Synthesis of some Novel Nitrogenous Heterocyclic Compounds with Expected Biological Activity as Antimicrobial and Cytotoxic Agents.

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Abstract

This study includes synthesis of some nitrogenous heterocyclic compounds linked to amino acid esters or heterocyclic amines that may have a potential activity as antimicrobial and/or cytotoxic. Quinolines are an important group of organic compounds that possess useful biological activity as antibacterial, antifungal and antitumor .8-Hydroxyquinoline (8-HQ) and numerous of its derivatives exhibit potent activities against fungi and bacteria which make them good candidates for the treatment of many parasitic and microbial infection diseases.

These pharmacological properties of quinolones aroused our interest in synthesizing several new compounds featuring heterocyclic rings of the quinoline derivatives linked to amino acid ester or heterocyclic amine with the aim of obtaining a pharmacologically active compounds.O-alkylation has been done on(8-hydroxyquinoline) to get (O-alkylated ester) derivatives which are deesterfied to get acetic acid derivatives, then coupled with amino acid that have protected carboxyl group (amino acid esters) or heterocyclic amine by using conventional solution method for peptide synthesis as a coupling method.

The proposed analogues were successfully synthesized and the processing of the reactions confirmed by TLC ,the synthesized analogues with the proposed structures as they were characterized and proved by melting point, infrared spectroscopy (IR) and elemental microanalysis.

The tested analogues showed cytotoxic activity on the HEp-2 cell line (tumor of larynx) with inhibitory concentration percent of (IC %) range (32.43 % - 49.55%) and showed that the tested compounds had variable antimicrobial activities against selected bacteria and yeast when compared with selected standard drugs.

Keywords: Quinolones, 8-hydroxyquinoline , N-heterocycles biological activity.

تخليق بعض المركبات الحلقية الجديدة الغير متجانسة والحاوية على النتروجين مع توقع الفعالية الحيوية لها كمضادات ميكروبية وسرطانية

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الخلاصة

تتضمن هذه الدراسة تخليق بعض المركبات الحلقية غير المتجانسة المقترنة بأسترات الأحماض الامينية او مركبات حلقية غير متجانسة حاوية على مجموعة امينية والتي قد تمتلك فعالية نشيطة كمضادات للجراثيم و/ أو مضادات للسرطان
تمتلك الكوينولينات اهمية مميزة كأحد المركبات العضوية والمستحضرات الطبية والتي تستخدم كمضادات بكتيرية وفطرية وسرطانية. اظهر ال (٨-هيدروكسيكوينولين) والعديد من مشتقاته أنشطة فعالة ضد الفطريات و البكتيريا التي جعلها مرشحة جيدة لعلاج العديد من الأمراض الطفيلية و الجرثومية . من جميع مشتقات الهيدروكسيكوينولين (٨-هيدروكسيكوينولين) هو الأكثر إثارة للاهتمام ، وذلك بسبب خصائصه المتعددة الوظائف ، هذه الخصائص الدوائية أثارت اهتمامنا بتحضير عدة مركبات جديدة تتميز بحلقات الغير متجانسة لمشتقات الكوينولين مرتبطة بإسترات الأحماض الأمينية او مركبات حلقية غير متجانسة حاوية على مجموعة امينية بهدف الحصول على المركبات الصيدلانية الفعالة.

تم إجراء O-Alkylationعلى(٨-هيدروكسيكوينولين)للحصول على مشتقات (O-Alkylated ester) و من ثم تحلل الاستر للحصول على مشتقات حامض ألخليك ومن ثم ربطها مع مختلف استرات الأحماض الامينية او مركبات حلقية غير متجانسة حاوية على مجموعة امينية باستخدام طريقة المحلول التقليدية لصناعة الببتيدات كطريقة للاقتران وتكوين أصرة اميدية للحصول على المركبات النهائية.ان عملية تخليق المركبات المطلوبة قد تمت باتباع طريقة التفاعل متعدد الخطوات .وقد تمت مراقبة سير التفاعلات باستخدام كروماتوغرافيا الطبقة الرقيقة .تم تشخيص التركيب الكيميائي للمركبات الوسطية والنهائية والتاكد منها من خلال قياس درجات الانصهار والتحليل الطيفي للأشعة تحت الحمراء والتحليل الدقيق للعناصر .
أظهرت النتائج التي اجريت على المركبات المصنعة نشاط سمي على نوع من الخلايا السرطانية (ورم الحنجرة) بنسبة تركيز مئوية مثبطة تتراوح ما بين ال (٣٢,٤٣ % - ٤٩,٥٥%) وكذلك اظهرت النتائج ان المركبات المصنعة لاتمتلك اوتمتلك فعاليات ميكروبية مختلفة ضد البكتريا والفطريات المختارة بالمقارنة مع الادوية القياسية المختارة ايضا في هذه الدراسة

الكلمات المفتاحية :كوينولون، ٨- هيدروكسيكوينولين ، الفعالية الحيوية للمركبات الحلقية الغير متجانسة والحاوية على النتروجين.

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Introduction

Heterocyclic compounds are acquiring more importance in recent years which display biological activities. [1] Heterocyclic compounds particularly five and six member heterocyclic have attracted the attention of pharmaceutical community over the years due to their therapeutic values. [2] Poly functionalized heterocyclic compounds containing nitrogen, sulphur, oxygen as heteroatom play important roles in the drug discovery process. [3] Analysis of drugs in late development stages or in the market shows that 68% of them are heterocycles. [4]

N-heterocycles have found wide-spread applications as key components of a large number of biologically-active natural products. Examples include antibiotics such as penicillin and cephalosporin, alkaloids such as vinblastine and morphine and fungal natural product like cyclosporine A , all exhibiting interesting biological properties. However, synthetic N-heterocyclics have also found widespread use in pharmaceuticals as anticancer agents, analeptics, analgesics, hypnotics and antioxidants [5].Quinoline is one of the most popular N-heteroaromatic compounds incorporated into the structures of many pharmaceuticals. Many quinoline-containing compounds exhibit a wide spectrum of pharmacological activities, such as antiplasmodial [6], cytotoxic [7], antibacterial [8], antiproliferative [9] ,antimalarial [10] , and anticancer activity[11] . These pharmacological properties of quinolines and their derivatives had attracted worldwide attention in the last few decades because of their wide occurrence in natural products and drugs [12,13] . Quinoline derivatives also have been shown to exhibit awide variety of pharmacological activities including effects on cancer and nowadays it is reported that the incorporation of quinolone nucleus could alter the course of reaction as well as the biological properties of the synthesized compounds [14, 15].

Materials and Methods

Materials

8-hydroxyquinoline was purchased from AVONCHEM (U.K). Absolute ethanol, Absolute methanol, Acetone, and Chloroform were purchased from GCC (Germany). Absolute isopropyl alcohol, ethylBromoacetate , Diethyl ether, Ethyl acetate, Hydrazine hydrate, Hydrochlorica cid, N-methyl morpholine (NMM), Petroleum spirit and Thionyl chloride were purchased from BDH (U.K).Coumarin was purchased from Himedia (India). D-Alanine, 1-Hydroxy benzotriazole (HOBt), and N,NDimethyl formamide (DMF), were purchased from Fluka AG(Switzerland). All other reagents were of analytical grade.

Methods of Identification

General methods were used for purification and identification of the synthesized analogues including:

- Thin Layer Chromatography:
- Melting Points:
- Infrared Spectra:
- Elemental Microanalysis

Synthesis

Esterification of amino acids synthesis of D-alanine methyl ester HCl compound (A.1) [16]

A suspension of D-Alanine (5 mmol, 0.445g) in (100ml) of absolute methanol, was cooled down to –15 ºC then thionyl chloride (5 mmol, 0.37 ml) was added drop by drop(the temperature should be kept below –10 ^{0}C) , the reaction mixture was left at 40 ^{0}C for 3hr, then reflux started for other 3hr and left at room temperature overnight, the solvent was evaporated to dryness under vacuum, redissolved in methanol and evaporated, this process was repeated several times and recrystallize from methanol–ethyl acetate (3:1)

Synthesis of 1-aminoquinolin-2(1H)-one (B.1) [17]

A solution of (2.96g, 20 mmol) coumarin and excess hydrazine hydrate (99%), in 25 ml of absolute ethanol was refluxed for 24 h, left at room temperature(R.T.) for one week , then the solvent was evaporated to dryness and the solid product was washed with cold ethanole, and recrystallized from chloroform to give yellow crystals. TLC was done indicate the disappearance or lightening of coumarin spot and new spot was resulted.

Synthesis of ethyl-quinoline-8-yl oxyacetate. compound 1 [18]

A mixture of 8- hydroxyquinoline(12.5 mmol), ethyl bromoacetate (12.5 mmol) and K_2CO_3 (17.9 mmol) in 50ml dry acetone was refluxed for about 20 hr at 70 ^{0}c. After cooling, the mixture was evaporated to dryness and the residue was partitioned between chloroform (50 ml) and water (50 ml). The organic phase was dried with anhydrous sodium sulphate (Na_2SO_4), filtered and evaporated to dryness. The residue was recrystallized from ethanol.

Synthesis of 2(quinoline-8-yloxy) acetic acid (QAA) compound 2 [19]

A solution of compound **1** (1.5g, 7.38mmol) in methanol (25 ml) and 5% NaOH (6 ml) was heated under reflux for 4 h. After cooling, the solution was evaporated to dryness and the residue was dissolved in 10 ml water and acidified with 1N HCl(6 ml). The

white precipitate was filtered, dried and crystallized from methanol.

Coupling method and reagents

Conventional solution method for peptide synthesis was used as a coupling method between the carboxy protected amino acids or heterocyclic amine and carboxy derivatives of quinolone.

Dicyclohexylcarbodiimide (DCC) was used in the peptide bond formation as the coupling reagent, while 1-Hydroxybenzotriazole(HOBt) was used to decrease racemization and to increase the yields [20] .

Synthesis of methyl 2-(2-(quinolin-8-yloxy)acetamido)propanoate (Comp.3)

To a stirred solution of D-Ala. methyl ester HCl (comp. **A.1**) (2mmol, 0.28g) in (25ml) of Dimethylformamide (DMF), (2mmol, 0.22ml) of N-Methylmorpholine (NMM) was added with stirring for 10 min., then (2mmol,0.406 g) of (comp. **2**) was also added, and the mixture was cooled down to (-10 ^{0}C) then (4.0mmol, 0.540g) of HOBt and (2.0mmol,0.412 g) of DCC were added with stirring, which was continued for 2 days at 0 ^{0}C and then at room temperature for 5days.

The reaction mixture evaporated to exclude DMF and redissolved in chloroform from which the N,N–Dicyclohexyl urea (DCU) was filtered off. The clear filterate washed three times with 5% sodium bicarbonate solution, 0.1N HCl, once with distilled water, and with saturated sodium chloride solution. The chloroform layer was dried with anhydrous magnesium sulphate and evaporated under vacuum; the resulted product was collected, recrystallized from (methanol: chloroform) (5:1).

Synthesis of N-(2-oxoquinolin-1(2H)-yl)-2-(quinolin-8-yloxy)acetamide (comp. 4)

To a stirred solution of compound **2** (0.406g, 2.0 mmol) in (20ml) of DMF, (0.32 g, 2.0 mmol) of compound **B1** was added, the mixture was cooled down to (-10 ^{0}C) then (0.56g, 0.4 mmol) of HOBt and (0.412g ,2 mmol) of DCC were added with stirring, which was continued for 2days at 0 ^{0}C and then at room temperature for 5 days.

The reaction mixture evaporated to exclude DMF and re dissolved in chloroform from which the N,N–Dicyclohexyl urea (DCU) was filtered off. The clear filterate washed three times with 5% sodium bicarbonate solution, 0.1N HCl, once with distilled water, and with saturated sodium chloride solution. The chloroform layer was dried with anhydrous magnesium sulphate and evaporated under vacuum; the resulted product was collected, recrystallized from (methanol: acetone) (2.5:1) .

Biological activity [21,22]

Antimicrobial activity

The antimicrobial of the synthesized final products was done in IBN SENA Research Center / Research and Development Authority/ Ministry of Industry and Minerals_IRAQ.

A preliminary antibacterial and antifungal activity has been carried out according to Agar well Diffusion

Method

The prepared compounds had been studied for their antimicrobial activity *in vitro* against three tested bacteria (*Staphylococcus aureus)* as gram positive bacteria *and(E. Coli. ,Pseudomonas aeruginosa*) as gram negative bacteria *and* the yeast *(Candida albicans*) were clinical isolated and maintained on nutrient agar medium for testing antibacterial activity and sabaroud agar medium for antifungal activity. Compounds were dissolved in DMSO.Ciprofloxacin, Gentamycin and Cephalexin were used as a standard antibiotic for antibacterial activity and Clotrimazole was used as a standard drug for antifungal activity.

Sensitivity assay

Agar well diffusion assay was carried out by using bacterial suspension of about (1.5×10^8 CFU/ml) obtained from McFarland turbidity standard (number 0.5).This was used to inoculate by swabbing the surface of Mueller Hinton agar (MHA) plates. Excess was air-dried under a sterile hood, and in each agar plate of tested bacteria five wells were made and (30µl) of each concentration was added in it. The plates were incubated at 28 °C for 48 hours (Fungi spp.) or 37 °C for 24 hours (bacteria) and the antimicrobial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the disc in mm. The assessment of antibacterial activity was based on measurement of the diameter of inhibition zone formed around the well, and show that the zone of inhibition varied with the increasing of concentration of the tested compounds, as show in Table (4) .

Cytotoxic activity study [23]

A preliminary *in vitro* cytotoxicity assay (Cell Viability Assay on Cancer Cell Line) for some of the final compounds (**3** and **4**) has been carried out in the Biotechnology Research Center / University of AL-Nahrain. Actually, the *in vitro* cytotoxicity assays with cultured cells are widely used to evaluate chemicals including cancer chemotherapeutics, pharmaceuticals, biomaterials , natural toxins, antimicrobial agents and industrial chemicals because they are rapid and economical.

Cytotoxicity assay has been done by using Neutral Red uptake assay method. This method has been used for cytotoxicity assay of compounds (3 and 4). A set of two fold in four concentrations (3, 1.5, 0.75, 0.375 µg/ml) was made for each product and the exposure time of the assay was 24hrs.

Result and Discussion

Synthetic part

A. The reaction pathways

The aim of our research is to synthesize quinoline derivatives coupled to different amino acid ester or heterocyclic amine (figure 1).

The overall synthesis strategy based one four major lines:

1. Amino acid derivatives

The amino acids were activated by thionyl chloride to get acyl chloride that attacks either ethanol or methanol to get ethyl or methyl esters of the selected amino acids.

2. alkylation of 8-hydroxyquinoline by ethyl bromoacetate in presence of anhydrous potassium carbonate base to get (O-alkylated ester) derivatives which are deesterfied by using NaOH solution to get acetic acid derivatives.

3. Peptide bond formation

Conventional solution method for peptide synthesis used as a coupling method between the carboxy-protected amino acids or 1-amino quinolone derivatives with acetic acid side chain of quinolone. The DCC/ HOBt coupling reagents used for peptide bond formation.

The overall reaction pathway is shown in the following figure 1 .

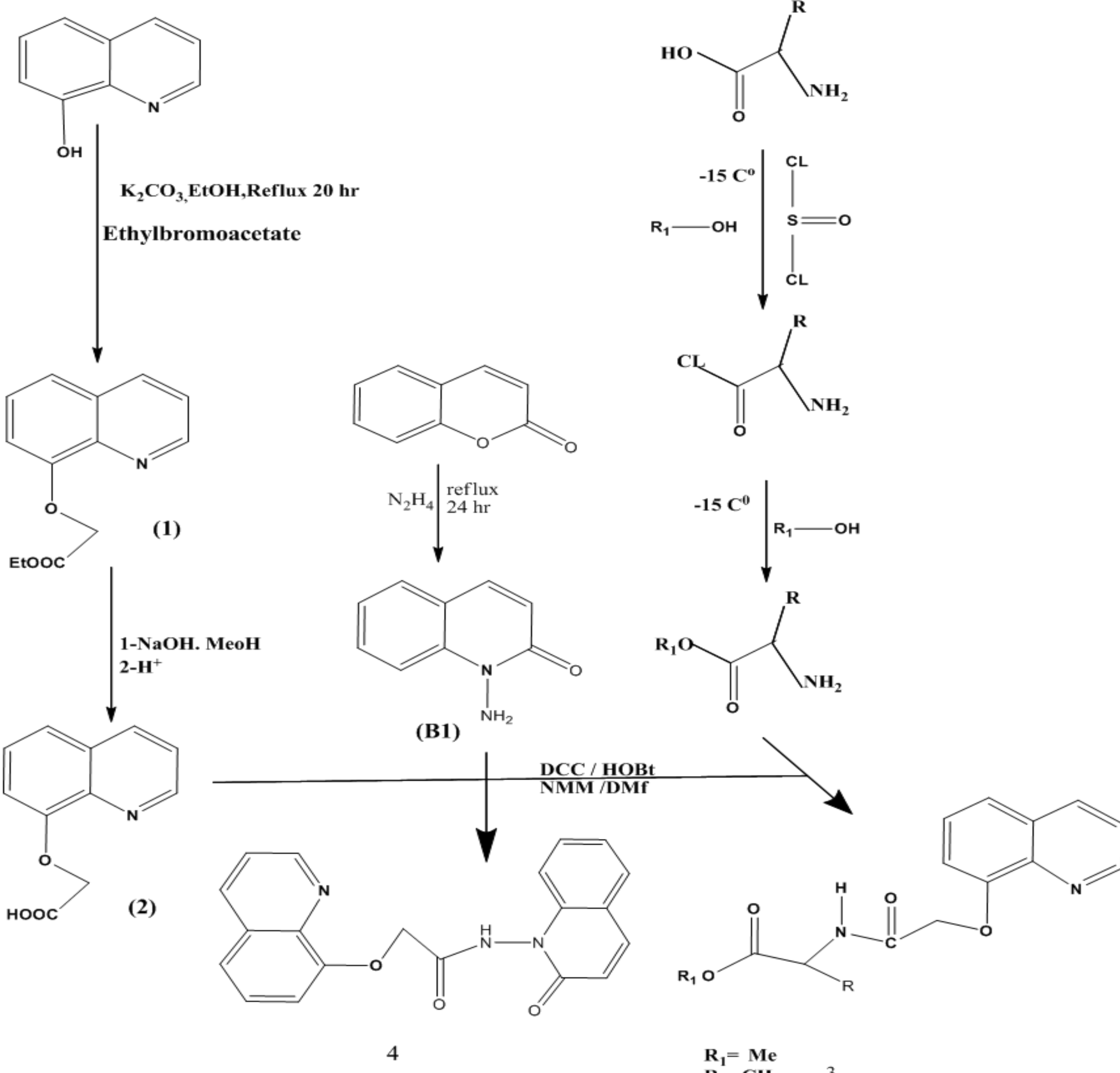

Figure (1): Scheme of overall pathway of synthesis of N-heterocyclic compounds.

B. Strategy of synthesis

The strategy of synthesis started from the esterification of amino acids then proceeded to the synthesis of quinolone-8-yloxy acetic acid from 8-hydroxyquinoline and finally coupling between the acid and the amino group of the amino acid esters, and as follows:

1) Esterification of amino acids

The esterification of carboxyl group of amino acids is normally used as an amino acid protecting group. Esterification of carboxyl group enhances the nucleophilic character of amine group and allows its subsequent acylation [(24)] .

2) 8-hydroxyquinoline can be alkylated at hydroxyl group by alpha-haloester of ethylbromoacetate which is widely used as alkylating agent to O-, N- and S- groups. Triethylamine or potassium carbonate act as a base which make a nucleophilic attack to the hydroxl group (-OH) of the above heterocyclic and deprotonate it and convert it to negative charge group which will then make a nucleophilic attack to the ethylbromoacetate which have bromide group that is a good leaving group to get ethyl acetate ester derivative of the above heterocyclic (compound 1). [(25)]

The mechanism of O-alkylation by alkyl halide is a nucleophilic substitution as shown in figure (2) [(26)] **.**

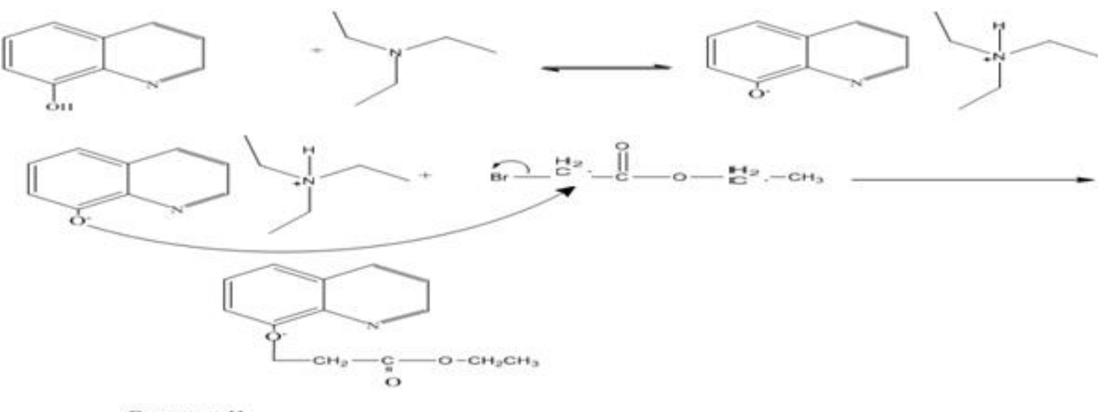

Figure (2): Scheme of the O-alkylation of 8-hydroxyquinoline

3) Peptide Coupling Method:

There is many ways to form a peptide bond [(30)] , however in this work; the direct coupling with DCC/HOBt method was used. This method is characterized as being simple, efficient, no racemization, rapid, and leading to a good yield at R. T. [(31)]. The overall reaction resembles the dehydration process to form the amide bond (figure 1) .The properties of synthesized new compounds are shown in table (1) while the data obtained from elemental microanalysis are shown in table (2).On the other hand the characteristic IR bands are shown in table (3).

4) Removal of ethoxy group from compounds 1 were done by alkaline catalyzed de-esterification (saponification) to get the corresponding conjugated bases, that acidified by mineral acid (HCl) to get the corresponding carboxylic acid[(27,28)] (compound 2). The mechanism of saponification was proposed by Ingold as shown in figure (3) [29]**).**

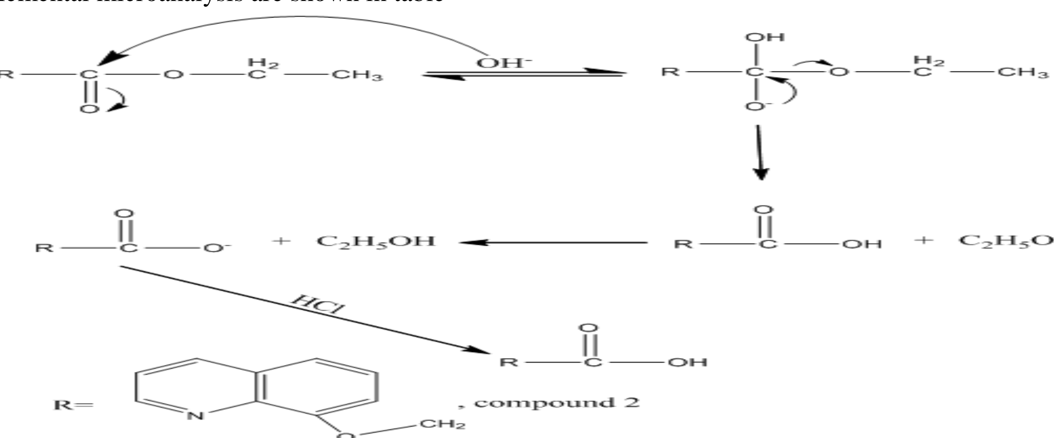

Figure (3): Scheme of saponification of compound 1.

Table (1): The identification parameters of the synthesized compounds

Symp.	Compound Name	Yield %	Physical appearance	m.p. ^{o}C	R_f value
A1	D-Alanine methyl ester. HCl	78	Off-white crystals	98-102	0.73A 0.69B
B1	1-amino-quinoline-2-(*1H*)-one	73	yellow crystals	128-130	0.90A 0.63C
1	ethyl 2-(quinolin-8-yloxy)acetate	77	yellow crystals	52-54 52-53	0.74C 0.79D
2	2-(quinolin-8-yloxy)acetic acid	68	Palle-yellow crystals	210-214	0.30C 0.25D
3	methyl 2-(2-(quinolin-8-yloxy)acetamido)propanoate	60	White crystals	208-210	0.88B 0.36C
4	N-(2-oxoquinolin-1(2H)-yl)-2-(quinolin-8-yloxy)acetamide	50	Faint-yellow powder	217-220	0.90D

Table(2): The Elemental microanalysis of the synthesized compounds

Cpd No.	M. Wt.	Chemical Formula	Calculated ⁄ Found			
			C%	H%	N%	O%
3	288.30	$C_{15}H_{16}N_2O_4$	62.49 61.94	5.59 6.10	9.72 10.58	22.20 21.38
4	345.35	$C_{20}H_{15}N_3O_3$	69.56 70.44	4.38 4.25	12.17 12.26	13.90 13.05

Table (3): The characteristic IR bands of the synthesized compounds; (measured in cm^{-1})

A1	CH_3O, CH_3, O, NH_2 .HCL	3325, 3277 asym. and sym. str. of NH_2 ; 2928, 2850 asym. and sym. CH_3, CH_2 str.;1743 C=O Str. Ester; 1570NH bend.; 1215 C-O str. Ester; 1435 CH_2 bend.; 1375 CH_3 bend. .
B1	N, O, NH_2	3306, 3186 asym. and sym. str. of NH_2; 3057 C-H ar. str of benzene; 1662 C=O str. of amide; 1514NHbend.; 1118 C-N str.;1597,1548,1458 C=Car. Str. .
1	N, O, EtOOC	(3053 C-H_{ar} Str.), (2989$_{assym}$, 2875 $_{sym.}$ C-$H_{alif.}$ Str.), (1743.7 C=O Str. ester), (1504 , 1446 C=C_{ar} Str.), (1219 C-O Str. ester), (1118 Ar-O-C str.),1653 C=N str. .

Table (3): Continued the characteristic IR bands of the synthesized compounds; (measured in cm^{-1})

2		(3462 OH str. Of COOH), (3053 C-$H_{ar.}$ Str.), (2937_{assym},2833_{sym} C-$H_{alif.}$ Str.), (1716 C=O Str. of COOH), (1600 , 1562,1454 C=C_{ar} Str.), (1118 Ar-O-C str.).
3		3323 NH str.), (3036 CH $_{ar.}$ Str.), (2928_{assym},2850_{sym}C$H_{alif.}$Str.), (1743.7 C=O Str. Ester), (1678C=O Str. amid) ,(1624 C=N Str.), (1535-N-H bend.) ,(1572,1564,1502 C=C_{ar} Str.), (1242 Ar-O-C str.).
4		(3323 NH str.), (3032 CH $_{ar}$ str.), (2928 $_{assym.}$, 2850 $_{sym}$ CH_{alif}. Str.), (1683 C=O str. amide), (1537 NH bend. amide II), (1570-1435 C=C str.), (1242 Ar-O-C str.).

Asym=asymmetric , sym.=symmetric , str.=stretching , bend.=bending , ar.=aromatic, alif=alifatic

Table (4): The antibacterial activity of the tested compounds.(3 and 4)

Compound No.		**Zone of Inhibition in mm**		
		E. Coli ATCC10536	**Staphy. Aureus ATCC6538**	**Pseud. aurogenosa ATCC15442**
3	100µg/ml	No activity	No activity	13
	200µg/ml	13	No activity	14.5
4	100µg/ml	No activity	No activity	15
	200µg/ml	13.5	No activity	15
ciprofloxacin	100µg/ml	20.5	22	30
	200µg/ml	26	32	35
Gentamycin	200µg/ml	20	20	14
Cephalexin	600µg/ml	24	36	No activity

Table (5): The antifungal activity of the tested compounds.(3 and 4)

Compound No.		Zone of Inhibition in mm
		Candidia albicans 10231
3	100µg/ml	14
	200µg/ml	11
4	100µg/ml	10
	200µg/ml	11
Clotrimazole	100µg/ml	12.75
	200µg/ml	14.5

According to the following equation, the cytotoxic effect for tested compounds expressed as IR % at transmittance wave length 492 nm as showed in table 6

$$\text{Inhibition Rate\%} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Table (6): The initial cytotoxic effect (cell viability assay) on Hep - 2 cell line of compounds (3 and 4) by Neutral red assay method.

Cpd. No.	Concentration					time 24 hrs.	
	3µg/ml	**1.5µg/ml**	**0.75µg/ml**	**0.375µg/ml**	**Control/ 0µg/ml**		
3	0.1396 88.49 11.51	0.1353 85.768 14.23	0.1066 67.575 32.43	0.1093 69.286 30.71	0.15775 100 0	**Abs. at 492nm** **cell viability%** **I.R.%**	
4	0.1026 65.05 34.97	0.09 57.05 42.95	0.0796 50.45 49.55	0.0836 52.99 47.01	0.15775 100 0	**Abs. at 492nm** **cell viability%** **I.R.%**	

Abs.= Absorbance I.R.%= Inhibition Rate Percent

Summary of the Results

1)From the result in table (4), all tested compounds had low or no activity against *E.Coli* and no activity against *Staphylococcus aureus* when compared with Ciprofloxacin, Gentamycin and Cephalexin. While all tested compounds showed high activity against *Pseudomonas aeruginosa* when compared to Cephalexin, and comparable activity against same bacteria when compared to Gentamycin and finally ,the tested compounds showed low activity against *Pseudomonas aeruginosa* when compared to Ciprofloxacin. From the results in Table (5), the tested compounds showed a moderate to good activity against *Candida albicans.* when compared to Clotrimazole.

2)The cytotoxic study was done on Hep-2 cell line passage (75), Exposure time =24hrs.
Staining is Neutral red stain. When the cancer cell line (Hep-2) was treated with these products the result showed significant cytotoxic effect in tested samples in comparison with the control. The toxic effect varied from one sample to another, all samples showed a significant toxicity ($P < 0.05$) started from 3µg/ml to the 0.375µg/ml.
The inhibitory concentration percent (IC %) was estimated, and the result was varied among samples as shown in table (6).

Conclusions

From the antimicrobial and cytotoxic activity studies, compound 4 showed the best activity**.** according to pharmacokinetics properties and cationic nature of comp.4, comp.4_may cause DNA intercalation through DNA topoisomerase/gerase chelation or other binding sites.

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