protonation sites for compound **25** are shown in Figure 2.16



25

Figure 2.16: Possible protonation sites for compound **25**

2.3 Testing for Antibacterial Activity

2.3.1 Materials

Mueller-Hinton agar (MHA), Nutrient broth, Eosin methylene blue agar (EMB), 0.5Mcfarland standard, sterile normal saline and sterile 10% Dimethyl sulfoxide (DMSO) solution.

2.3.2 Microorganisms used

Bacterial strains used in this study are summarized in Table 2.1.

Table 2.1: Bacterial strains used in this study and their types according to Gram stain activity.

Bacterial strains	**Type of bacteria**
Staphylococcus aureus	Gram positive
Staphylococcus epidermidis	Gram positive
pseudomonas aeruginosa	Gram negative
Escherichia coli	Gram negative

2.3.3 Preparation of McFarland turbidity standard No. 0.5

McFarland 0.5 turbidity standard was prepared by mixing 50µl of a 1.175% (w/v) barium chloride dihydrate ($BaCl_2 \bullet 2H_2O$) solution and 9.95ml of 1% (v/v) sulfuric acid. The tube which had the 0.5McFarland standard was then sealed with parafilm to prevent evaporation and stored in the dark at room temperature. The 0.5McFarland standard was vigorously mixed on a vortex mixer before use. A 0.5McFarland Standard is comparable to a bacterial suspension of 1.5 X 10^8 colony-forming units (cfu/mL).[50]

Three to four colonies of each bacterium were transferred into tubes that had 5.0-10mL of sterile normal saline, the turbidity of the bacterial suspensions was adjusted to have similar turbidity of 0.5McFarland standard with bacterial suspension of about 1.5 x10^8 cfu/mL.

2.3.4 Antibacterial Susceptibility Test

Antimicrobial susceptibility for the four bacterial strains used in this study was determined according to the Clinical and Laboratory Standard Institute (CLSI) using the disk diffusion method.[51]The antibiotic disks (purchased from Oxoid company) used in this study were Aztreonam (ATM,30 µg), Clindamycin (DA, 2µg), Ciprofloxacin (CIP, 5µg), Nalidixic acid (NA, 30µg), Tetracycline (TE, 30µg), Cotrimoxazole (SXT, 30µg), Kanamycin (K, 30µg), Cefoxitin (FOX, 30µg), Norfloxacin (NX, 10µg), Levofloxacin (LEV, 5µg).

Normal saline suspensions of all bacterial strains were adjusted to the McFarland 0.5 standard and used to inoculate MHA plates with a sterile cotton swab. The disks were placed on the seeded MHA plate. The plates then incubated at 37°C for 24 hours. The inhibition zones (if any) were measured in millimeters, and the isolates were classified as resistant (R) or susceptible (S), according to CLSI guidelines. [51] The results are shown in Table 3.1 (chapter 3).

2.3.5 Determination of Minimum Inhibitory concentration (MIC)

MIC of curcumin derivatives was determined by the broth microdilution method in sterile 96- wells microtiter plates according to the CLSI.[51]The curcumin derivatives (400µg/ml of 10% DMSO) and 10% DMSO (negative control) were two fold-serially diluted in nutrient broth in the wells of the plates in a final volume of 100µL. After that, a bacterial inoculum size of 10^4CFU/ml was added to each well. Negative control

wells containing either 100µL NB only, or 100µL DMSO with bacterial inoculum, or curcumin derivatives and nutrient broth without bacteria were included in these experiments. Each curcumin derivative compound was run in duplicate. The microtiter plates were then covered and incubated at 37°C for 24 h.

The MIC was considered as the lowest concentration of the compound which inhibited the bacterial growth. The MIC values (µg/ml) are revealed in Tables 3.2-3.5 (chapter 3).

2.3.6 Determination of Minimum Bactericidal Concentration (MBC)

The wells that showed inhibition to bacterial growth, 10µL from these wells were transferred by disposable inoculating loop and subcultured on MHA plates. Then these plates were incubated at 37ºC for 24 hours.

The lowest concentration of curcumin derivative that is required to kill a specific bacterial strain was considered as MBC, Table 3.6 (chapter 3).

2.4 Evaluation of the Genotoxic Potential of curcumin derivative on *E. coli* strain

2.4.1 Inoculation of *E. coli*

From 24-hourold *E. coli* strain growth culture plated on EMB agar medium, few colonies were sub-cultured under sterile conditions into a bottle containing 20mL of nutrient broth and incubated at 37°C for 1 hour with continuous shaking. After that, under aseptic conditions, 1mL of one hour old *E. coli* culture was added to each of 4 sterile bottles each

containing 25mL of nutrient broth medium. These bottles were incubated at 37°C for 1 hour with continuous shaking. Then different concentrations of compound **25** (100µg/ml, 50µg/ml and 25µg/ml of 10% DMSO) were added into 3 of the *E. coli* broth culture. The fourth bottle was considered as a negative or untreated control by adding a specific volume of 10% DMSO.

2.4.2 DNA extraction

Genome of *E. coli* was prepared for enterobacterial repetitive intergenic consensus (ERIC) PCR according to the method described previously.[52]Three mL samples were taken from the *E. coli* growth culture after 2h, 4h, and 24h, centrifuged for five minutes at 14000rpm where the supernatant of each sample was discarded. Each bacterial sample pellet was re-suspended in 1mL of Tris-EDTA (10mM Tris-HCl, 1mM EDTA [pH 8]), centrifuged for five minutes at 14000rpm, then the supernatant was discarded. The pellet of each bacterial sample was re-suspended in a 200µl of sterile distilled water, boiled for 15 min, then the mixture was incubated in ice for five minutes. Then the samples were pelleted by centrifugation at 14000rpm for five minutes, and each sample supernatant was transferred to new eppendorf tube.

After that, the DNA concentration for each sample was determined by using nanodrop spectrophotometer (GenovaNano, Jenway) and the DNA samples were stored at -20ºC for ERIC-PCR analysis.

2.4.3 Enterobacterial repetitive intergenic consensus (ERIC) PCRanalysis

The ERIC-PCR was performed using Primer ERIC1: 5`-ATG TAA GCT CCT GGG GAT TCA C-3` and Primer ERIC2: 5-AAG TAA GTG ACT GGG GTG AGC G-3`. Each PCR reaction mix (25µl) was composed of 10mM PCR buffer pH 8.3; 3mM $MgCl_2$; 0.4mM of each dNTP; 0.8µM primer; 1.5U of Taq DNA polymerase and fixed amount of DNA template (depended on the DNA concentration of negative control).

Then, DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 3 min at 94ºC; followed by 40 cycles of denaturation at 94ºC for 50 s, annealing at 50ºC for 1 min and extension at 72ºC for 1 min, followed by a final extension step at 72ºC for 5 min. The PCR products were analyzed by electrophoresis through 2% agarose gel.

2.4.4 Agarose gel electrophoresis

A 2% agarose gel was used to analyze ERIC-PCR products. A DNA marker of 100bp was also used on the same gel including ERIC-PCR products. Electrophoresis was run using 1X TAE electrophoresis working buffer (50x: 242g Tris base, 57.1 ml glacial acetic acid and 100ml of 0.5M EDTA [pH 8.0]) for 75 min at 80 V. Then the gel was stained with Ethidium bromide (0.5µg/ml of water).

The ERIC-PCR profile was visualized using UV trans-illuminator and photographed. Changes in ERIC-PCR banding pattern profiles following curcumin derivativenumber**25**treatments, including variations in band intensity as well as gain or loss of bands, were taken into consideration.[53, 54]

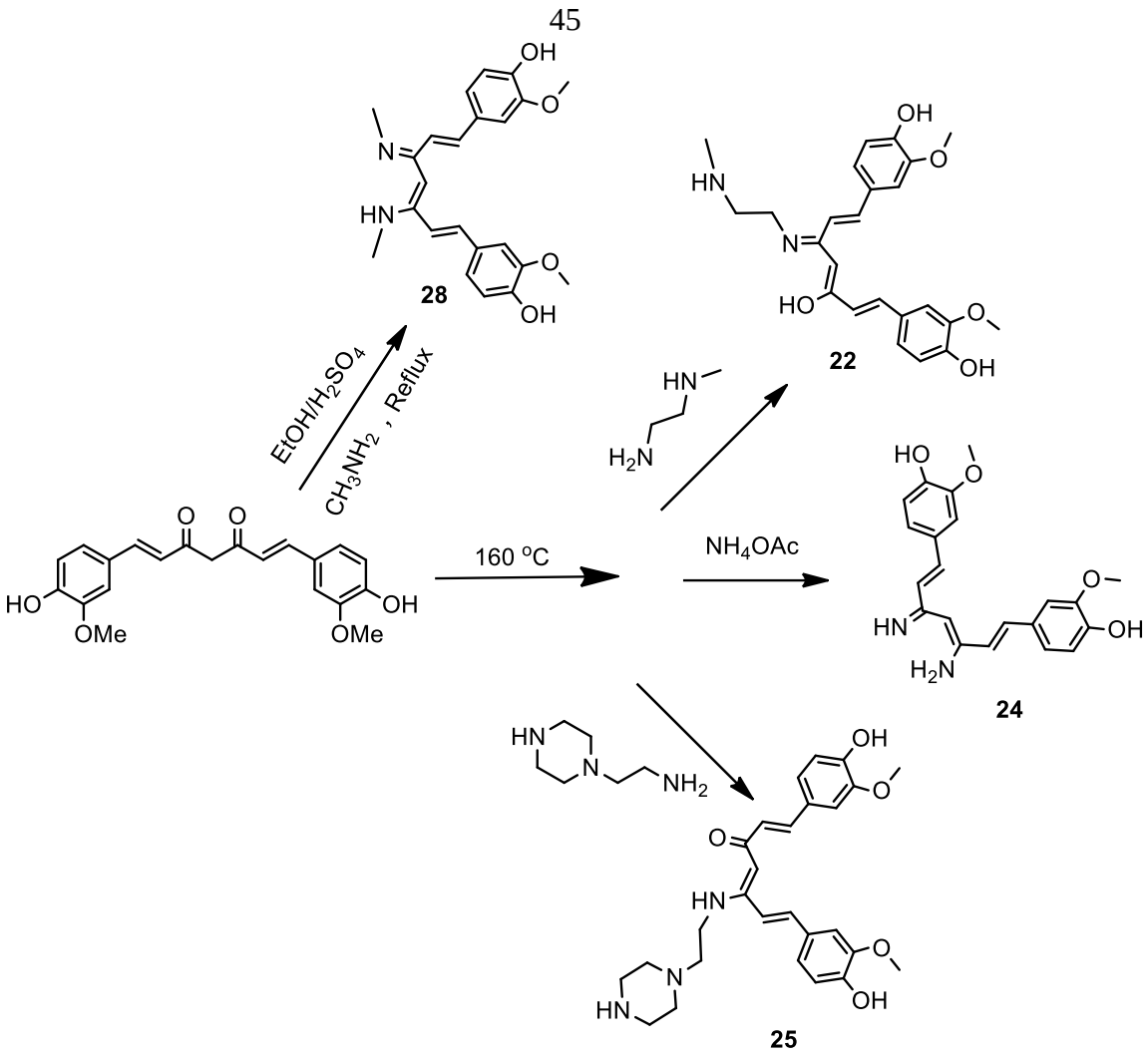
3 RESULTS AND DISCUSSION

In a previous work that was carried out at our laboratories, various curcumin based heterocycles were synthesized.[55] from reacting curcumin with various diamino compounds. The synthesis was carried out by reacting curcumin with the diamino compound in ethanol and acetic acid under reflux for two hours. Acetic acid played a dual function as a catalyst and as a solvent. Some of the prepared curcumin based excellent heterocycles showed excellent antimicrobial activities against gram positive bacteria and mild activity against gram negative. In continuation of this work and in order to develop curcumin based alkaloids with high efficiency against bacteria, we have synthesized for the first time ever a series of novel curcumin based alkaloids.

The compounds were synthesized using two new methods, where the yields in some cases as shown in the experimental part were quantitative. In the first method, curcumin was reacted with the diamine compounds in ethanol and a catalytic amount of sulfuric acid (**procedure 1**). The second method involved mixing the reactants, curcumin and diamine compounds, heating them gradually to melt, then heating them at 160^{o}C for 30 min (**procedure 2**). The structures of the prepared compounds are shown in schemes 3.1 and 3.3.

3.1 Preparation of curcumin based alkaloids with alkyl amine chain

Various amines were used for preparing alkaloid with alkyl amine chain, they are summarized in scheme 3.1.



Scheme 3.1: Chemical structures of the prepared curcumin based alkaloids

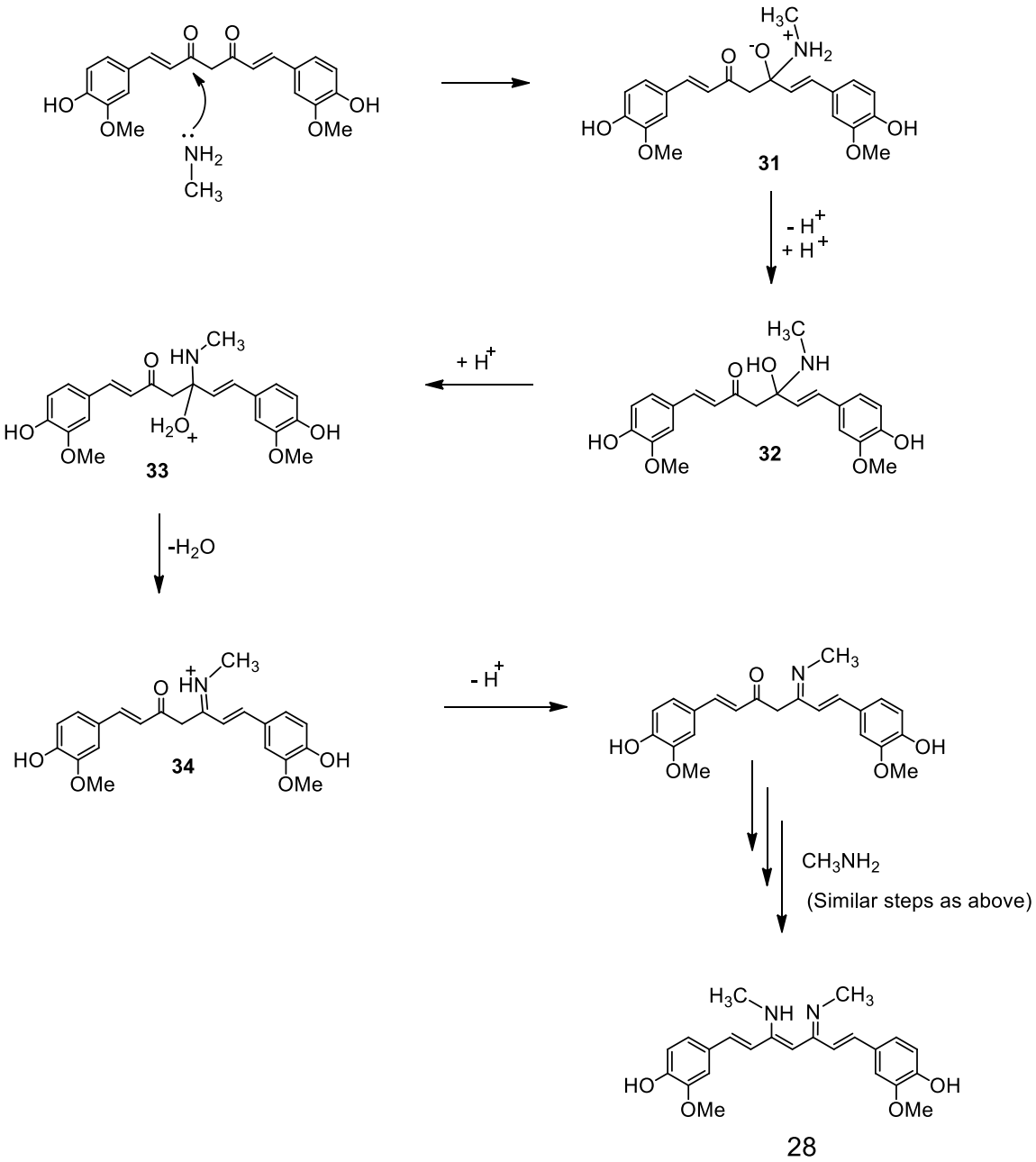
The structures of all prepared alkaloids were confirmed by FT-IR, ^{1}H and $^{13}CNMR$. Alkaloid **22** was prepared as shown in scheme 3.1 from reacting curcumin with N-methylethylene diamine. Both procedures 1 and 2 gave same product **22.** The^{1}H NMR of compound **22** is shown in Figure 2.1, the N-CH_3 appear at a chemical shift of 3.05, the two methylene groups are covered with water peak which usually present in DMSO. The two peaks at 4.6 and 5.1 represent the protons of the vinylic hydroxyl group and the aryl one, respectively.

In Compound **25,** the alkaloids with piperazinyl ring was prepared from reacting curcumin with 1-(2-aminoethyl) piperazine with 1:1 mol ratio. Procedure 2 was followed. Compound **25** could also be prepared using procedure 1.

Compound **28** was prepared from reacting curcumin with methyl amine in 1: 2 mol ratio using procedure 1. The proton NMR of compound 28 is shown in Figure 2.7. The aromatic groups are consistent with the structure, the O-CH_3 and N-CH_3 is buried under the water peak, which as mentioned earlier present in DMSO solvent.

Compound **24** was prepared using procedure 2, in this reaction curcumin was heated at 160^oC with excess ammonium acetate in solvent free reaction. The ^{1}HNMR of compound 24 is shown in Figure 2.3, the spectrum shows the NH_2 protons at chemical shift of 1.89. The OCH_3 group of the aromatic ring are covered with the water peak. The Rest of the peaks are consistent with the structure as shown in Chapter 2.

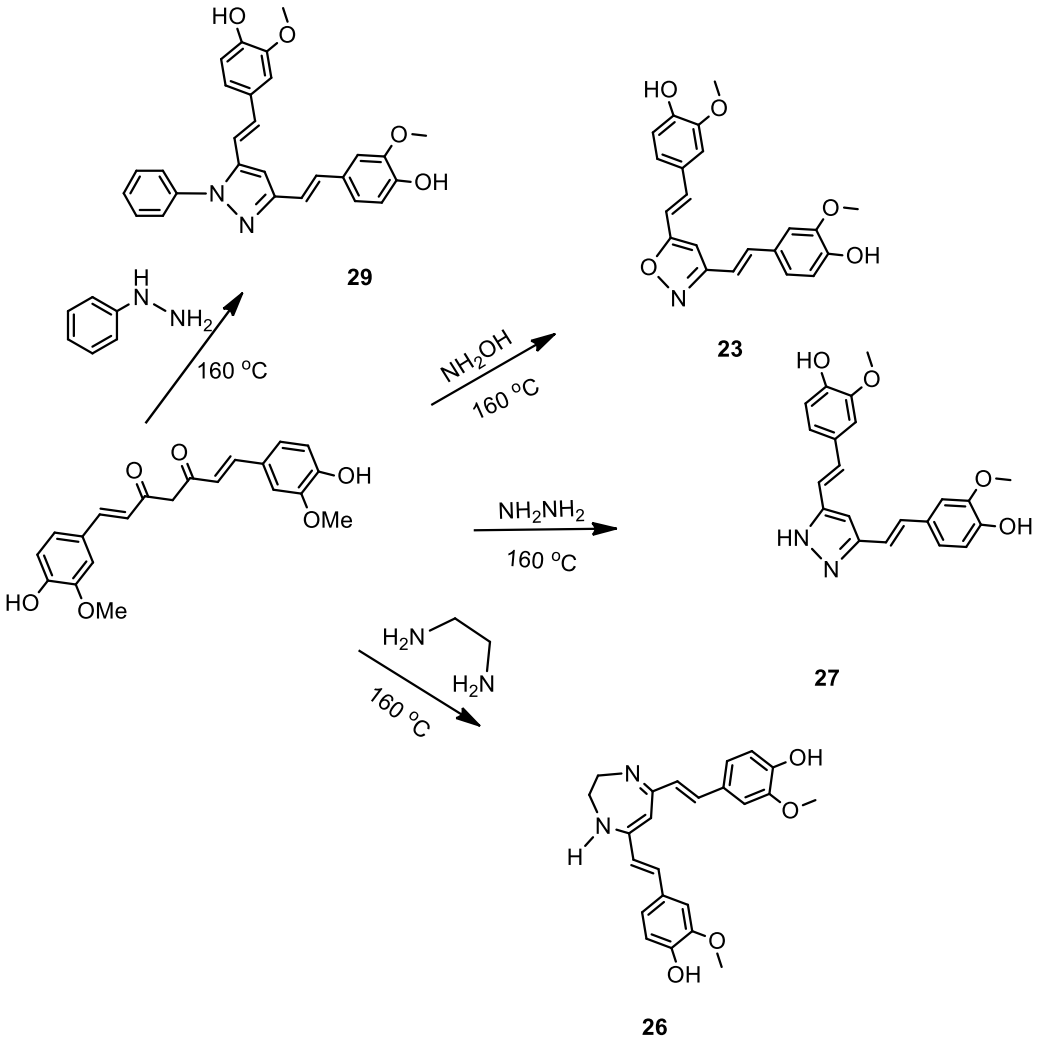
A detailed mechanism on the condensation reaction between curcumin (**19**) and the amino compounds (methylamine) to form alkaloids is shown in scheme 3.2. An amino group of the amino compound makes a nucleophilic addition on one of the curcumin carbonyl groups followed by loss of a water molecule. Nucleophilic attack by a second amino group followed again with a loss of a second water molecule results on the formation of the final product.



Scheme 3.2: Mechanism of coupling between curcumin and methylamine

3.2 Preparation of curcumin based alkaloids with heterocyclic rings

Several compounds of curcumin based alkaloids with heterocyclic rings were prepared. The chemical structures for the prepared compounds and the reaction conditions for synthesizing them are summarized in scheme 3.3.



Scheme 3.3: Chemical structures of the prepared curcumin based alkaloids with heterocyclic rings

The synthesis of 1,4-Diazepine, compound number **26,** could be carried out by either method. The yield was quantitative. 1,4-diazepine **26** was prepared from reacting curcumin with ethylene diamine.

Curcumin based alkaloid with pyrazol ring **27** can be prepared following procedure 1or 2, by reacting curcumin with hydrazine.

The ^{1}HNMR of **27** is shown in Figure 2.6. The peak at 8.8 could be attributed to NH proton. Using excess ethylene diamine 1:4 mol ratio gave

the product4,4'-((3Z,5E)-3-((2-aminoethyl)amino)-5-((2-aminoethyl)imino) hept-3-ene-1,7-diyl)bis(2-methoxyphenol) shown in Figure 3.1. The structure of the product was confirmed by ^{1}HNMR, the spectrum is shown in Fig 3.2. The spectrum shows four NH_2 protons at chemical shift of 3.3 and 2 NH protons at chemical shift of about 3.7.

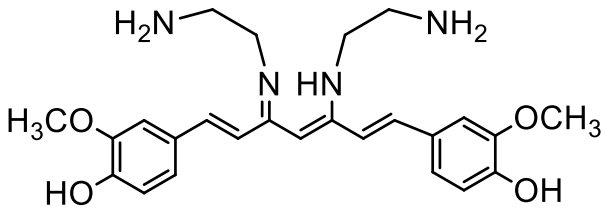


Figure 3.1: Chemical structure of4,4'-((3Z,5E)-3-((2-aminoethyl)amino)-5-((2-aminoethyl)imino)hept-3-ene-1,7-diyl)bis(2-methoxyphenol)

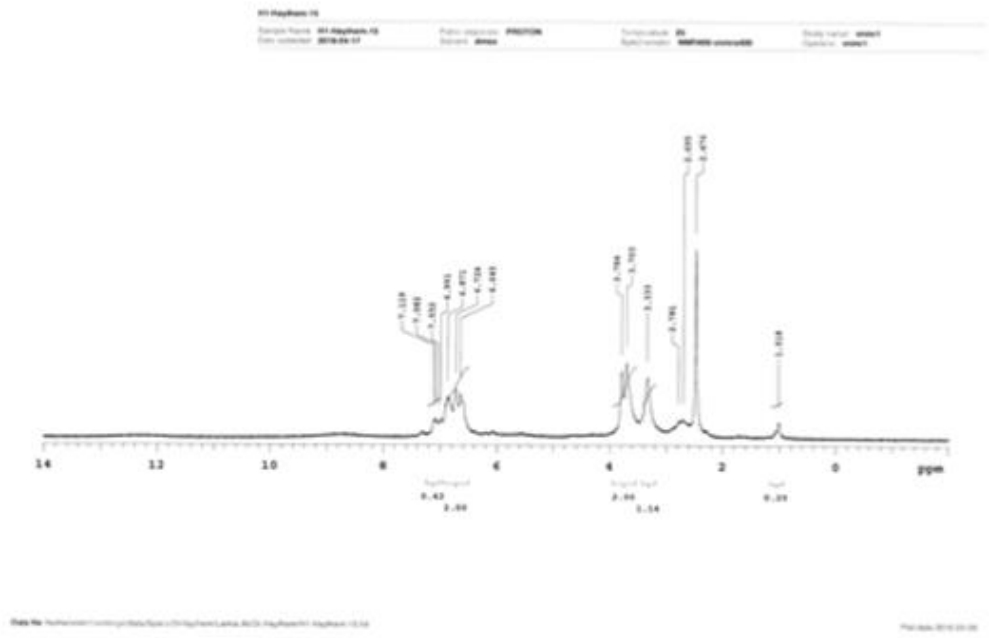


Figure 3.2: ^{1}HNMR of 4,4'-((3Z,5E)-3-((2-aminoethyl)amino)-5-((2-aminoethyl)imino)hept-3-ene-1,7-diyl)bis(2-methoxyphenol)

Another alkaloid with isoxazole ring **23** was prepared from reacting curcumin with hydroxylamine using procedure number 1, procedure 2 works too. The ^{1}HNMR spectrum of compound **23** is shown in Figure 2.2. The spectrum clearly shows the aromatic protons and OCH_3 protons, in addition a peak shows at a chemical shift of 9.5, the peaks could be related to vinylic proton, as shown in Figure 3.3.

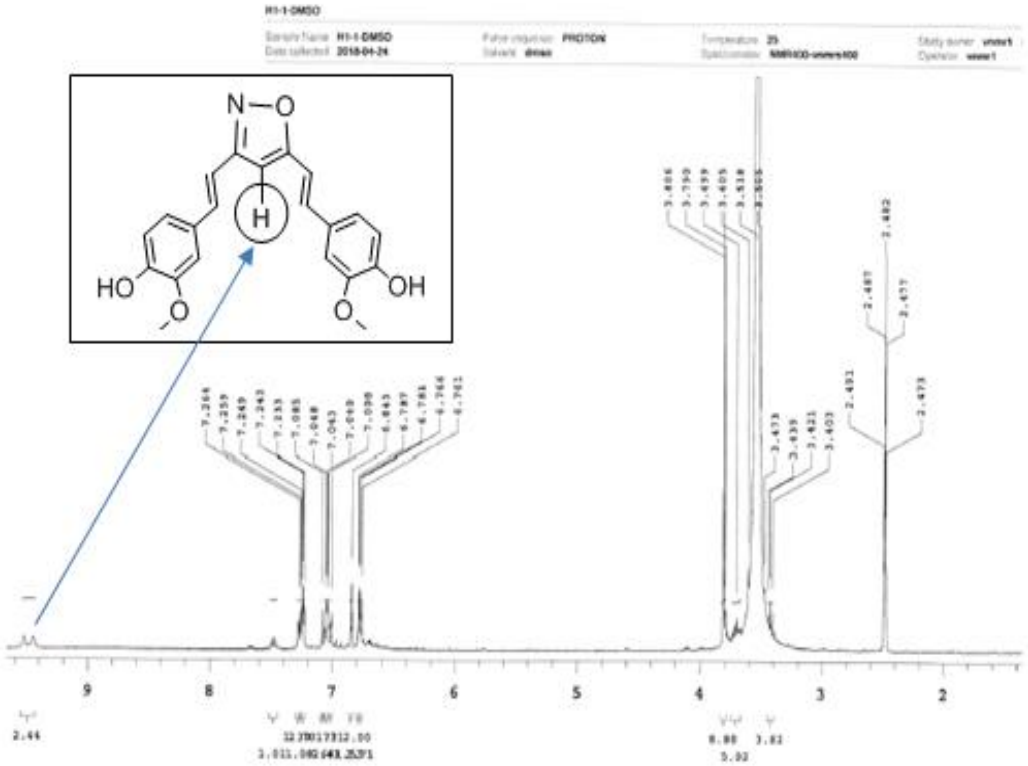
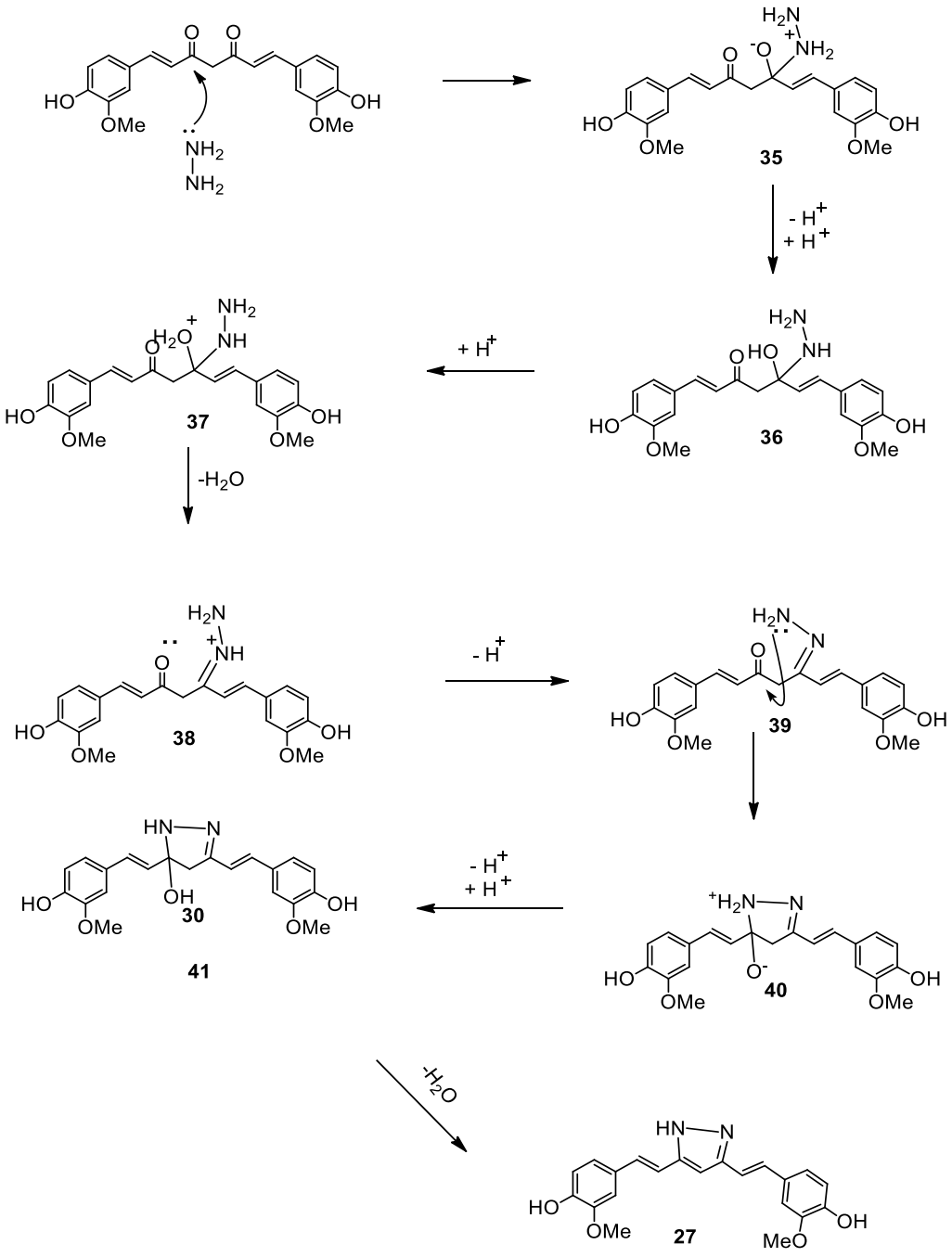


Figure 3.3: ^{1}H NMR of compound alkaloids **23** showing vinylic proton

An alkaloid with phenyl pyrazole was also prepared from reacting phenyl hydrazine and curcumin following procedure number 2. The structure of the product was confirmed by ^{1}H and ^{13}CNMR.

A detailed mechanism on the condensation reaction between curcumin (**19**) and diamino compounds (hydrazine) to form alkaloids with heterocyclic ring is shown in scheme 3.4. An amino group of hydrazine makes a

nucleophilic addition on one of the curcumin carbonyl groups followed by loss of a water molecules. Nucleophilic attack by the second amino group on the intermediate **41** followed with a loss of a second molecule of water results on the formation of the product **27**.



Scheme 3.4: Mechanism of coupling between curcumin and hydrazine

3.3 Antibacterial activities of the prepared curcumin derivatives

3.3.1 Antibiotic Resistance Test

Antibiotic resistance test showed that the bacterial strains used in this study were susceptible to different antibiotics. It was found that the *Staphylococcus aureus* isolate was sensitive to most of the tested antibiotics. However, *Pseudomonas aeruginosa* isolate was resistant against most of the antibiotics used. *Staphylococcus epidermidis* isolate was resistant against Cotrimoxazole and Tetracycline antibiotics. *Escherichiacoli* isolate was resistant against Nalidixic acid and Aztreonam. Results of antibiotic resistance against four species of bacteria that used in this study are presented in Table 3.1.

Table 3.1: Antibiotic sensitivity profile of four bacterial species used in this study

Antibiotic*	**Bacterial species**			
	S. aureus	***P. aeruginosa***	***S. epidermidis***	***E. coli***
ATM	R	S	R	R
DA	S	R	S	R
CIP	S	S	S	S
NA	R	R	R	R
TE	S	R	R	S
SXT	S	R	R	S
K	S	R	S	S
FOX	S	R	S	S
NX	S	S	S	S
LEV	S	S	S	S

*S: Sensitive, R: Resistant

3.3.2 Interpretation of MIC results

The minimal inhibitory concentration (MIC) of each curcumin derivative against bacteria was determined by using broth microdilution method. Results of the current study revealed that the semisynthetic compounds (curcumin derivatives) showed different inhibition degrees against the tested bacteria. *Staphylococcus aureus* was highly susceptible to compounds **23**, **26**, **27**, and **28** with MIC less than 1.56µg/mL. *P*seudomonas *aeruginosa* strain was sensitive to compounds **22**, **23**, **24**, **26** and **29** at MIC 100µg/mL. While *S. epidermidis* and *E. coli* strains were sensitive to compounds **23** and **25** at MIC 50µg/mL and 100µg/mL, respectively. The results of MIC using curcumin derivatives against the four tested bacterial species are shown in tables 3.2-3.5. The DMSO solution (negative control) which had a concentration of 10%, showed non antibacterial activity against these bacterial strains.

Protonated version of compound **25**, which was treated with HCl, (compound **31**) did not show any improvement against bacterial growth, where it showed no activity against *S. aureus*, *S. epidermidis* and *E. coli*. However, compound **25** (un protonated) showed excellent activities against these organisms and it showed low MIC value against *E. coli*. The results indicate that compound **25** in the amine form is more active against bacteria than in the ammonium form (protonated form).

Table 3.2: MIC values for compounds (22-31) against *Staphylococcus aureus*

Concentration (µg/mL)*	22	23	24	25	26	27	28	29	30	31
200	-	-	-	-	-	-	-	-	-	+
100	-	-	-	-	-	-	-	-	-	+
50	-	-	-	-	-	-	-	-	-	+
25	-	-	-	-	-	-	-	-	-	+
12.5	-	-	-	-	-	-	-	-	+	+
6.25	-	-	-	+	-	-	-	-	+	+
3.125	-	-	-	+	-	-	-	+	+	+
1.563	+	-	+	+	-	-	-	+	+	+

*(+): Bacterial growth, (-): no bacterial growth

Table 3.3: MIC values of compounds (22-31) against *Pseudomonas*

Concentration (µg/mL)*	22	23	24	25	26	27	28	29	30	31
200	-	-	-	-	-	-	-	-	-	-
100	-	-	-	+	-	+	+	-	+	+
50	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+
12.5	+	+	+	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+	+	+	+
3.125	+	+	+	+	+	+	+	+	+	+
1.563	+	+	+	+	+	+	+	+	+	+

*(+): Bacterial growth, (-): no bacterial growth

Table 3.4: MIC values of compounds (22-31) against Staphylococcus epidermidis

Concentration (µg/mL)*	22	23	24	25	26	27	28	29	30	31
200	+	-	+	-	+	+	+	+	-	+
100	+	-	+	-	+	+	+	+	-	+
50	+	-	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+
12.5	+	+	+	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+	+	+	+
3.125	+	+	+	+	+	+	+	+	+	+
1.563	+	+	+	+	+	+	+	+	+	+

*(+): Bacterial growth, (-): no bacterial growth

Table 3.5: MIC values of compounds (22-31) against E. coli

Concentration (µg/mL)*	22	23	24	25	26	27	28	29	30	31
200	+	+	+	-	+	+	+	+	+	+
100	+	+	+	-	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+
12.5	+	+	+	+	+	+	+	+	+	+
6.25	+	+	+	+	+	+	+	+	+	+
3.125	+	+	+	+	+	+	+	+	+	+
1.563	+	+	+	+	+	+	+	+	+	+

*(+): Bacterial growth, (-): no bacterial growth

3.3.3 Results of MBC test

Results of the current study showed that compound **26** had high bactericidal activity against *S. aureus* strain with minimum bactericidal concentration (MBC) value of less than 1.56µg/mL. Compounds **23**, **25**, **26**, **27**, **29** and **30** showed MBC value of 200 µg/mL against *P. aeruginosa*.

Compound **25** had MBC value of 100µg/mL against *E. coli*. The minimum bactericidal concentration (MBC) values of each derivative against the four tested bacterial strains are shown in Table 3.6.

Table 3.6: MIC and MBC values (µg/ml) for studied curcumin derivatives against different bacterial species

Curcumin derivative	**Bacterial species**							
	***S. aureus* (µg/mL)**		***P. aeruginosa* (µg/mL)**		***S. epidermidis* (µg/mL)**		***E. coli* (µg/mL)**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
22	3.125	50	100	-	-	-	-	-
23	<1.5	50	100	200	50	-	-	-
24	3.125	3.125	100	-	-	-	-	-
25	12.5	12.5	200	200	100	-	100	100
26	<1.56	1.56	100	200	-	-	-	-
27	<1.56	50	200	200	-	-	-	-
28	<1.56	100	200	-	-	-	-	-
29	6.25	50	100	200	-	-	-	-
30	25	100	200	200	100	-	-	-
31	-	-	200	-	-	-	-	-

3.3.4 Genotoxic potential of compound 25 on the DNA of *E. coli*

Profile of ERIC-PCR product for DNA extracted from *E. coli* treated and untreated with compound **25** for various time intervals is presented in Fig. 3.4.

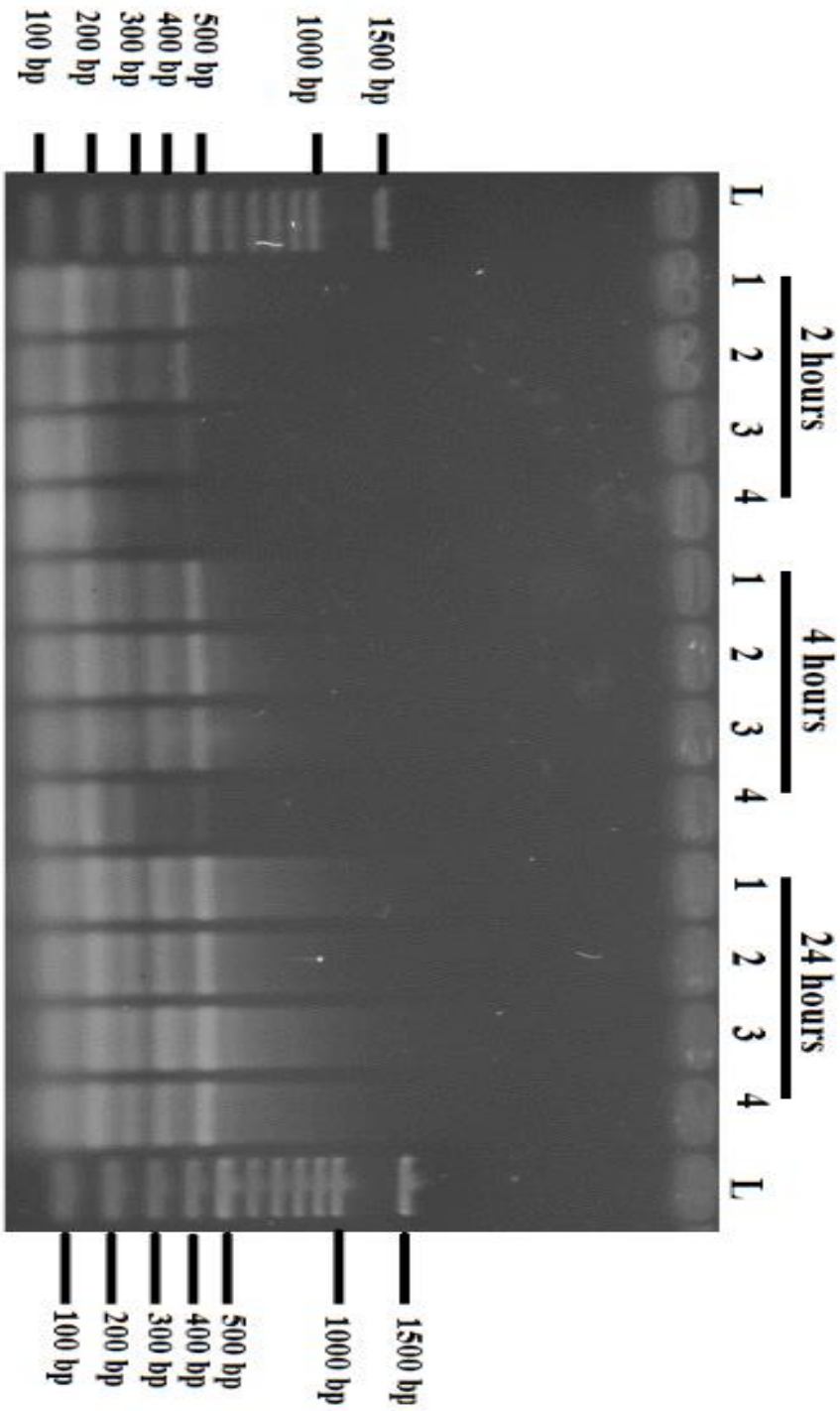


Figure 3.4: ERIC-PCR profile of *E. coli* strain treated and untreated with different concentrations of compound **25** at different time intervals. Lanes L are 100-bp DNA ladder (negative control). Lanes 1, 2, 3 and 4 are ERIC-PCR products from E. coli treated with compound **25** using 100µg/ml, 50µg/ml and 25µg/ml, respectively. Lane 4: untreated *E. coli* strain or negative control.

Changes in the extracted DNA genome from E. coli strain were evaluated in comparison with untreated controls at the same time interval.

After 2 hours, results of ERIC-PCR showed no change in the number of bands in both treated and untreated *E. coli*. However, the ERIC-PCR bands produced from *E. coli* strain treated with compound **25** with concentrations of 100µg/mL and 50µg/mL are more intense than the bands from the untreated control. But at low dose (25µg/mL), there was no change in the intensity of bands in both ERIC-PCR product of *E. coli* strain treated and untreated with compound **25**.

In general, after 4 hours, results of ERIC-PCR products showed that bands produced from *E. coli* strain treated with compound **25** with a concentrations of 100µg/mL, 50µg/mL and 25µg/mL are more intense than the bands from the untreated control. In E. coli treated with compound **25** with a concentration 25µg/mL, a band with approximately 250-bp amplicon size was faint in comparison with the same band in untreated control. In addition, a new band with an amplicon length above 500-bp had appeared in treatment with 50µg/mL.

After 24 hours, it was observed that the number and intensity of bands of each concentration (100µg/mL, 50µg/mL, 25µg/mL) are the same as well as in the control bands, and the intensity of the bands was not affected. This may be explained by certain mechanisms used in bacteria to be able to repair the damage in bacterial DNA, or may be due to compound degradation by bacterial enzymes.

The main changes in the treated *E. coli* with compound **25** included the differences in band intensity as well as appearing of certain bands in comparison with untreated control. Genotoxic compounds can produce DNA alterations such as single and/or double strand breaks, point mutations and/or chromosomal rearrangements. These alterations or damages in the bacterial DNA may have a potential effect on the sites of primer annealing and or inter-priming distances.[53, 56, 57]

Point mutations, large deletions, and/or homologous recombination are considered as mechanisms that can produce new primer annealing sites, thus resulting in the appearance of extra new bands or change the amplicon size.[56]However, understanding and defining the specific mechanisms which lead to variations in ERIC-PCR profile is difficult. Other techniques are required that can support and assist in understanding the proposed mechanisms such as analysis of amplicons using DNA sequencing or probing.[53]

Based on the results of the genotoxicity study, the main recommendation is making *in vivo* genotoxicity studies using animal models and human cell lines for proper assessment of the safety of using compound **25** for therapeutic purposes.

4 CONCLUSIONS

1) Isoxazole **23**, diazepine **26**, pyrazol **27**, amines **28** and piperazine **25** exhibited remarkable potency against gram-positive *S. aureus*.
2) Isoxazole **23** and piperazine **25** were the most effective against gram -positive *S. epidermidis*.
3) Compound **25** showed activity against both gram-positive and gram-negative bacteria used.
4) Compound **25** showed the highest activity in its unprotonated version (alkali).
5) The genotoxic study showed that compound **25** with the piperazine group damaged the DNA of E. coli strains.
6) It is recommended to make *in vivo* genotoxicity studies using animal models and human cell lines for proper assessment of the safety of using compound **25** for therapeutic purposes.
7) Further studies and tests are needed in order to assist in understanding the proposed mechanism of the action of compound **25** in damaging the *E. coli* DNA.
8) An extensive study is underway to optimize the effectiveness of diazepine type of compounds and to determine their mode of action.

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جامعة النجاح الوطنية

كلية الدراسات العليا

تصميم وتحضير ألكالويدات من الكركمين ودراسة النشاط الحيوي لها

اعداد

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اشراف

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أ.د.شحدة جودة

قدمت هذه الأطروحة استكمالا لمتطلبات الحصول على درجة الماجستير في الكيمياء بكلية الدراسات العليا في جامعة النجاح الوطنية، نابلس – فلسطين.

2018

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الملخص

تم تحضير مجموعة جديدة من الألكالويدات باستخدام الكركمين, حيث أن هذه الألكالويدات تنتمي إلى فئتين: الفئة الأولى تحتوي على سلسلة من الألكيل أمين, والفئة الثانية تحتوي على حلقة غير متجانسة مثل: (pyrazole), (diazepine) و (isoxazole). وتم تحليل المركبات الناتجة باستخدام تقنيات التحليل الطيفي المختلفة، مثل:

(FT-IR), (^{13}CNMR) و (^{1}HNMR).

تم تحضير المركبات باستخدام طريقتين جديدتين بحيث أن النسب المئوية لكميات النواتج كانت نوعية. في الطريقة الأولى تمت مفاعلة الكركمين مع مركبات ثنائي الأمين بوجود الايثانول وكمية محفزة من حمض الكبريتيك. أما الطريقة الثانية فتضمنت خلط الكركمين مع مركبات ثنائي الأمين ثم التسخين تدريجيا للوصول إلى درجة الانصهار, وبعدها تم التسخين على درجة حرارة 30 درجة مئوية لمدة 30 دقيقة.

تم اختبار النشاط المضاد للبكتيريا لهذه المركبات ضد أربعة أنواع مختلفة من البكتيريا, وأظهرت النتائج أن هذه المركبات لها قدرة على منع النمو البكتيري لبكتيريا جرام الموجبة بقيم(MIC) تتراوح ما بين 1.5 و6.25 (µg/mL) لبكتيريا (*S. aureus*). ومن بين كل المركبات التي تم تحضيرها, تم ملاحظة أن مركبات (isoxazole **23**), (diazepine **26**), (pyrazole **27**), (amines with methyl **28**) و(piperazine **25**) لها قوة هائلة ضد بكتيريا جرام الموجبة (*S.aureus*). بالاضافة الى ذلك, تم ملاحظة الفعالية العالية لمركبات (soxazole **23**) و(piperazine**25**) على البكتيريا موجبة جرام الاخرى (*S. epidermidis*).أما بكتيريا سالبة

جرام فقد أظهرتمقاومة أعلى ضد المركبات من بكتيريا موجبة جرام, حيث أن بكتيريا (*E. coli*) السالبة جرام كانت حساسة لمركب (**25** piperazine)فقط.

أظهرت دراسة السمية الوراثية أن المركب **25** المحتوي على مجموعة (piperazine) قد تمكن من تدمير (DNA) لبكتيريا (*E.coli*). ونتيجة لذلك, من المستحسن دراسة السمية الوراثية على خلايا حيوانية وخلايا الانسان للتقييم الصحيح لسلامة استخدام مركب **25** لأغراض علاجية. هناك دراسة مستفيضة جارية لتحسين فعالية نوع (piperazine) من المركبات ولتحديد طريقة عملها.