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Synthesis, characterization of 2-amino-pyran and derivatives evaluation of the bacterial, antioxidant and anticancer activity

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Abstract--Using ethanol as a solvent, heterocyclic hexagonal rings for 2-amino-pyran derivatives (I₃₃-I₄₀) were produced by reacting a mole of chalcones derivatives with a mole of malononitrile. Physical properties such as melting point, colour, and molecular weight and spectroscopic measurements such as the infrared spectrum, [¹H-NMR], [¹³C-NMR] spectrum were used to confirm the accuracy of the prepared compounds' compositions. The biological activity of various produced compounds was investigated in two species of pathogenic bacteria, one of which is Gram-positive, *Staphylococcus aureus*, and the other, Gram-negative, *Escherichia coli*. Chemical solutions for the two substances (I₂₅, I₃₂) with concentrations (0,01, 0.001, 0.0001) mg /ml were created using a solvent DMSO and Muller Hinton Agar growth medium. The antibiotic Ciprofloxacin was utilized as a control sample for the diffusion sensitivity test of the bacterial isolates used in the investigation. Using DPPH root and varied concentrations, the impact of chemical (I₃₂) on the elimination of free radicals was also investigated. Breast cancer cells were taken from Baghdad's Medical City and used to test the efficiency of several produced chemicals against them. 200 µl of the prepared concentrations of the compounds produced for the research (25, 50, 100, 200) g/ml were added to the pits at three concentrations, cytotoxicity tests were conducted in three duplicates, and (IC₅₀) values were computed. The cytotoxicity impact of the compounds was evaluated and produced using MTT and solute solutions on breast cancer cells and the ordinary cell line WRL68 for comparison.

Keywords--2-amino-pyran, *staphylococcus aureus*, *escherichia coli*, antioxidant activity, breast cancer.

Introduction

Pyrene or pyran is a heterocyclic, unsaturated organic compound consisting of six central atoms, five of which are carbon atoms and one oxygen atom, and contains two double bonds. It has the chemical formula C_5H_6O , and the molar mass is 82.10 g/mol [1]. Its common name is (IUPAC) 2H-Pyran, 4H-Pyran, and its other names are 2H-Oxine and 4H-Oxine [2]. There are two isomers of pyran that differ in the location of the double bonds. In 2H- pyran, the saturated carbon is in position 2 [3]. In contrast, in 4H-pyran, the saturated carbon is in position 4. 4H-pyran was first isolated, prepared and distinguished in 1962 by the pyrolysis of 2-acetoxy-3,4-dihydro-2H-pyran [4], and found to be very unstable, particularly in the presence of air, 4H-pyran does not readily match the corresponding dihydropyran and beryllium ion, which readily decomposes in an aqueous medium [5]. Although pyrenes themselves are of little importance in chemistry, many of their derivatives are essential biological molecules [6], such as pyranoflavonoids, and pyranones are also important derivatives, which are natural products, an example of which is coumarin [7]. As the term pyran is applied to its saturated ring analog, which is more appropriately referred to as tetrahydropyran (oxane), in this context, monosaccharides containing the six-membered ring system are known as pyranose [8]. Piran compounds are prepared in many ways, including through a series of reactions as the response begins with propylene oxide, which was converted directly through three steps to acetylene [9]. Then, heating gave chitin dimethyl acetal diene, which regularly reacts with 2-chloro-1,4- naphthoquinone to form anthraquinone, an aromatic compound, which reacts with sulfuric acid to produce lactone [10]. Bacteria are microorganisms that can only be seen with a microscope. Bacteria are found everywhere, in the air, in the water, in the human body, and inside the digestive canal and respiratory system [11]. Bacteria may persist for many years, enduring all kinds of improper circumstances including extreme heat or cold, as well as other severe environmental factors. When the bacteria's environment improves, they shed the thick membrane and resume their prior activity and vigor [12]. *Staphylococcus aureus* is a Gram-positive bacterium that has spherical germ cells with a diameter of approximately 3 m, is extremely thick, immobile, non-sporulating, and ferments sugars [13, 14]. *Escherichia coli* is a Gram-negative bacillus that thrives on ordinary culture medium without the need of growth factors. These bacteria are killed by heating them to 60°C for 30 minutes. On MacConkey agar culture, this type of bacterium may thrive, and some strains of this bacteria can produce capsules [15]. This bacterium is one of the most well-known forms of intestinal bacteria in people and animals, and it causes bladder, urinary tract, meningitis, and bile sac inflammation [16]. Cancer is a group of diseases characterized by the unrestricted growth and division of cells in the body's tissues, as well as the ability of these cells to invade and destroy neighboring tissues or spread to distant tissues via the blood or lymphatic system, which are the characteristics of a malignant tumor. They differ from Benign's adenoma in that they have a distinct development pattern, are unable to penetrate, and cannot metastasize. A benign tumor may become cancerous in certain cases [17]. Cancer may strike anybody at any age, but the risk rises with age, and it is one of the top causes of mortality in both industrialized and developing nations [18]. Breast cancer is a kind of malignant tumor that affects the breast tissue and manifests itself in the ducts of the tubes that transport milk

to the nipple and milk glands [19]. It affects both men and women, although the frequency in men is uncommon, with just one male injury for every 200 female injuries [20]. Breast cancer is classed as invasive or non-invasive, depending on whether it is found in the duct or the lobes [21]. Modifiable risk factors (such as alcoholic beverage intake) and fixed risk factors (such as age and biological sex) are the two types of risk factors [22]. The greatest risk factor for breast cancer is gender, since women are more likely than men to acquire it and the risk increases with age [23].

Experimental

Material: All chemicals used in this work were purchased from Fluka, Aldrich, and BDH and used without further purification.

Devices used: The melting points were measured using Electrothermal Melting Apparatus 9300. The FT-IR spectra were captured using a Shimadzu FT-IR 8400S spectrophotometer with a scale of (400-4000) cm^{-1} by KBr disc. DMSO- d_6 as solvents were used to capture $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra on Bruker instruments running at 400 MHz.

Preparation of 2-amino-pyran derivatives (I₃₃-I₄₀) [24]

In a round flask of 100 ml volume, 0.01 mole of the prepared chalcone derivatives (I₁-I₈) is dissolved in 10 ml of ethanol. A solution of 0.01 mole of malononitrile dissolved in 10 ml of ethanol is added, then 10 ml of ethanol is added. 10% sodium hydroxide solution, and the mixture rises for 6 hours with continuous stirring, then concentrates the solvent, cools the answer, and is added to crushed ice. It is equalized by adding drops of concentrated hydrochloric acid HCl and noting the product's precipitation. The precipitate is separated by filtering, washed with cold water, and recrystallized methanol, and table (1) shows some physical properties of 2-amino-pyrene derivatives (I₂₅-I₃₂).

Table (1): Physical properties of 2-amino-pyran derivatives (I₃₃-I₄₀)

Comp. No.	X	R	Molecular Formula/ g/mol	M.Wt	Color	M.P. (°C)	Yield (%)
I ₂₅	F	Cl	C ₁₈ H ₁₂ FN ₂ OCl	326.76	Drack yellow	139-141	78
I ₂₆	F	Br	C ₁₈ H ₁₂ N ₂ OBr	371.21	Light brown	134-135	59
I ₂₇	F	CH ₃	C ₁₉ H ₁₅ N ₂ OF	306.34	Light brown	171-173	71
I ₂₈	F	OCH ₃	C ₁₉ H ₁₅ N ₂ O ₂ F	322.34	Yellow	185-187	63
I ₂₉	Cl	Cl	C ₁₈ H ₁₂ N ₂ OCl ₂	343.21	Brown	99-101	55
I ₃₀	Cl	Br	C ₁₈ H ₁₂ N ₂ OClBr	387.66	Light brown	159-160	60
I ₃₁	Cl	CH ₃	C ₁₉ H ₁₅ N ₂ OCl	322.79	Light brown	157-158	66
I ₃₂	Cl	OCH ₃	C ₁₉ H ₁₅ N ₂ O ₂ Cl	338.79	White	191-193	69

Biological activity study

This research employed two kinds of harmful bacteria, one of which is Gram-positive, *Staphylococcus aureus*, and the other of which is Gram-negative, *Escherichia coli*; and these microorganisms. It is essential in medicine because it

is resistant to antibiotics. These bacteria were collected from the Department of Life Sciences at the College of Education for Pure Sciences. The culture medium was utilized as a form of Muller Hinton Agar, which is used to test the biological activity of antibiotics and other chemicals. Chemicals have therapeutic potential. Chemical solutions of (I_{25} , I_{32}) were prepared in concentrations of (0,01, 0.001, 0.0001) mg/ml and using a solvent Dimethyl sulfoxide to measure and determine the minimum inhibitory concentration (MIC) (DMSO). The bacteria isolates used in the study were sensitivity tested using the diffusion method in the nutrient medium Mueller- Hinton agar, which is a transparent food medium with a dark yellow color that is useful in testing the sensitivity of microorganisms to antibiotics because it contains casein and starch extracted from an animal infusion. It allows most bacteria and germs to thrive. The medium was prepared and sterilized in an autoclave, then distributed in dishes, and allowed to harden before making four small pits in each plate. It was then incubated for 48 hours at 37 degrees Celsius (24 hrs). The results were read the next day to show the sensitivity derivatives used, which are dependent on the diameter of the inhibition visible in the dishes around the holes used as the diameter of the inhibition increases. Inhibition refers to the rise in the biological activity of the prepared compounds, as opposed to antibiotics' diameter of inhibition [25, 26].

Measuring the antioxidant activity of some compounds prepared ex vivo (DPPH inhibition activity test)

DPPH root (2,2-Diphenyl-2-Picryl Hydrazyl) was used to evaluate natural oxidation's free radical scavenging activity. At a concentration of 0.1 mM by dissolving 4 mg in 100 mL of methanol, then 3 mL of DPPH prepared solution was added to 1 ml of the prepared compounds of several concentrations ranging from (25, 50, 100, 200) $\mu\text{g}/\text{mL}$, as well as Ascorbic acid prepared from dilute concentrations of (25, 50, 100, 200) $\mu\text{g}/\text{ml}$, left the mixture in the dark for 30 minutes, then read the absorbance at a wavelength of 516 nm, and calculated the percentage of scavenging ability of the prepared compounds DPPH for free radicals for each compound as well as for Ascorbic acid AA [27], which represents the positive witness for comparison through the following equation:

$$I\% = (A_{bc_0} - A_{bc_1}) / A_{bc_0} \times 100$$

The standard solution was made by adding DPPH solution without ascorbic acid or extracts.

Testing the cytotoxicity of I_{32} on breast cancer cells (MCF-7)

Cancer cells were obtained from the Medical City - Baghdad, and the cancer cells were preserved in liquid nitrogen; they were perpetuated, grown, and tested at the Biotechnology Research Center at Al-Nahrain University; and work began with steps:

First: The natural cell line WRL 68 Cell Line: The human liver cell line represents a tank similar to hepatocytes and primary liver transplants. It has been proven that the cells secrete albumin and alpha-fetoprotein and express enzymes specific to the liver. Like alanine aminotransferase

Second: Solutions Used in Tissue Culture Technique: Several solutions used for cell culture were prepared, namely: Antibiotic Solution (Streptomycin (1g/vial),

Benzyl Penicillin, Sodium Bicarbonate Solution, Phosphate Buffer Saline (PBS), Trypsin Solution, EDTA, Trypsin - EDTA

Third: Media: several media attended, namely: Roswell Park Memorial Institute - 1640 Medium (RPMI), Serum-Free Medium, and Freezing Medium [28].

Breast Cancer Cell Growth (MCF-7): The freshener method was used to grow cancer cells as follows

The tumor cells were thawed using a water bath at 37 °C. Then, the tumor cells were placed in a 25 cm² animal cell culture vessel (Falcon) containing culture medium (RPMI-1640) and 10% calf serum. Then the culture vessels containing the cell suspension and medium were incubated in a 5% CO₂ incubator at 37 °C for 24 hours. After incubation, and when it was confirmed that there was growth on the farm and that it was free from pollution, secondary farms were conducted for it. The cells were examined using an inverted microscope to ensure their viability, freedom from contamination, and growth to approximately 500000-800000 cells/ml. Then the cells were transferred to the growth booth, and the used culture medium was discarded. Cells were washed using PBS solution and discarded, and the process was repeated twice for 10 minutes each time. A sufficient amount of trypsin/EDTA enzyme solution was added to the cells and incubated for (30-60) seconds at a temperature of 37 °C and monitored until they changed from a monolayer of cells to single cells. Then, the enzyme was stopped by adding a new development medium containing serum. Then the cells were collected in centrifugal tubes and placed in the apparatus at a speed of 2000 rpm for 10 minutes at room temperature to precipitate the cells and get rid of the trypsin and the used medium. The filtrate was discarded, and the cells were suspended in a fresh medium containing 10% serum [29]. The cell number was examined by taking a specific volume of the cell suspension and adding to it the same volume of Trypan Blue dye to find out the number of cells and their vitality percentage using a Haemocytometer chip, according to the equation:

$$C = N \times 10^4 \times F/ml$$

The cell suspension was distributed in new containers and then incubated in a 5% CO₂ incubator at 37 °C for 24 hours: Total Cell Count/ ml = Cell Count x dilution factor (Sample Volume) x 10⁴.

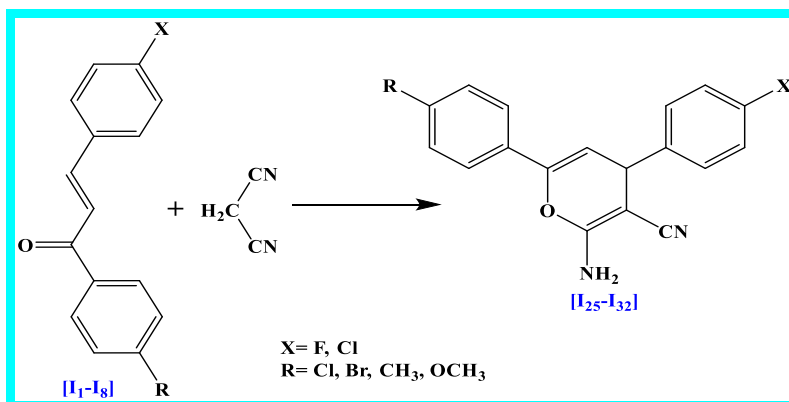
MTT Cytotoxicity Assay

Cytotoxicity tests were performed in three replicates, and the (IC₅₀) values were calculated. 3-(4,5 dimethylthiazol - 2-yl) 2,5-Diphenyl tetrazolium bromide (MW = 414), and solute solution [30]. The manufacturer's instructions were followed as they prepared breast cancer cells as previously mentioned, then placed the cell suspension at a concentration of (1 x 10⁴ to 1 x 10⁶) cells/ml in a 96-hole newspaper to a final volume of 200 µl of complete culture medium for each hole and the plates were covered with sterile parafilm, gently stirred and incubated. After incubation, the medium was removed, and 200 µl of the prepared concentrations of the studied compounds (25, 50, 100, 200) µg/ml were added to the pits at three pits for each engagement with the control sample and incubated in a 5% incubator. CO₂ at 37 °C for 24 hours. After exposure to the compounds under study, ten µl of MTT solution was added to each hole; then, the plate was incubated in a 5% CO₂ incubator at 37 °C for 4 hours. 100 µl of DMSO was added

to each pit and set for 5 minutes. Then, the absorbance was read using an ELISA device at a wavelength of 570 nm [31]. Statistical analysis was performed on the optical density readings to calculate the IC₅₀.

Results and Discussion

The 2-amino-pyran derivatives (I₃₃-I₄₀) were prepared by reacting a mole of chalcone derivatives with a mole of malononitrile and using ethanol as a solvent, as in the following equation:



Scheme (1): Route of prepared compounds (I₂₅-I₃₂)

Diagnosis of 2-amino-pyran derivatives (I₃₃-I₄₀)

The reaction to the 2-amino-pyran derivatives (I₃₃-I₄₀) was confirmed by observing the changes in the physical properties of the melting point and the significant color difference. During measurements of infrared (IR), (¹H-NMR), and (¹³C-NMR) spectra.

When studying the infrared (FT-IR) spectrum of 2-amino-pyran derivatives (I₂₅-I₃₂), two absorption bands were observed at the frequency (3239-3270) cm⁻¹, and (3176-3195) cm⁻¹ due to the stretching of the (NH₂) bond, and the appearance of an absorption band at the frequency (3007-3098) cm⁻¹ due to the extension of the aromatic (CH) bond, as well as the appearance of two absorption bands at the frequency (2913-2997) cm⁻¹ and (2818-2891) cm⁻¹, are due to the stretching of the aliphatic (CH) bond, in addition to the appearance of an absorption band at the frequency (2254-2264) cm⁻¹ due to the stretching of the (CN) bond, as well as the appearance of two absorption bands at the frequency (1581-1599) cm⁻¹ and (1477-1499) cm⁻¹ were due to the stretching of the aromatic (C=C) bond, and there was an absorption band at the frequency (1302-1387) cm⁻¹ that was due to stretching (C-O) group bond, as shown in table (2), and these bundles were close to what is found in the literature [32, 33].

Table (2): Infrared absorption results (cm-1) 2-amino-pyran derivatives (I₃₃-I₄₀)

Comp. No.	X	R	ν NH ₂	ν (CH) Arom.	ν (CH) Aliph.	ν (CN)	ν (C=C) Arom.	ν C-O	Others
I ₂₅	F	Cl	3261 3180	3043	2966 2825	2262	1583 1491	1332	ν (C-F) 1093 ν (C-Cl) 700
I ₂₆	F	Br	3255 3198	3012	2945 2879	2256	1599 1481	1387	ν (C-F) 1061 ν (C-Br) 633
I ₂₇	F	CH ₃	3270 3183	3032	2913 2865	2260	1583 1497	1302	ν (C-F) 1070
I ₂₈	F	OCH ₃	3239 3177	3071	2997 2818	2264	1594 1486	1365	ν (C-F) 1087
I ₂₉	Cl	Cl	3254 3191	3007	2920 2891	2257	1581 1477	1366	ν (C-Cl) 734
I ₃₀	Cl	Br	3259 3182	3066	2943 2820	2254	1587 1485	1340	ν (C-Cl) 773 ν (C-Br) 678
I ₃₁	Cl	CH ₃	3241 3195	3098	2942 2857	2261	1590 1499	1349	ν (C-Cl) 769
I ₃₂	Cl	OCH ₃	3260 3176	3011	2990 2831	2259	1592 1488	1358	ν (C-Cl) 741

When studying the nuclear magnetic resonance spectrum of the proton for the compound (I₃₀), it was observed that multiple signals appeared in the range (7.037-7.903) ppm attributed to the aromatic ring protons, and the appearance of a binary signal in the position (6.864 and 6.872) part of The ppm is attributed to the proton of the (CH) group of the double bond, the appearance of a binary signal at the position (6.829 and 6.853) ppm is attributed to the proton of the (CH) group adjacent to the benzene ring, and the appearance of a single signal at the site (3.406) ppm attributed to the protonation of the (NH₂) group, and the appearance of a signal at the site (2.502-2.512) ppm attributed to the protons of the solvent (DMSO-d₆) [34], and as in figure (3).

When studying the nuclear magnetic resonance spectrum of carbon for the compound (I₃₀), it was observed that a signal appeared at the site (187.60) ppm attributed to the carbon of the carbonyl group (C=O) far from the group (NH₂). The appearance of a signal at the site (142.45) ppm was attributed to the group carbon (CN), and the formation of a signal at the site (135.79) ppm was attributed to the group carbon (=C-NH₂), as well as the appearance of signals At the location (127.37-134.66) ppm attributed to the carbons of the aromatic benzene ring, and the formation of a signal at the site (126.85) ppm attributed to the carbon of the group (=CH) of the double bond in the pyran ring, and a signal appeared at the site (121.81) ppm attributed to the carbon of the pyran ring which is related to the benzene ring, and the appearance of signals at the range (39.19-41.23) ppm attributed to the solvent carbonate (DMSO-d₆) [35], and as in figure (4).

Biological activity of some prepared compounds

The study of the biological activity of the compounds prepared at certain concentrations showed that most of these compounds contain antagonistic

activity against the types of bacteria studied compared with the antibiotic (Ciprofloxacin), which is a broad-based antibiotic, especially these two types of bacteria studied in addition to many types. It also has an inhibitory diameter. It is great as it gives a high selectivity when studying the sensitivity of bacteria to the prepared compounds since this antibiotic is used to treat many infections and diseases such as infections of the urinary tract, especially those that occur as a result of infection with colon bacteria and *Staphylococcus aureus* bacteria. It also treats simple cystitis in females caused by bacteria Colon. It treats chronic bacterial prostatitis caused by colon bacteria and *Staphylococcus aureus* and infections of the lower respiratory tract, sinusitis, arthritis, and bones. It is also used to treat diarrhea caused by colon bacteria and effectively treat typhoid. Therefore, two compounds of the compounds prepared in this research (I_{25} , I_{32}) were studied on different types of chromium-positive and negative bacteria, which recorded a global antagonistic activity against the bacteria studied and compared with the mentioned antibiotic, it is possible to use this Compounds as a treatment for the same infections and pathological conditions above after investigating the biological pathway of these compounds, their side effects, and the amount of their accumulation in animal tissues (0.01, 0.001, 0.0001) mg/ml where the inhibition diameter ranges between (10 mm the lowest inhibition diameter, to 35 mm, the highest measured inhibition diameter) and the table below shows the inhibitory activity of some of the prepared compounds [36], and as in table (3).

Table (3): The inhibitory activity of the two compounds (I_9 , I_{16}) in the growth of several positive and negative bacteria (the diameter of inhibition is measured in mm)

Comp. No	<i>E. coil</i>			<i>Staph. aur</i>		
	0.0001	0.001	0.01	0.0001	0.001	0.01
I_{25}	18	23	29	10	17	21
I_{32}	16	24	36	15	19	26
Ciprofloxacin	18	25	27	10	15	20

Antioxidant Activity

The qualitative evaluation of the anti-oxidant activity of free radical scavenging is done by utilizing the TLC method to determine the prepared compound (I_{32}) ability to reduce the oxidative stress, besides studying the quantitative free radical scavenging activity. Any compound's action is shown when it changes the purple color of DPPH to yellow color, and the intensity of the spot color stated the positive marker. The results indicate that the prepared compound (I_{32}) has an excellent scavenging ability. There is a change in color for yellow, and that is detected that (I_{32}) was a good source of anti-oxidant, but with the least activity than ascorbic acid (as standard). It is clear from the collected data that the free radical scavenging activity of the compound under search increased with increasing concentration exhibiting; for that, the highest free radical scavenging percentage is given with 200 $\mu\text{g}\text{mL}$ of with 80 %, and had the lowest percentage of 40% when 25 $\mu\text{g}\text{mL}$. Also, this search displays that 2-amino-pyran derivatives were a leading source of anti-oxidant, besides it has an excellent free radical scavenging activity and functions as an anti-bacterial [37,38]. As shown in figure (6).

Results of the breast cancer cell cytotoxicity (MCF-7) test

The test results for compound (I₃₂) showed good inhibitory activity against (MCF-7) breast cancer cells and normal cell line (HdFn). When calculating the IC₅₀ of the compound (I₃₂) against breast cancer cells was 161.4. Its value was 231.4 against normal cells. When calculating IC₅₀, the results showed significant differences, $P \leq 0.0001$, when treated with the compound (I₃₂) for breast cancer cells and normal cells [39], as shown in figures (6, 7, and 8).

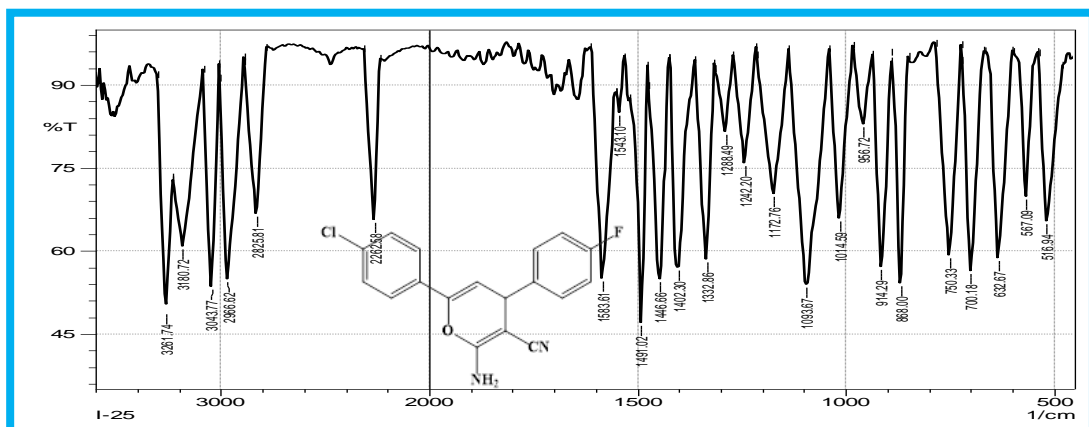


Figure (1): Infrared spectrum of the compound (I₂₅)

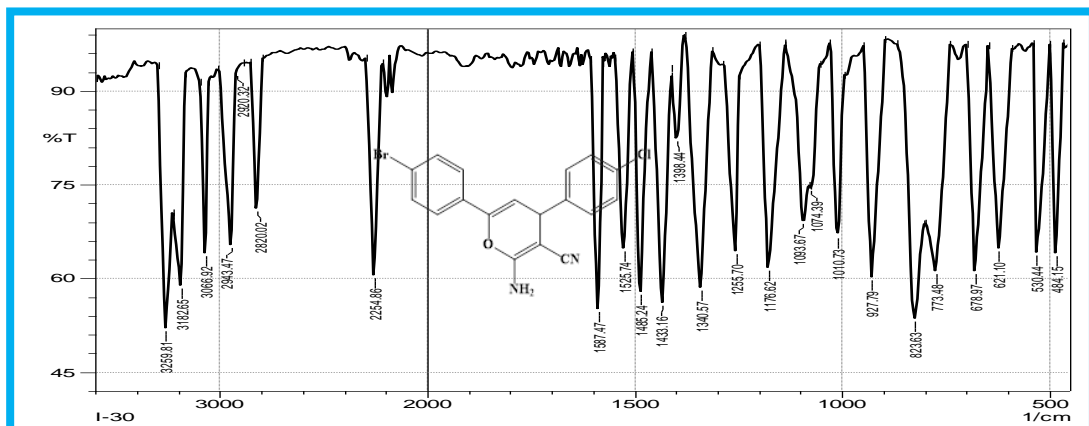
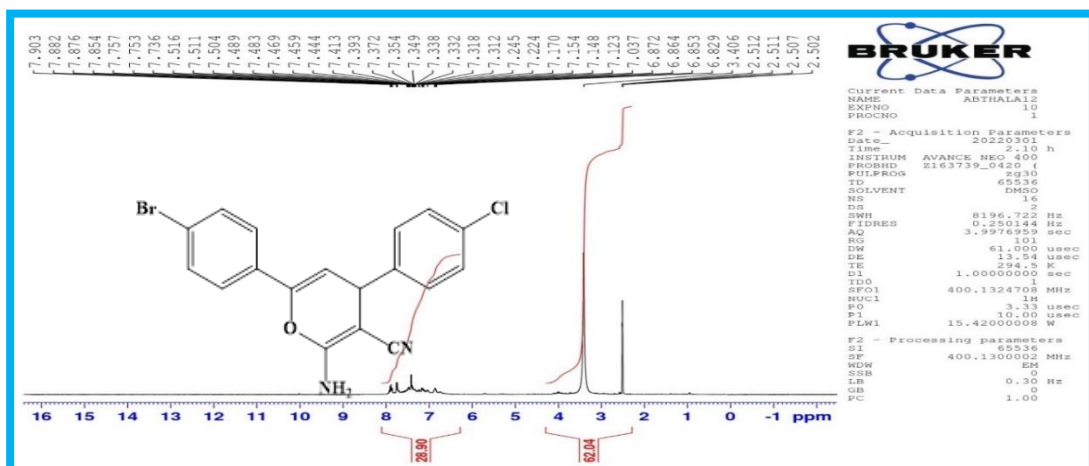
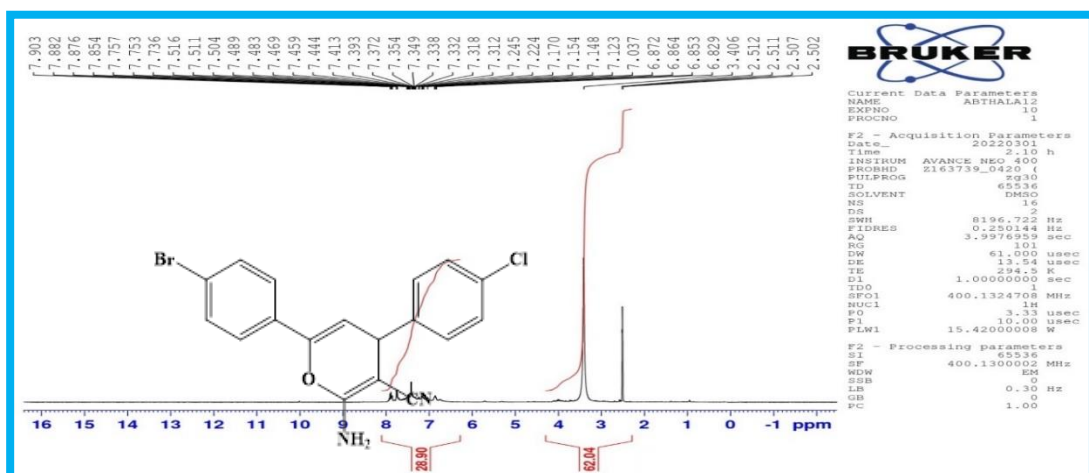
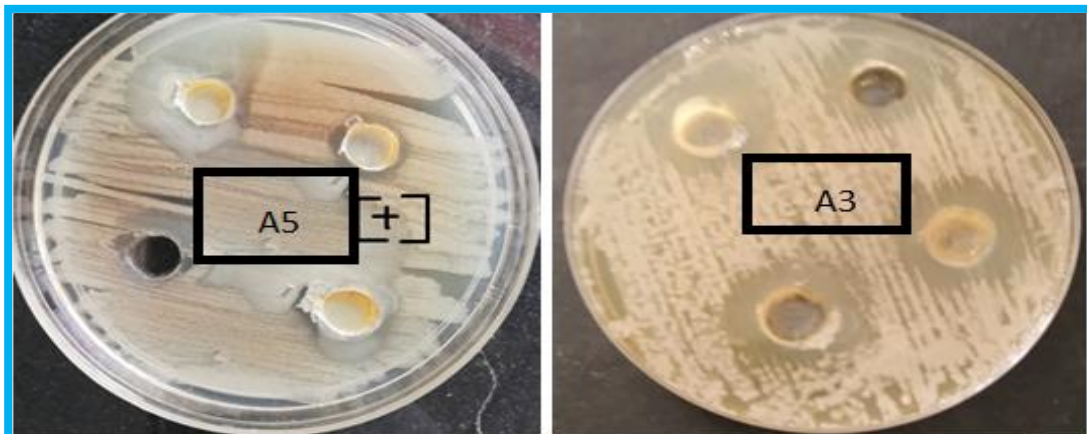


Figure (2): Infrared spectrum of the compound (I₃₀)

Figure (3): The $^1\text{H-NMR}$ spectrum of compound (I₃₀)Figure (4): The $^{13}\text{C-NMR}$ spectrum of compound (I₃₀)Figure (5): Inhibition of compound (I₂₅) against *Escherichia coli* and inhibition of compound (I₃₂) against *Staphylococcus aureus*

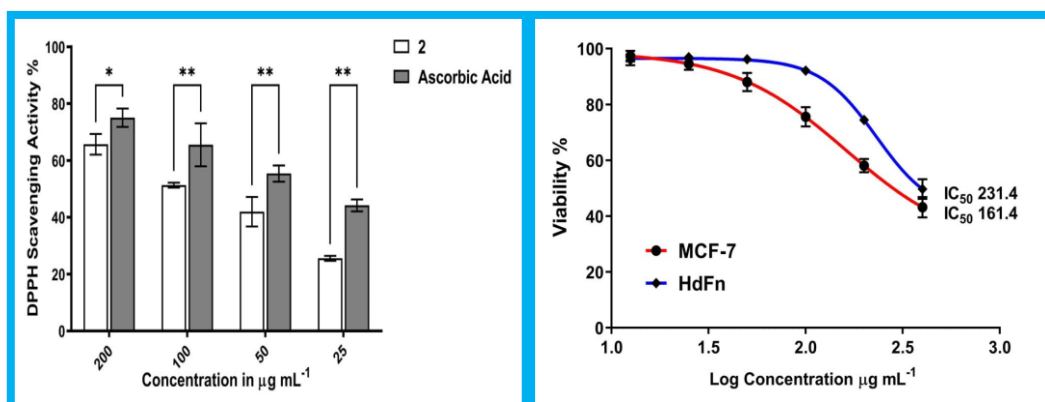


Figure (6): Antioxidant activity for compound (I_{32}) and anticancer efficacy of MCF-7 and HdFn for compound (I_{32})

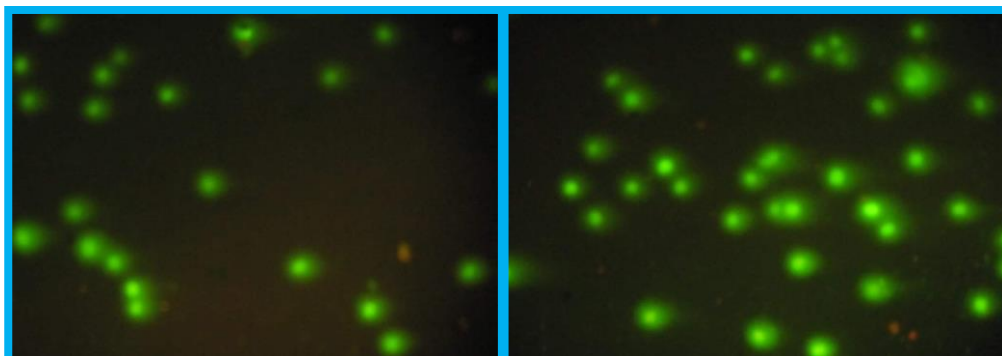


Figure (7): Anticancer efficacy of control sample

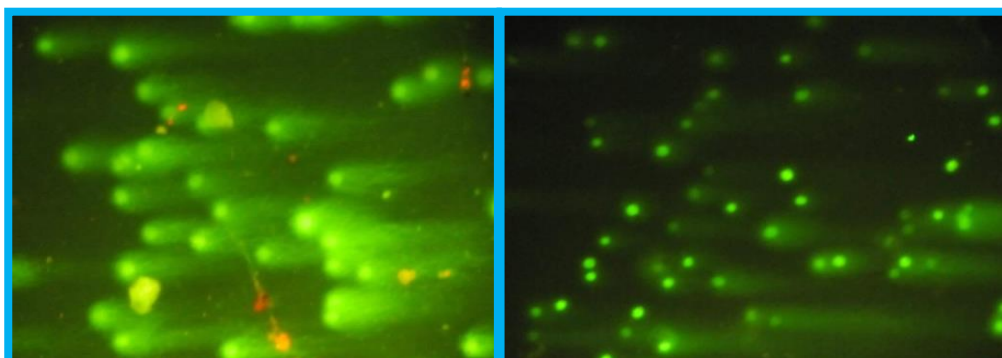


Figure (8): Anticancer efficacy of compound (I_{32})

Conclusions

Through spectroscopic and physical measurements, it was found that the accuracy and validity of the prepared compounds. The biological activity study showed that the compounds had the effect of inhibiting *Gedo* compared to the control sample. (I_{32}) compound gave the highest percentage of 35 mm against *E. coli* bacteria, and the two compounds gave the same percentage of inhibition of 26 mm against *Staphylococcus aureus* bacteria. The results indicate that the

prepared compound (I₃₂) has an excellent scavenging ability. That the free radical scavenging activity of the compound under search increased with increasing concentration exhibiting; for that, the highest free radical scavenging percentage is given with 200 µg/mL of with 80 %, and had the lowest percentage of 40% when 25 µg/mL. The test results for compound (I₃₂) showed good inhibitory activity against (MCF-7) breast cancer cells and normal cell line (HdFn). When calculating the IC₅₀ of the compound (I₃₂) against breast cancer cells was 183.4. Its value was 561.5 against normal cells. When calculating IC₅₀, the results showed significant differences, P<0.0001, when treated with the compound (I₃₂) for breast cancer cells and normal cells.

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