Synthesis, Characterization of a Novel 1,1'-[1,4-phenylenebis(1,3,4-thiadiazol-5,2-diyl)] bis (3-chloro-4-(4-hydroxyphenyl) azetidin-2-one and evaluation its Biological activities

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Synthesis, Characterization of a Novel 1,1'-[1,4-phenylenebis(1,3,4-thiadiazol-5,2-diyl)] bis (3-chloro-4-(4-hydroxyphenyl) azetidin-2-one and evaluation its Biological activities

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Abstract

In current study, synthesis of a novel azetidin-2-one compound, i.e 1,1'-[1,4-phenylenebis(1,3,4-thiadiazol-5,2-diyl)] bis (3-chloro-4-(4-hydroxyphenyl) azetidin-2-one from reaction of 0.002mole of 4,4'-[1,4-phenylene bis(1,3,4-thiadiazole-5,2-diyl)] bis (azaneylylidene) bis (methaneylylidene) diphenol with 0.004mole of 2-chloroacetylchloride in dioxane as a solvent in the presence of trimethylamine. The biological activity of new azetidin-2-one compound was evaluated at 100 mg/ml against four types of bacteria i.e. *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp. and its minimum inhibitory concentration (MIC) was 2, 2 ,3 ,3 mg/ml for *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp. respectively. Median lethal dose (LD_{50}) and cytotoxicity activity were also investigated. The LD_{50} for this compound is 1.4 gm/kg bw. The results showed that this compound did not affect the red blood cell until the concentration reach 25 mg/ml or above. The new compound was characterized by spectral data, i.e., FT-IR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis which confirmed its proposed structure.

Keywords: Azetidin-2-one compound, Biological activity, Cytotoxicity, LD_{50}

Introduction

Azetidin-2-one, a four-membered cyclic lactam (β-lactam) skeleton has been recognized a useful building block for the synthesis of a large number of organic molecules by exploiting the strain energy associated with it [1]. Azetidin-2-one and its derivatives constitute an important class, four membered heterocyclic compounds possessing diverse biological activities. The 2-Carbonyl derivatives of azetidines are called 2-azetidinones or more commonly named as β-lactams [2]. They have been shown to possess a broad spectrum of biological activity. 2-Azetidinone skeleton is well established as the pharmacophore of β-lactam antibiotics, the most

widely employed class of antibiotics [3, 4]. The toxicity of a substance can be determined before use in humans by using in vitro tests and animal studies [5].

Previously several compound were synthesized [6,7]. The compound synthesized in this study was a novel compound and there was no data on its biological activity, cytotoxicity and LD50. This study aims at synthesizing a novel (Azetidine) and evaluating its biological activities such as antibacterial, MIC, cytotoxicity assay and it is LD_{50}.

Materials and methods

Synthesis of 1,1'-[1,4-phenylenebis(1,3,4-thiadiazol-5,2-diyl)] bis(3-chloro-4-(4-hydroxy phenyl) azetidin-2-one.

This new compound was prepared according to [8] method. (1.00 gm, 0.002 mole) of 4,4'-[1,4-phenylene bis(1,3,4-thiadiazole-5,2-diyl)] bis (azaneylylidene) bis (methaneylylidene) diphenol [7] was dissolved in 10 ml of dioxane with constant stirring, (0.209 gm, 0.002 mole) of triethylamine was added, then (0.464 gm, 0.004 mole) of 2-chloroacetylchloride was added drop wise. The mixture was stirred vigorously for 15 minutes and refluxed for 5hrs. Cooled to room temperature, filtered off, washed with ice cooled water, brown precipitate was formed, dried, recrystallization from ethanol gave brown powder with m.p. 320°C dec., wt. 0.18 gm, yield (14%), R_f value = 0.45 (3:7, chloroform/methanol).

Physical measurements

Melting point: The product compound's melting point was expressed in degree (0 ^{o}C). It was measured in the Department of Chemistry, University of Basrah College of Science. Using Gallenkamp Thermal Point apparatus.

FT-IR Spectra: FT-IR spectrum was recorded, using Shimadzu FT-IR-8400 affinity spectraphotometer (Japan), in Department of Chemistry, College of Education for pure Science, University of Basrah using KBr disc, and expressed in (400-4000) cm^{-1}.

Thin Layer Chromatography: Thin layer chromatography of the starting materials and products performed by using Eastman chromatography sheet (Germany) with suitable eluent (methanol: ethyl acetate) ratio (3:7); the spot was visualized by exposing the dry plate in UV light.

Elemental Analysis: Elemental micro analysis of Carbon, Hydrogen, Nitrogen and Sulfur were carried out in Al al-Bayt University, Al-Mafraq, Jordan using a Euro vector EA 3000A Elemental analysis (Italy).

Nuclear Magnetic Resonance Measurements: ^{1}H-NMR and ^{13}C-NMR spectra of prepared compound were recorded using BRUCKER Ultra shield spectrophotometer (300 MHz), University of Al al-Bayt, Jordan. The Chemical shift was expressed in υ\ (ppm) unit. Using tetramethylsilane (TMS) as internal standard and DMSO-d_6 as a solvent.

Biological activity of chemical compounds:

Bacterial isolates: Four bacterial isolates have been used. These are *Bacillus cereus*, *Staphylococcus aureus, Escherichia coli* and *Salmonella* sp. The *Bacillus cereus* was published in Gen bank according to [9]. The other bacterial strains were collected from the Central Lab. of College of Veterinary Medicine University of Basrah. The turbidity of the actively growing bacterial suspension was adjusted with 0.5 McFarland standard [6,7,10].

Antimicrobial activity test of the prepared compounds: The concentration 100mg/ml from the synthesis compounds were prepared and were used in this study [11]. The antimicrobial susceptibility was tested by agar well diffusion method according to [12].

Minimum inhibitory concentration (MIC) for the compound: Using concentrations (1, 2, 3, 4, 5, 10) mg/ml. Well diffusion method was used to determine the MIC for prepared compound on the fourth bacterial cultures. The prepared compound was dissolved in DMSO into different concentrations [13]. Bacterial suspensions was adjusted to MacFarland standard 0.5($1.5*10^{8}$) CFU.

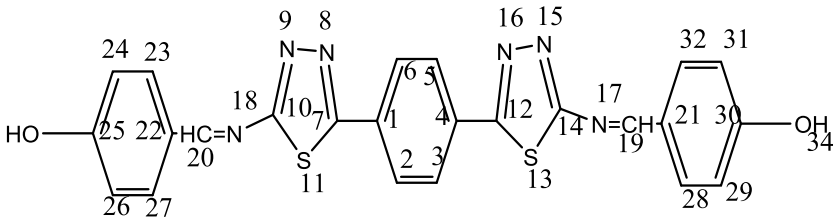
Blood cell cytotoxicity assay: The method of studying the cell toxicity of the prepared compound [14]. A physiological saline (1.0 ml blood suspension in 20 ml saline) has been prepared. Different levels of prepared compound have been used in DMSO. Two ml of erythrocyte suspension prepared in the first step were added to the sterile tubes, adding 0.1 ml of each concentration. Two ml of tab water with 0.1 ml of erythrocyte were used as positive control, and two of normal saline with 0.1 ml of erythrocyte were used as negative control. The turbidity was read at 10, 30 and 60 min. at 37 ° C. The concentrations that provided a clear solution due to RBC lysing are an indication of the degree of toxicity to the erythrocytes of the test compound. [6,7,14].

Median Lethal Dose (LD_{50}) assay: To determine the LD_{50} for the compound, a total number of seven male and female rats (*Rattus norvegicus*) were used. In the animal house of the College of Veterinary Medicine / University of Basrah, the animals aged 8-10 weeks and their body weight between 190-200 gm, each animal was isolated in one cage in a good air condition room. The animals fed on dried bread, pellet and given R.O. water [6,7]. The LD_{50} steps used in this experiment was "up and down" method described by Dixon [15].

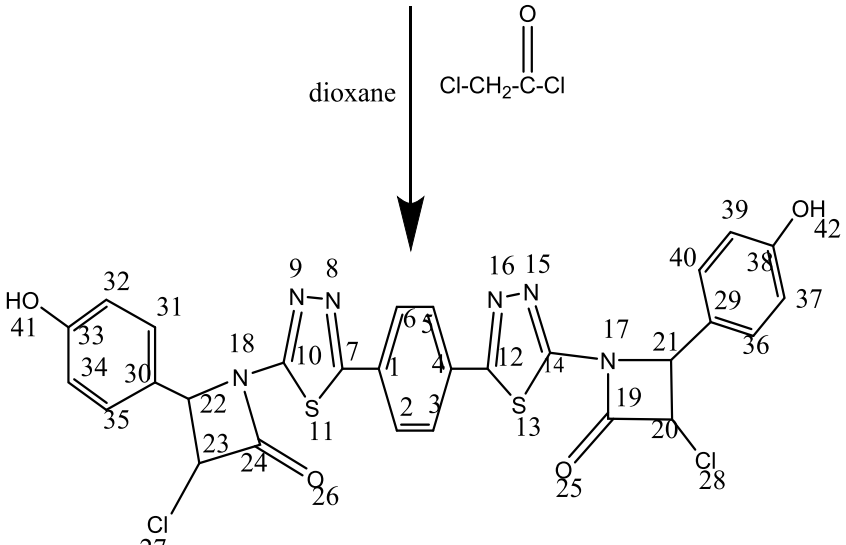
Results

Synthesis of a Novel 1,1'-[1,4-phenylenebis(1,3,4-thiadiazol-5,2-diyl)] bis(3-chloro-4-(4-hydroxyphenyl) azetidin-2-one

Azetidin-2-one compound was synthesized. The reaction equation for the synthesis of this compound is shown in Figure1



4,4'-((1*E*,1'*E*)-((1,4-phenylenebis(1,3,4-thiadiazole-5,2-diyl))bis(azaneylylidene))bis(methaneylylidene))diphenol



1,1'-(1,4-phenylenebis(1,3,4-thiadiazole-5,2-diyl))bis(3-chloro-4-(4-hydroxyphenyl)azetidin-2-one)

Figure 1:- Chemical equation for synthesis of a novel azetiden-2-one.

Elemental analysis Found (calculated)= **C**: 52.38(52.76), **H**: 2.52(2.85), **N**: 13.45(13.19), **S**: 10.26(10.06).

The IR spectrum of new compound (KBr disc) showed characteristic bands at ν3199 cm^{-1} due to O-H stretching. Weak band at ν C-H 3045cm^{-1}attributed to stretching of aromatic hydrogen atoms, Weak band at ν2968 cm^{-1} belong to stretching of aliphatic hydrogen atoms, Very strong band at ν1707 cm^{-1} may attributed to carbonyl of β-lactam, very strong band at ν1670cm^{-1}may have assigned to C=N stretching, while very strong band at νOH1600cm^{-1} due to O-H bending, tow strong bands at ν1514 cm^{-1} and ν1454 cm^{-1} may attributed to stretching of asymmetrical and symmetrical of C=C bond, respectively, strong band at ν1388 cm^{-1}due to stretching of C-N bond, while very strong band appears at ν1116cm^{-1} may be attributed to stretching of N-N bonds, strong band at ν833 cm^{-1} may be attributed to stretching of C-Cl bonds ,medium band appears at ν707cm^{-1}attributed to cyclic C-S-C bonds. See Figure (2).

The ^{1}H-NMR spectrum for new compound was recorded using DMSO-d_6 as a solvent (300Mz). In general, ^{1}H-NMR spectrum shows signal at 5.35ppm(d) attributed to 2H (C21, C22), (*J*=5.27Hz), signal at 5.54ppm(d) may assigned to 2H (C20, C23), (*J*=5.00Hz), signal at 6.94ppm(d) may assigned to 4H (C40, C36 and C31, C35), (*J*=8.44) the signal at 7.77ppm(d) may attributed to 4H (C39, C37 and C32, C34), (J=8.37Hz) and broad signal at 9.79ppm(s) may assigned to 4H (C3, C2 and C5, C6). See Figures (3, 4)

The^{13}C-NMR spectrum for new compound was recorded using DMSO –d_6. 47.19ppm (C20, C23), 66.31ppm (C21, C22), 115.80ppm (C32, C34, C37, C39), 129.41ppm (C31, C35, C36, C40, C30, C29), 132.04ppm (C2, C3, C5, C6), 134.41ppm (C1, C4), 163.28ppm (C14, C10), 165.52ppm (C33, C38), 166.62ppm (C7, C12) and 169.70ppm (C19, C24). See Figure (5).

Biological activity of new compound: The antimicrobial activity was determined against four types of bacteria in the concentration 100 mg/ml Table (1), Figure (6).

Table (1) Antimicrobial activity of new compound

Chemical compound	**Types of bacteria**	**Inhibition zone (mm)** **100 mg/ml**
Azetidinone compound	*Bacillus cereus*	20
	Staphylococcus aureus	16
	Escherichia coli	18
	Salmonella sp.	17

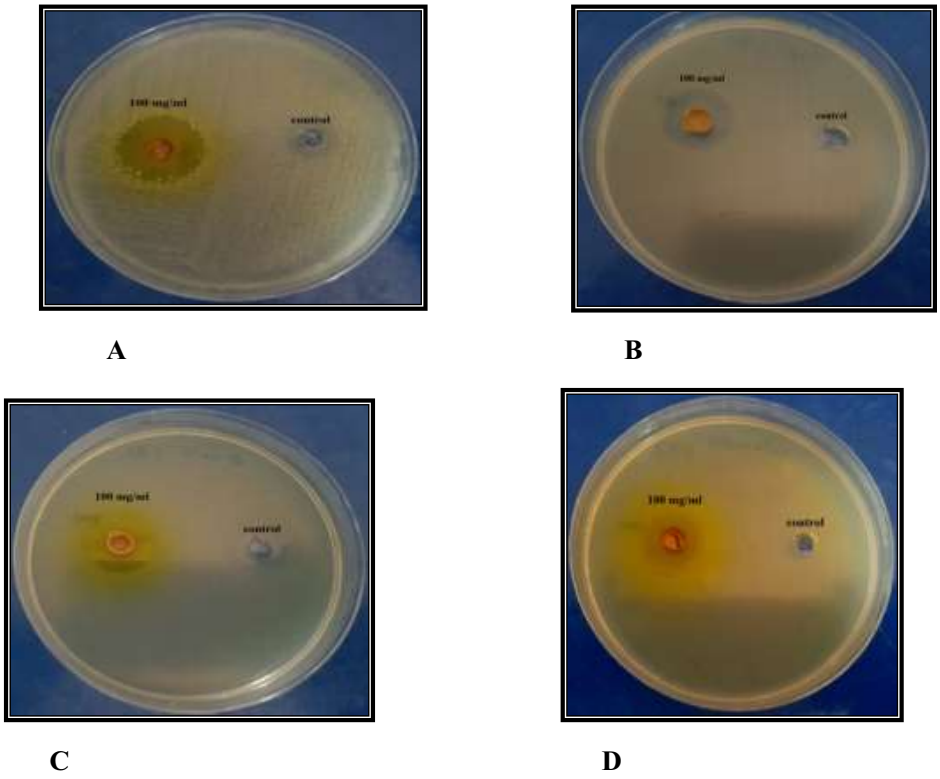


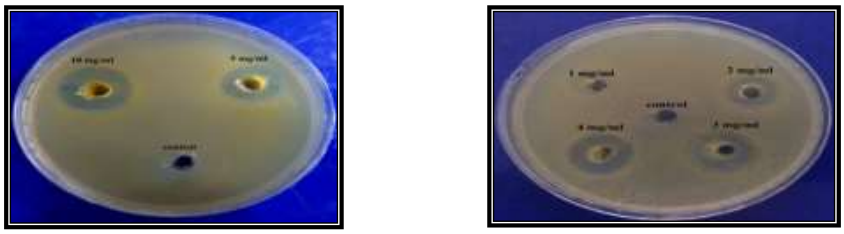
Figure (6) Biological activity of Azetidinone compound against different types of bacteria. A= *B. cereus* , B= *S. aureus* ,C= *E. coli* , D= *Salmonella* sp.

Minimum inhibitory concentration (MIC) of the new compound: The MIC of this new compound was 2 mg/ml for both *B. cereus* and *S. aureus*, but it was 3 mg/ml for *E. coli.* and *Salmonella* sp. Table (2), Figure (7).

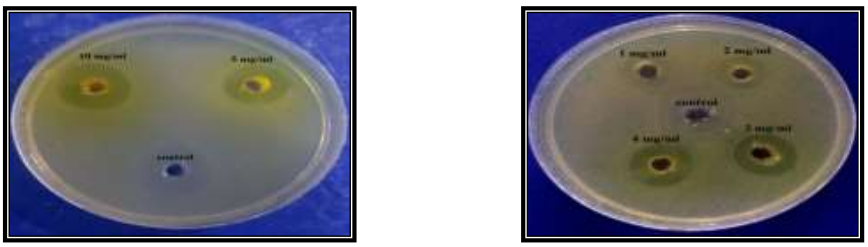
Table (2) The MIC for the compound against different types of bacteria

Conc. mg/ml	Inhibition zone (mm)			
	B. cereus	***S. aureus***	***E. coli***	***Salmonella* sp.**
10	18	16	18	16
5	17	15	16	14
4	15	14	16	12
3	14	13	12	11
2	13	6	0	0
1	0	0	0	0

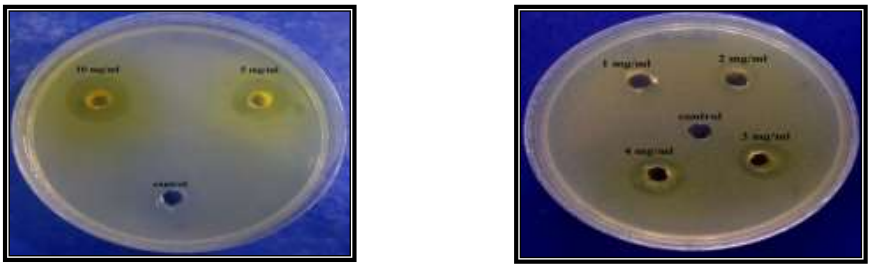
A) 1 **2**



B) 1 **2**



C) 1 **2**



D) 1 **2**

Figure (7) The MIC of prepared compound against different types of bacteria. A= *B. cereus* , B= *S. aureus* ,C= *E. coli* , D= *Salmonella* sp.

Toxicity assay of the new compound: The produced compound did not affect the red blood cells at the concentration 5 and 15 mg/ml, but the concentration 25 and 40 mg/ml caused low

hemolysis in red blood cells, the concentrations 60 and 80 mg/ml cause moderate hemolysis and the concentrations 90 and 100 mg/ml cause high hemolysis in red blood cells. Table (4), Figure (8).

Table (4) Toxicity of prepared compound.

Conc. (mg/ml)	Toxicity
5,15	-
25, 40	+
60, 80	++
90, 100	+++

-= No hemolysis, + = few hemolysis, ++ = moderate hemolysis, +++ = high hemolysis

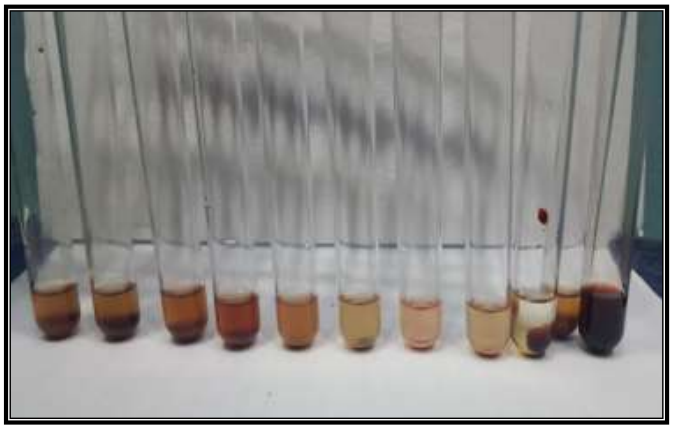


Figure (8) Toxicity of the new compound.

Median Lethal Dose (LD_{50}) assay: The code that found was (XXOX) for the new compound, and the LD_{50} was determined according to the formula described by Dixon [15]. The LD_{50} was 1.4 gm/kg bw

Discussion

New compound was prepared from reaction of one mole of 4,4′-[1,4-phenylenebis(1,3,4-thiadiazole-5,2-diyl)] bis(azaneylylidene) bis(methaneylylidene) diphenol and two moles of 2-chloroacetyle chloride which was added drop wise through 15 minutes in the presence of one mole of trimethyl amine, then the mixture was refluxed for 5hrs, cooled and the precipitate was recrystallized from ethanol to give brawn precipitate with high melting point decomposed at 320°C with low yield percent (14%), the reaction was monitored by TLC, The R_f value for the new compound equal to 0.45 (3:7 Chloroform/Methanol). The new compound was solid, unaffected by air, insoluble in water but soluble in common organic solvents. The new compound was elucidated by Elemental analysis, FT-IR, ^{1}H-NMR and ^{13}C-NMR. The elemental analysis of new compound is within ±0.5% of the theoritical values.

The IR spectrum of new compound (KBr disc) showed characteristic bands at ν3199 cm^{-1} due to O-H stretching. Weak band at νC-H 3045cm^{-1}attributed to stretching of aromatic hydrogen atoms, Weak band at ν2968 cm^{-1} belong to stretching of aliphatic hydrogen atoms, Very strong band at ν1707 cm^{-1} may attributed to carbonyl of β-lactam [16], very strong band at ν1670cm^{-1}may have assigned to C=N stretching, while very strong band at ν1600cm^{-1}due to O-H bending, tow strong bands at ν1514 cm^{-1} and ν1454 cm^{-1} may attributed to stretching of asymmetrical and symmetrical of C=C bond, respectively, strong band at ν1388 cm^{-1}due to stretching of C-N bond, while very strong band appears at ν1116cm^{-1}may be attributed to stretching of N-N bonds, strong band at ν833 cm^{-1} may be attributed to stretching of C-Cl bonds, medium band appears at ν707cm^{-1} attributed to cyclic C-S-C bonds (16). See Figure (2).

The ^{1}H-NMR spectrum for new compound was recorded using DMSO-d_6 as a solvent (300Mz). In general, ^{1}H-NMR spectrum shows band at 5.35ppm(d) attributed to 2H (C21, C22), (*J*=5.27Hz), signal at 5.54ppm(d) may assigned to 2H (C20, C23), (*J*=5.00Hz), which give indication that the tow aliphatic hydrogen atoms in β-lactam moiety were in cis-form [17], signal at 6.94ppm(d) may assigned to 4H (C40, C36 and C31, C35), (*J*=8.44), the signal at 7.77ppm(d) may attributed to 4H (C39, C37 and C32, C34), (*J*=8.37Hz) and broad signal 9.79ppm(s) may assigned to 4H (C3, C2 and C5, C6). See Figures (3, 4).

The ^{13}C-NMR spectrum for new compound was recorded using DMSO –d_6. The signal at 47.19ppm may attributed to aliphatic carbon atoms in β-lactam moiety (C20, C23), while the signal at 66.31ppm attributed to aliphatic carbon atoms attached directly to chloride atoms (C21, C22), signal at 115.80ppm may assigned to aromatic (C32, C34, C37, C39), signal at 129.41ppm may assigned to aromatic (C31, C35, C36, C40, C30, C29), while signal at 132.04ppm attributed

to aromatic (C2, C3, C5, C6), signal at 134.41ppm belong to aromatic (C1, C4), signal at 163.28ppm may assigned to aromatic carbons within the heterocyclic rings (C14, C10), while signal at 165.52ppm belong to aromatic carbons attached directly to hydroxyl groups (C33, C38), signal at166.62ppm attributed to aromatic carbon atoms within heterocyclic rings (C7, C12) and the last signal at 169.70ppm attributed to carbonyl atoms (C19, C24). See Figure (5).

The new compound at 100 mg/ml had high activity against *B. cereus* and low biological activity against *S. aureus* and its MIC was (2, 2, 3, 3) mg/ml on *B. cereus*, *Staph aureus*, *E. coli* and *Salmonella* spp., respectively.

The reason belongs to the difference in the nature of the general structure of the cell wall between the two types of the bacteria (gram positive and gram negative) that used in this study.

Azetidine and its derivatives, four membered heterocyclic compounds having diverse biological activities. The 2-Carbonyl derivatives of azetidines are called 2-azetidinone or more commonly called β-lactams. It had pharmacological activities. The ring system has a wide range of activities like antimicrobial, antitubercular, anticancer, antidiabetic [2].

The cell wall of the bacteria is very important as many antibiotics have a concentrated effect on cell wall. The cell wall differs in the chemical composition between gram positive and gram negative bacteria. In addition, number of the wall layers, wall thickness and wall content of fat Polyunsaturated fatty acids and multiple proteins. The severity of bacterial and fungal infections has become a major worldwide problem, and the World Health Organization has chosen antimicrobial resistance as the theme for World Health Day 2011[18]. Researchers are constantly trying to counteract infections by synthesizing new effective antibacterial and antifungal agents because of bacterial resistance [18, 19, 20, 21]. The thiadiazole ring acts like a pharmacophore. It is also a bio-isoster thiazole ring included in third and fourth generation cephalosporins and this observation makes it possible to use it in the synthesis of antimicrobials. [22].

The new compound is good antimicrobial agent on gram positive and gram negative bacteria. This is due to the presence of pharmaceutical beta-lactam ring in its structure [8]. Azetidinones are very important class of compounds possessing wide range of biological activities [23].

The new compound was caused hemolysis of red blood cells at 25-100 mg/ml. This cytotoxicity can be attributed to stereo genic aldol units which are especially common in polyketides (Polyketides are structurally a very diverse family of natural products with diverse biological activities and pharmacological properties) [24].

The new compound is a novel compound and no data on their toxicity were available; therefore, the experiment focused on determining its acute toxicity by measuring its LD_{50} in males and females rats of adults. There are a number of LD_{50} determination methods available. Acute toxicity study was conducted using the Dixon method to measure the median lethal dose (LD_{50}). (15).

Because of the new compound is not soluble in water, there were soluble in DMSO and used intraperitoneal injection (IP) as suspension to abdominal cavity of the rats for the determination of the LD_{50}.

For the new compound, the mortality recorded when rats are exposed to 1.4 gm/kg bw. The mortality was increased when the dose of the compound was increased. When the compound was injected IP in the rat with the lethal or nearest to the lethal dose, the rat lost his feeling with its extremities will paralysed, start crawling, fatigue and then lice the site of injection.

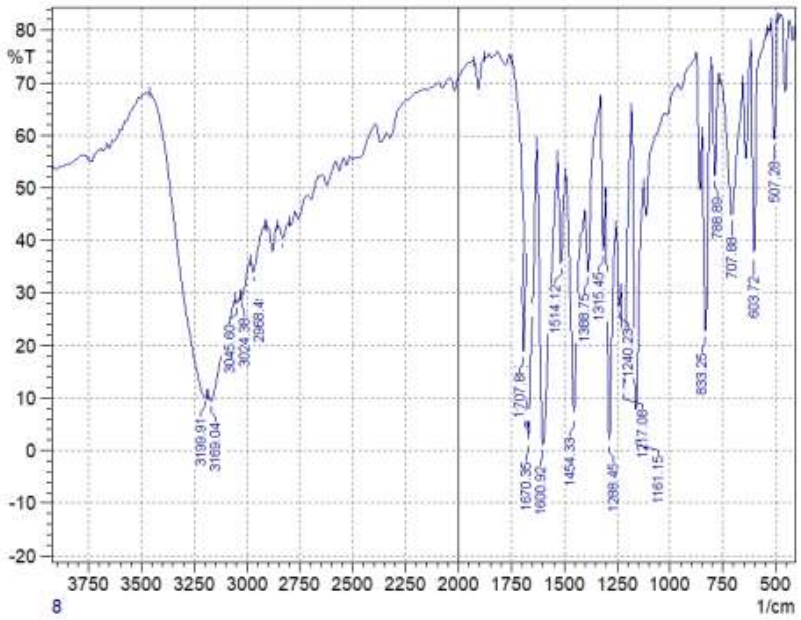


Figure (2) IR-spectrum for the new compound

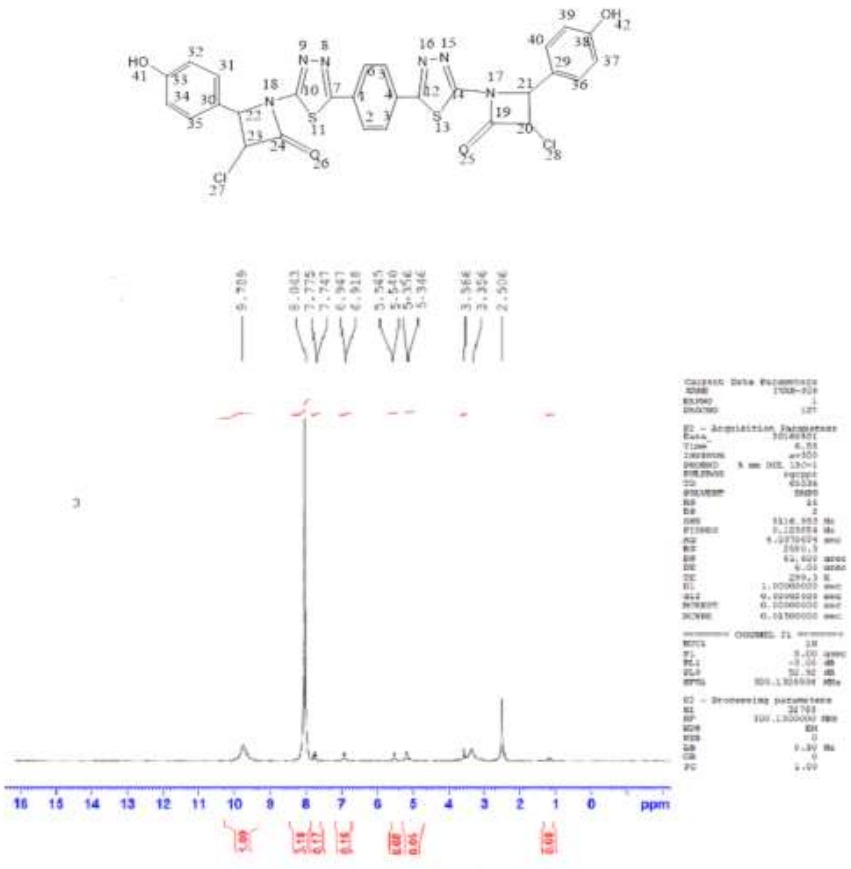


Figure (3) ^{1}H-NMR spectrum for the new compound

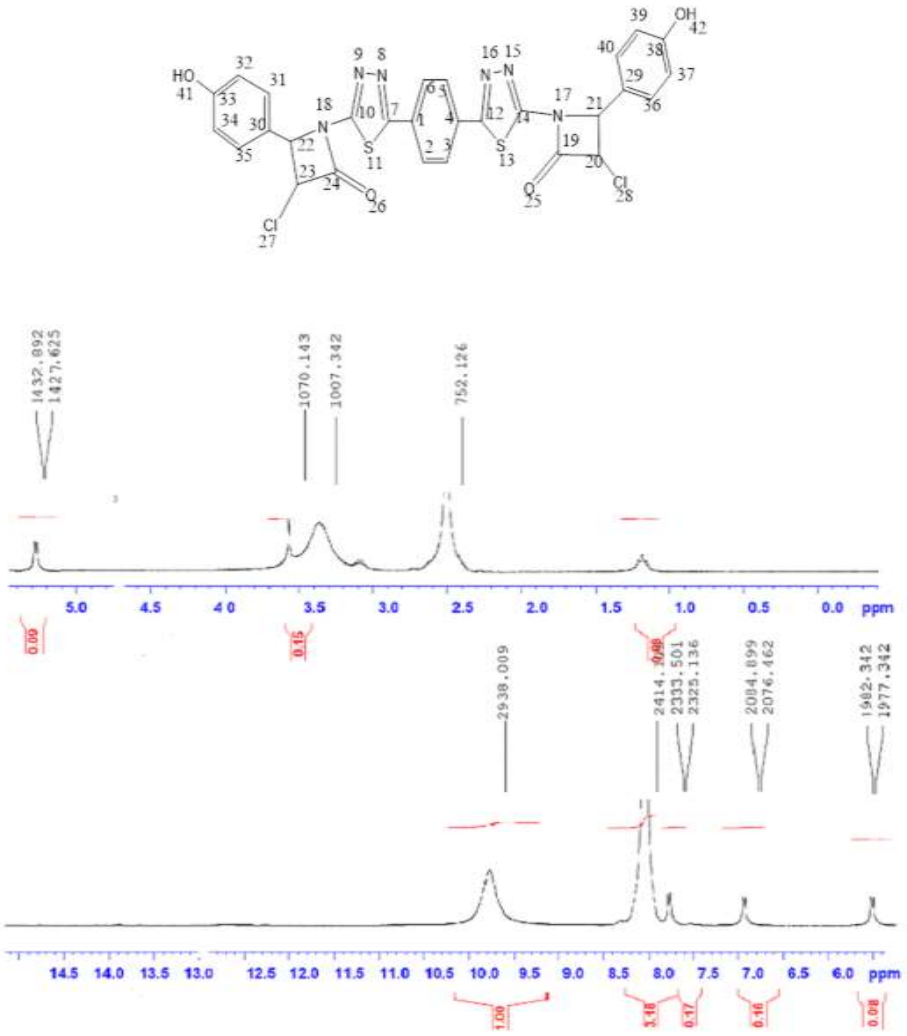


Figure (4) Expanded ^{1}H-NMR spectrum for the new compound

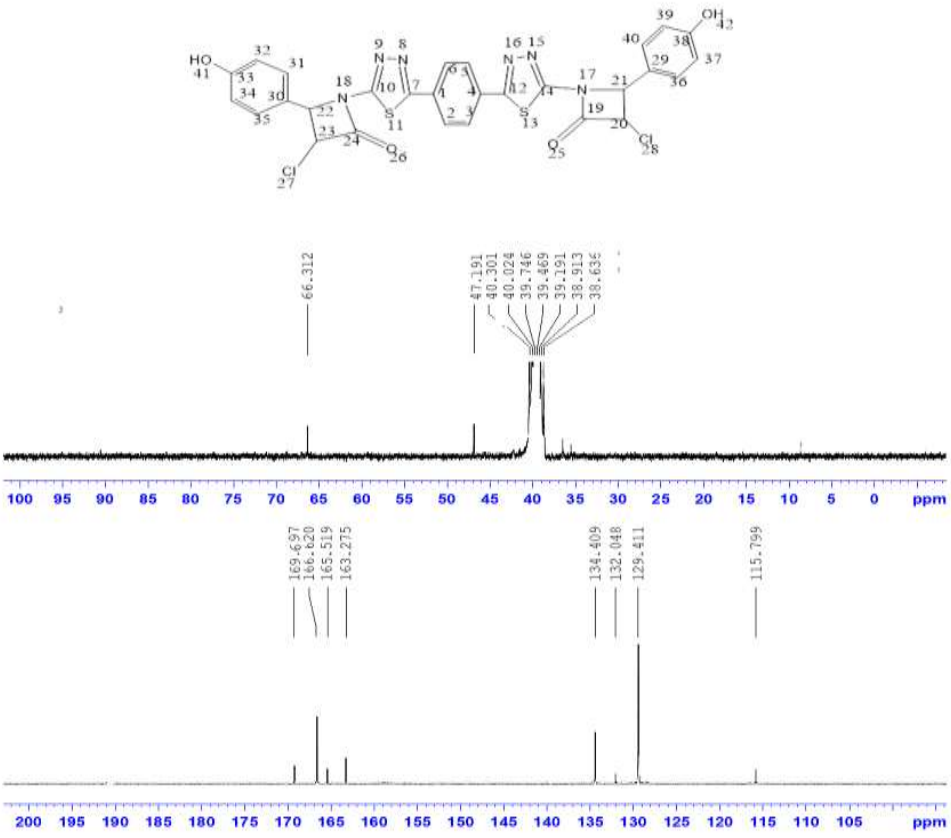


Figure (5) ^{13}C-NMR spectrum for the new compound

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