One Pot Synthesis, Quantum Chemical Stimulation and Anticancer Evaluation of Some New 8-Azacoumarin Derivatives

Sameh Rizk (samehrizk@sci.asu.edu.eg)  
Ain Shams University Faculty of Science  
https://orcid.org/0000-0002-8232-5399

Mohamed Megahed  
Ain Shams University Faculty of Science

Monda Badawy  
National center for Radiation research and technology

Mohamed Aly  
Port Said University

Research Article

Keywords: Azacoumarin, Pyrazole, Ionizing radiation, Anticancer Agents, p21 and Caspase-3

DOI: https://doi.org/10.21203/rs.3.rs-192965/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

New anticancer agents are highly needed to overcome cancer cell resistance. Synthesis of newly pyrazole, derivatives via heterocyclic ring opening of azacoumarin promoted with grinding and ultrasonic reaction conditions. Efficient solvent less one pot synthesis can be well progressed to afford the good yield of new heterocyclic products that were characterized by IR, 1H-NMR, MS and micro-analytical data. Anticancer evaluation for the synthesized compounds exhibited good cytotoxicity. The anti-liver cancer activity of all compounds was screened in vitro against hepatocellular carcinoma (HCC) cell lines (HepG-2) by viability assay. The synthesized compounds were evaluated for their anticancer activity and found to exhibit promising activities. All new compounds were tested for possible anti-cancer activity against HepG-2 cell lines in comparison to the reference drug doxorubicin (DOX). Compound 8 was the most active against the liver carcinoma cell line (HepG-2) giving promising half-maximal inhibitory concentration (IC50) value of 27.5 ± 1.3 µg/mL, compared with DOX with IC50 value of 0.36 ± 0.02 µg/mL. However it has weak cytotoxic effects against normal rat hepatocytes with 50% cytotoxic concentration (CC50) = 1820.5 µg/ml (= > 500 µg/ml). Compound 8 was selected to be tested in combination with ionizing gamma radiation. Gene expression levels of the cell cycle inhibitor p21 and caspase-3 was quantified. As well as, Oxidative stress was quantified by measuring the concentration of malondialdehyde (MDA), and antioxidant activity of reduced glutathione (GSH). This study concluded that the new derivative of the azacoumarin compound has an effective anti-cancer effect and it was found that using the new compound with ionizing radiation at a dose of 8 Gy improves the effectiveness of the compound on liver cancer cells.

1. Introduction

Many heterocyclic compounds encircling pyrazole ring are correlated with diverse pharmacological belongings. These classes of heterocyclic compounds have synchronized the cardiovascular system, antimicrobial [1,2,3], anticancer [4], anticonvulsant [5], antiviral [6,7,8], anti-HIV [9], antifungal activity [10] and anti-leishmanial [11]. Pyrano-[2,3-b]-pyridine (8-Azacoumarin) structure is considered a key starting material for many heterocyclic compounds and screening a broad spectrum of biological activity [12]. This prompted us a specific simple synthesis aiming to construct some new pyrazole derivatives in a single molecular framework as a unique key precursor designing new, potent, selective agent appear to be promising for anticancer evaluation. Cancer drug targeting is an effective way to treat malignancies or cancer growths. Chemotherapy can be used either individually or in conjunction with other interventions, such as surgery or radiation. Radiotherapy uses high frequency penetrating waves like x-rays or gamma rays, or particles like proton rays or neutron rays to kill or prevent the replication of cancer cells. A proliferation of naturally occurring coumarin and coumarin-related compounds and their wide therapeutic potential placed them among the most promising drugs [13]. For several years, natural and artificial coumarin derivatives have demonstrated a variety of and important therapeutic potential like antimicrobial, [14] anti-inflammatory [15], analgesic [16], anticancer [17], anticoagulant [18], antioxidant [19], antiHIV [20]. An outsized number of derivatives especially have demonstrated cytotoxic activity both in vitro and in vivo [21,22].

Coumarins can function on different tumor cells depending on their structure. There are various mechanisms to control the cancer cells; among this mechanisms inducing apoptosis and suppress the proliferation of cancer cells by arresting the cell cycle in phase G0 / G1, phase G2 / M [23,24].
Apoptosis is a crucial cytotoxicity condition caused by anticancer drugs\(^{25}\). Apoptosis or programmed cell death is a natural process that maintains a balance between cell proliferation and cell death and plays a regulatory role in regulating cell population size and homeostasis of tissues \(^{26}\). Apoptosis avoidance is known to be one of the hallmarks of cancer cells \(^{27}\). Caspases, a family of proteolytic enzymes is well known to play a crucial role in the apoptotic process. Activating these proteases which are usually present inside cells as inactive zymogens result in the cleavage of several protein substrates inside the cell leading to irreversible apoptotic cell death. Caspase-3 among these is one of the most powerful downstream caspases and is called Caspase Effector \(^{28}\). Also, cyclin-dependent kinase inhibitors p21 and p27 are proteins that bind and inhibit the activity of complexes of CDK2 / cycline E, CDK4 / cycline D1, and/or CDK6 / cycline D1 and thus regulate the progression of the cell cycle at phase G1\(^{29}\). Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. GSH is one of the major endogenous antioxidants produced by the cells, participating directly in the neutralization of free radicals and reactive oxygen compounds, as well as protecting cellular protein thiol groups and maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms \(^{30}\). Therefore, GSH is critical to fight against oxidative stress \(^{31}\). Malondialdehyde (MDA) is a lipid peroxide that can damage the plasma membrane of cells. By measuring its content, we can usually understand the degree of lipid peroxidation in the body, thereby indirectly evaluating the degree of cell damage \(^{32, 33}\).

2. Material And Methods

2.1 Chemistry experimental:

Melting points are uncorrected and measured in open glass capillaries. The FT-IR Shimadzu-8400S Spectrophotometer (USA, New York) in KBr pellets used to chart IR spectra \(\nu_{\text{max}} \text{ cm}^{-1}\). \(^{1}\)H-NMR and \(^{13}\)C-NMR spectra were registered on 300 spectrophotometer Germany, Rheinstetten, 300 and 125MHz respectively in DMSO-\(d_6\) solvents and TMS as internal standard. Using Shimadzu GCMS-QP-1000 EX mass spectrometer (Japan, Kyoto) to measure the mass spectra via EI technique (70 e.v.). CHN automatic analyzer used in elemental analyses and measured at Central forced armed Cairo, Egypt. Sonication (model SW 4 cleaner a Toshcon, 37 KHz, 150 W) was achieved the synthesized compounds. Check the purity of synthesized compounds with TLC. All chemical reagents and solvents were achieved from Ali-baba fine chemical company without further purification.

3.1. 1-([1.1- bi-phenyl]-4-yl)-3-(4-methoxy phenyl) prop-2-en-1-one (1):

Grinding a mixture of (0.01 mol, 1.96 g) of 4-acetyl biphenyl, (0.01 mol, 1.49g) 4- dimethylamino benzaldehyde and 2 g of KOH and few drops of water for 20 m until change color of colorless reaction mixture turned light yellow. Then add 50 mL water to the reaction mixture, the solid that separated was filtered, dried and crystallized from ethanol as light yellow crystals , yield: 98% , m.p:88-90\(^0\)C. IR(KBr): \(\nu\text{ cm}^{-1}\) 1679 for C=O and 1600 C=C. \(^{1}\)H NMR (DMSO-\(d_6\)): \(\delta\) (ppm): 2.99 (s, 6H, -N(CH\(_3\))\(^2\)); 6.8 - 8.02 (m, 13H, Ar-H; biphenyl and phenyl groups);7.82 (dd,1H, H-C=, \(J=16.2\) Hz); 8.02 (dd, 1H, H-C=, \(J=16.2\) Hz). \(^{13}\)C-NMR (DMSO) 41.9, 111.3, 121.5, 128.1, 129.4, 130.1, 136.6, 140.9, 145.3, 146.4, 150.5, 188.9. Ms: m/z (% abundance): 327 M\(^+\), (100 %); 329 [M+2]\(^+\), (3.3%). Anal. For: C\(_{23}\)H\(_{21}\)NO (327). Cal: %C, 84.37; %H, 6.46; %N, 4.28. Found: %C, 84.23; %H, 6.24; %N, 4.12.

3.2. General procedure for synthesis of Azacoumarin derivatives (4a-f)
3.2.1. Method (i): Sonication a mixture of chalcone (1) (1mmol), ethyl cyanoacetate, ethyl acetoacetate or diethyl malonate (1mmol), and ammonium acetate (0.04 mol) together in a mortar by transfer into 10 mL ethanol in RBF that was located in an ultrasonic cleaning bath E\textsubscript{max} measured at 30°C. Reaction progress sustained until disappear the reactants by TLC. Irradiation 20–25 m, afforded yellow solid product, decanted with crushed ice, dried and was recrystallized.

3.2.2. Method (ii): Grinding a mixture of chalcone (1mmol), ethyl cyanoacetate, ethyl acetoacetate or diethylmalonate (1 mmol), and ammonium acetate (0.04 mol) together in an agate mortar and pestle checked by TLC for 25–30 m until the color of reaction mixture turned into yellow, left overnight and was recrystallized from ethanol.

4-Amino-7-(3,4-dichlorophenyl)-5-(4-methoxy phenyl)-2-oxo-2H-pyrano[2,3-b] pyridine-3-carbonitrile (4a): Yield 1.80 g (82%), light yellow finely crystalline, m.p. 176–178 °C. IR (KBr), ν cm\textsuperscript{-1}: 3404, 3324 (NH), 3055 (CH), 2270 (CN), 1734 (CO), MS (m/z) 437. \textsuperscript{1}H-NMR (DMSO-d\textsubscript{6}), δ, ppm, (J, Hz): 7.48–7.86 (12ArH, m, aromatic protons), 8.14 (2H, s, NH\textsubscript{2} which exchanged in D\textsubscript{2}O), and Cal., %: C 60.29, H 2.99, Cl 16.18, N 9.59. for C\textsubscript{22}H\textsubscript{13}Cl\textsubscript{2}N\textsubscript{3}O\textsubscript{3}. Found, %: C 60.07, H 2.73, Cl 16.00, N 9.36.

7-([1,1’-Biphenyl]-4-yl)-4-amino-5-(4-methoxy phenyl)-2-oxo-2H-pyrano[2,3-b] pyridine-3-carbonitrile (4b): Yield 2.08 g (84%), light yellow finely crystalline, m.p. 184–186 °C. IR (KBr), ν cm\textsuperscript{-1}: 3211 (NH), 3045 (ArH), 1720 (C=O), MS (m/z) 445. \textsuperscript{1}H-NMR (DMSO-d\textsubscript{6}), δ, ppm, (J, Hz): δ: 3.35 (s, 3H, OMe), 7.47–8.19 (m, 13H, ArH), 9.41 (1H, s, acidic NH proton which exchanged in D\textsubscript{2}O), and Cal., %: C 75.50, H 4.30, N 9.43, for C\textsubscript{28}H\textsubscript{19}N\textsubscript{3}O\textsubscript{3}. Found, %: C 74.95, H 4.04, N 9.32.

3-Acetyl-7-(3,4-dichlorophenyl)-5-(4-methoxyphenyl)-4-methyl-2H-pyrano[2,3-b] pyridin-2-one (4c): Yield 2.08 g (84%), light yellow finely crystalline, m.p. 184–186 °C. IR (KBr), ν cm\textsuperscript{-1}: 3428 (OH, enol due to intramolecular H bond), 3045 (ArH), 1736, 1676 (C=O), MS (m/z) 453. \textsuperscript{1}H-NMR (DMSO-d\textsubscript{6}), δ, ppm, (J, Hz): δ: 2.28 (s, 3H, Me), 2.67 (s,3H, CH\textsubscript{3}), 7.47–8.19 (m, 12H, ArH), 9.41 (1H, s, acidic OH proton which exchanged in D\textsubscript{2}O), and found, %: C 63.45, H 3.77, Cl 15.16, N 3.08, for C\textsubscript{24}H\textsubscript{17}Cl\textsubscript{2}NO\textsubscript{4}. Calculated, %: C 63.15, H 3.54, N Cl 14.92, N 3.00.

7-([1,1’-Biphenyl]-4-yl)-3-acetyl-5-(4-methoxyphenyl)-4-methyl-2H-pyrano[2,3-b] pyridin-2-one (4d): Yield 1.2 g (71%), light yellow finely crystalline, m.p. 176–178 °C. IR (KBr), ν cm\textsuperscript{-1}: 3479 (OH enol due to intramolecular H bond), 3045 (ArH), 1732, 1669 (C=O), \textsuperscript{1}H-NMR (DMSO-d\textsubscript{6}), δ, ppm, (J, Hz): δ: 1.21 (t, 3H, CH\textsubscript{3}), 4.14 (q, 2H, CH\textsubscript{2}), 7.63–8.54 (m, 12H, ArH), 9.41 (1H, s, acidic OH proton which exchanged in D\textsubscript{2}O), and found, %: C 78.08, H 5.02, N 3.03 for C\textsubscript{30}H\textsubscript{23}NO\textsubscript{4}. Calculated, %: C 71.52, H 4.22, N 3.09.

Ethyl 7-(3,4-dichlorophenyl)-4-hydroxy-5-(4-methoxyphenyl)-2-oxo-2H-pyrano[2,3-b] pyridine-3-carboxylate (4e): Yield 2.08 g (84%), light yellow finely crystalline, m.p. 184–186 °C. IR (KBr), ν cm\textsuperscript{-1}: 3316 (OH), 3045 (ArH), 1733, 1725, 1674 (C=O), \textsuperscript{1}H-NMR (DMSO-d\textsubscript{6}), δ, ppm, (J, Hz): δ: 2.28 (s, 3H, Me), 2.67 (s,3H, CH\textsubscript{3}), 7.47–8.19 (m, 12H, ArH), 9.41 (1H, s, acidic OH proton which exchanged in D\textsubscript{2}O), and found, %: C 59.28, H 3.52, Cl 14.58, N 3.08, for C\textsubscript{24}H\textsubscript{17}Cl\textsubscript{2}NO\textsubscript{6}. Found, %: C 63.15, H 3.54, N Cl 14.92, N 3.00.

Ethyl 7-([1,1’-biphenyl]-4-yl)-4-hydroxy-5-(4-methoxyphenyl)-2-oxo-2H-pyrano[2,3-b] pyridine-3-carboxylate (4f): Yield 1.2 g (71%), light yellow finely crystalline, m.p. 176–178 °C. IR (KBr), ν cm\textsuperscript{-1}: 3411 (OH), 3045 (ArH), 1733
(C=O), $^1$H-NMR (DMSO-d$_6$), $\delta$, ppm, (J, Hz): $\delta$: 1.21 (t, 3H, CH$_3$), 4.14 (q, 2H, CH$_2$), 7.63–8.54 (m, 12H, ArH), 9.41 (1H, s, acidic OH proton which exchanged in D$_2$O), and Calculated %: C 73.01, H 4.70, N 2.84 for C$_{30}$H$_{23}$NO$_6$. Found, %: C 72.52, H 4.22, N 2.59.

3-(Amino(3-amino-5-oxo-1,5-dihydro-4H-pyrazol-4-ylidene) methyl)-6-(3,4-dichlorophenyl)-4-(4-methoxyphenyl) pyridin-2(1H)-one (5a): Yield 1.80 g (77%), light yellow finely crystalline, m.p. 176–178 °C. IR (KBr), $\nu$ cm$^{-1}$: 3468, 3345 (NH), 3055 (CH), 2220 (CN), 1742 (CO), 1613 (C=N). $^1$H-NMR (DMSO-d$_6$), $\delta$, ppm, (J, Hz): 7.48–7.86 (12ArH, m, aromatic protons), 8.14 (2H, s, NH$_2$ which exchanged in D$_2$O), and Cal., %: C 56.18, H 3.64, Cl 15.08, N 14.89. for C$_{22}$H$_{17}$Cl$_2$N$_5$O$_3$. Found, %: C 56.07, H 3.43, Cl 14.90, N 14.36.

6-(3,4-Dichlorophenyl)-3-(1-(3-hydroxy-5-methyl-4H-pyrazol-4-ylidene) ethyl)-4-(4-methoxyphenyl) pyridin-2(1H)-one (5b): Yield 2.21 g (82%), light yellow finely crystalline, m.p. 184–186 °C. IR (KBr), $\nu$, cm$^{-1}$: 3045 (ArH), 1744, 1674 (C=O), MS (m/z) 445. $^1$H-NMR (DMSO-d$_6$), $\delta$, ppm, (J, Hz): $\delta$: 3.35 (s, 3H, OMe), 7.47–8.19 (m, 13H, ArH), 9.41 (2H, s, acidic NH proton which exchanged in D$_2$O), and Cal., %: C 61.55, H 4.09, Cl 15.14, N 8.97 for C$_{24}$H$_{19}$Cl$_2$N$_3$O$_3$. Found, %: C 61.35, H 3.82, Cl 14.92, N 8.73.

4-(6-(3,4-dichlorophenyl)-4-(4-methoxyphenyl)-2-oxo-1,2-dihydropyridin-3-yl)-6-methyl-2,5-dihydro-3H-pyrazolo[3,4-d] pyrimidin-3-one (6): Yield 1.45 g (68%), light yellow finely crystalline, m.p. 176–178 °C. IR (KBr), $\nu$ cm$^{-1}$: 3468, 3345 (NH), 3055 (CH), 1742 (CO), 1613 (C=N). $^1$H-NMR (DMSO-d$_6$), $\delta$, ppm, (J, Hz): 2.54 (s, 1H, CH$_3$), 6.80 (s, 1H, PyH), 7.48–7.86 (7ArH, m, aromatic protons), 8.14, 10.02 (2H, s, 2NH which exchanged in D$_2$O), 12.22 (s, 1H, OH which exchanged in D$_2$O), and Cal., %: C 58.31, H 3.47, Cl 14.34, N 14.17. for C$_{24}$H$_{17}$Cl$_2$N$_5$O$_3$. Found, %: C 56.07, H 3.43, Cl 14.90, N 14.36.

2.2 Biological evaluation:

Cell Culture:

Liver Cancer cell line of human origin HepG2 (human hepatocellular carcinoma) Cells were routinely cultured in DMEM media (Lonza), supplemented with 10%FBS (Lonza), 1%100u/ml penicillin and 100ug/ml streptomycin (Lonza) in a humidified incubator at 37 °C with an atmosphere containing 5%CO$_2$. Every experiment was carried out in triplicate. At 85% confluence cells were harvested using 0.25% trypsin and were subculture into 75 cm$^2$ and six–well plates or 96–well plates according to selection of experiments. Cells were allowed to attach to the surface for 24 hours prior to treatment. 8-Azacoumarin derivatives were dissolved in DMSO and diluted to appropriate concentrations.

Cytotoxicity evaluation against HepG-2 cell line:

The viability of control and treated cells were evaluated using the MTT assay in triplicate. MTT assay is a laboratory test and a standard colorimetric assay (an assay which measures changes in color) for measuring cellular growth, Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) was reduced to purpleformazan in the mitochondria of living cells. A solubilization solution (dimethyl sulfoxide) was added to dissolving the insoluble purple formazan product into a colored solution. Briefly, HepG2 (hepatocellular carcinoma cell line) was seeded in 96-well plates containing 100µl of the growth medium at a density of (1x10$^4$)
cells/well. Cells were permitted to adhere for 24h till confluence, washed with PBS, and then treated with different concentration of compounds in fresh maintenance medium from 500 to 15.63 µg and incubated at 37°C for 24h. A control of untreated cells was made in the absence of the test compound. Untreated cells used as negative control. Serial two-fold dilutions of the tested compounds were added into a 96-well tissue culture plate using a multichannel pipette (Eppendorff, Germany). After treatment (24h), the culture supernatant was replaced by fresh medium. Then, the cells in each well were incubated at 37°C with 100µl of MTT solution (5mg/ml) for 4h. After the end of incubation, the MTT solution was removed, and then 100µl of DMSO was added to each well. The absorbance was detected at 570 nm using a microplate reader (SunRise TECAN, Inc, USA) [34].

**Gamma Irradiation with the derivative:**

Gamma irradiation was performed at the National Centre for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Cairo, Egypt, using Canadian Gamma Cell-40 biological irradiator (137 Cesium), manufactured by the Atomic Energy of Canada Limited, Ontario, Canada. The radiation dose rate was 0.61 Gy/min at the time of exposure. HepG2 cells were irradiated with 8 Gy with or without the selected derivative. The dose calculated according to the Dosimeter department in the NCRRT.

**Quantification of p21 and caspase-3 gene expression & Oxidative stress evaluation:** The parameters were estimated in the following groups:

1. Control (HepG-2 cells without treatment).
2. IC50 x 2 (HepG-2 cells treated with IC50 x 2 = 55 µg/ml, then the cells were harvested after two time intervals 24 hr and 48 hr).
3. IC50 (HepG-2 cells treated with IC50 = 27.5 µg/ml, then the cells were harvested after two time intervals 24 hr and 48 hr).
4. ½ IC50 (HepG-2 cells treated with IC50 = 13.75 µg/ml, then the cells were harvested after two time intervals 24 hr and 48 hr).
5. RAD (HepG-2 cells irradiated with 8 Gy, then the cells were harvested after two time intervals 24 hr and 48 hr).
6. RAD + IC50 x2 (HepG-2 cells irradiated with 8 Gy + treated with IC50 = 55 µg/ml, then the cells were harvested after two time intervals 24 hr and 48 hr).
7. RAD + IC50 (HepG-2 cells irradiated with 8 Gy + treated with IC50 = 27.5 µg/ml, then the cells were harvested after two time intervals 24 hr and 48 hr).
8. RAD + ½ IC50 (HepG-2 cells irradiated with 8 Gy + treated with IC50 = 13.75 µg/ml, then the cells were harvested after two time intervals 24 hr and 48 hr).

**Real-time polymerase chain reaction (RT-PCR):**

To analyze gene expression of the cell cycle inhibitor p21 and caspase-3. Total RNA was extracted from the collected cell suspension using RNeasy Mini Kit (Qiagen, Cat. No. 74104) according to the manufacturer’s instructions. First strand complementary DNA (cDNA) synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen, Cat. No. 205311) according to the manufacturer’s instructions using 1µg RNA as a
RT-PCR were performed in a thermal cycler step one plus (Applied Biosystems, USA) using the Sequence Detection Software (PE Biosystems, CA). The primers utilized in these experiments are listed in Table 2. The reaction mixture of total volume 25 μl was consisting of 2X SYBR Green PCR Master Mix (Qiagen, Cat. No. 204143), 900 nM of each primer and 2μL of cDNA. PCR thermal-cycling conditions included an initial step at 95°C for 5 min; 40 cycles at 95°C for 20s, 60°C for 30s, and 72°C for 20s. The relative expression of the real-time reverse transcriptase PCR products was determined by the ΔΔCt method. This method calculates a relative expression to housekeeping gene using the equation: fold induction = 2-(ΔΔCt). Where ΔΔ Ct=Ct [gene of interest (unknown sample)-Cthousekeeping gene (unknown sample)] - [Ct gene of interest (calibrator sample) - Ct housekeeping gene (calibrator sample)] [35].

**Determination of plasma Malondialdehyde (MDA):**

Lipid peroxidation in cells was measured by spectrophotometric analysis of MDA based on its reaction with thiobarbituric acid using Colorimetric kit supplied by biodiaganostic, 29 El-Tahrer St. - Dokki- Giza – Egypt.

**Determination of Reduced Glutathione (GSH):**

GSH was assayed based upon the development of a relatively stable yellow color when 5,5'-dithiobis-(2-nitrobenzoic acid) is added to sulphdril compounds using Colorimetric kit supplied by biodiaganostic, 29 El-Tahrer St. - Dokki- Giza – Egypt.

Table 2: primer sequences for the genes amplified.

| Gene                          | Strand | Sequence 5—3                | Product length (bp) | Ref. Seq.    |
|-------------------------------|--------|-----------------------------|--------------------|--------------|
| cyclin-dependent kinase inhibitor p21 | F      | GCACAAGGGGTACAAGACAGTG      | 220                | NM_001291549 |
|                               | R      | CGATGGGAACCTTCGACCTTTGCA    |                    |              |
| caspase 3 (CASP3)             | F      | AGA ACT GGA CTG TGG CAT TGA G | 191                | NM_004346    |
|                               | R      | GCT TGT CGG CAT ACT GTT TCA G |                    |              |
| GAPDH                         | F      | AGGGGCCATCCACAGTCTTTC       | 258                | NM_001357943 |
|                               | R      | AGAAGGCTGGGGCTCATTG         |                    |              |

3. Result And Discussion

3.1 Chemistry

It was previously reported that the thermal and microwave reflux of chalcone 1 with diversity of active methylene for instance ethyl cyanoacetate, ethyl acetoacetate and diethyl malonate with ammonium acetate produced the pyridine esters 2a-c and 2-pyridone derivatives 3a-c in good yield that outlined in Scheme 1 [43-48].

Moreover, the authors can be reported the reaction of chalcone 1, ethylcyanoacetate in the presence of ammonium acetate in a multicomponent reaction (MCR) by mechano-grindstone all together or sonication using...
(THF) for 25-30 minutes afforded crude yellow solid products (4a-c). (Scheme 2). [49-55].

The IR spectrum of compound 4a reveals stretching absorption bands at 3434, 3329 cm\(^{-1}\) attributed to asymmetric and symmetric NH\(_2\) respectively, 2223 cm\(^{-1}\) for CN group and 1732 cm\(^{-1}\) for C=O group of the coumarin ring that assigned structure to this compound. The \(^1\)H-NMR spectrum of compound 4a shows \(\delta\) 6.80 ppm corresponding to H3 in pyridine nucleus and broad singlet at \(\delta\) 5.43 ppm corresponding to the NH\(_2\) protons with absence any band at 9.8 corresponding to NH of pyridine 3 are good evidence in formation of compound 4a. So, the ultrasonic and microwave irradiation cause via the isomerization of the 2-pyridone derivative 3 (not pyridine ester 2) to the reactive lactim intermediate which reacts with another ethyl cyanoacetate affording the pyrano[2,3-b] pyridine derivatives 4a-c as a sole product (Scheme 2). Reactivity of the 3-cyano pyridine-2-one derivatives were more than the pyridine ester quantum chemical parameter [18] as outlined in supplementary file. Reaction of azacoumarin 4a with various nitrogen precursors such as hydrazine hydrate yielded interesting pyrazole 5a incorporating heterocyclic compounds that have classy antibacterial activity (Scheme 3). Moreover, reaction of azacoumarin 4b with hydrazine hydrate afforded pyrazole 5b that outlined by the mass fragmentation to confirm the structure.

Reaction of the pyrazole 5a with boiling acetic anhydride is extremely flavored in the Z-configuration \(\rightleftharpoons\) E-configuration dynamic equilibrium as in the following scheme to afford the 1,3-diamino derivatives as intermediate. Bimolecular nucleophilic substitution (SN\(^2\)) of one of the amino groups followed by cyclization with the other amino group to afford the pyrazolo-pyrimidine derivative 6 (Scheme 4).

The appearance of broad bands at 3347-3179 attributed to OH and NH groups, 1668-1652 for carbonyl of amide groups in pyrazole moiety. The lower of hydroxy and carbonyl bands were due to intramolecular hydrogen bond which are respectable confirmation for the assigned structures of these compounds. \(^1\)H-NMR spectrum of compound 6 exhibits signals at \(\delta\) 6.80 ppm for H3 of pyridine proton, 12.22 ppm for broad singlet for acidic OH proton and signal at \(\delta\) 9.80 ppm of singlet NH proton.

### 3.2 Anticancer Activity

The results showed that the new azacoumarin derivative was able to cause cell cycle arrest at least partly through down-regulation the expression level of the cell cycle inhibitor p21 and induced cancer cell apoptosis via caspase-3 dependent pathway. The results indicated that the irradiation plus the derivative is more effective than the derivative only. The irradiation reinforced the effect of the derivative. As the lower dose from the derivative plus irradiation is more effective than the higher dose of derivative only. Many coumarin derivatives cause caspase dependent apoptosis [36]. P21 is recognized as a potent cyclin-dependent kinase inhibitor that facilitates cell-cycle arrest by interacting with different stimuli such as p53 and DNA repair process. P21 acts both as a tumor-suppressor gene and an inhibitor of apoptosis by interacting with various molecules and transition factors [37]. In fact, prolonged treatments of cancer cells with DNA damaging agents, determine a p21 inactivation mediated by caspase-3 cleavage and reduction of p21 levels resulted in apoptosis [38]. Caspase-3 is the most frequently activated cysteine protease, which plays a vital role in both intrinsic and extrinsic apoptotic pathways. Thus, caspase-3 has been recognized as a reliable molecular biomarker for cell apoptosis [39].

While, the results of the Oxidative stress markers (GSH and MDA) showed that GSH increase in the cells treated with the azacoumarin derivative only, but decrease with irradiation. However, the cells irradiated and treated with
the azacoumarin derivative showed higher GSH in compare with cells exposed to the ionizing radiation only. GSH is critical to fight against oxidative stress \(^{[40]}\). On the other hand, MDA decrease in the cells treated with the azacoumarin derivative only, but increase with irradiation. However, the cells irradiated and treated with the azacoumarin derivative showed lower MDA in compare with cells exposed to the ionizing radiation only. MDA indirectly evaluating the degree of cell damage \(^{[41, 42]}\).

**Table 3:** *In vitro* anticancer activity of azacoumarin derivatives 2,3,8,10 and doxorubicin as stander drug against HepG-2 (human hepatocellular carcinoma) Cells.

| Compounds | IC50(µg/ml) |
|-----------|-------------|
| 1b        | >500        |
| 4a        | >500        |
| 5b        | 27.5 ± 1.3  |
| 6         | >500        |
| Doxorubicin | 0.36±0.02   |

As Compound 6 was the most active against the liver carcinoma cell line (HepG-2) its cytotoxicity was evaluated against normal rat hepatocytes. it showed weak cytotoxic effects using MTT assay under the same experimental conditions for 48 hrs incubation with CC50 = 1820.5 µg/ml (≥ > 500 µg/ml).

**Table 4:** p21 gene expression, caspase-3 gene expression, GSH content and MDA in different groups after 24 hr and 48 hr.
| Parameters | P21 (fold) | Caspase 3 (fold) | GSH (mmol / g. tissue) | MDA (nmol / g.tissue) |
|------------|------------|-----------------|-----------------------|-----------------------|
| Groups     | 24 hr      | 48 hr           | 24 hr                 | 48 hr                 | 24 hr      | 48 hr   | 24 hr      | 48 hr   |
| Control    | 1.0±0.02   | 1.0±0.01        | 1.0±0.05              | 1.0±0.04              | 57±15     | 73±14   | 114±4.2   | 140±16  |
| IC50 x2    | 0.30±0.13*** | 0.17±0.07***   | 5.6±1.3***            | 7.3±1.3***            | 112±21**  | 135±16*** | 50±11**   | 55±22** |
| IC50       | 0.42±0.14*** | 0.32±0.03***   | 5.3±0.81***           | 6.8±1.2*              | 109±20**  | 127±14*** | 54±11**   | 66±3.5** |
| ½ IC50     | 0.54±0.06*** | 0.48±0.12***   | 3.4±0.31*             | 4.3±1.3               | 101±8.7*  | 116±9.4** | 70±10*    | 75±14* |
| RAD        | 0.26±0.12*** | 0.27±0.08***   | 7.0±0.85***           | 6.2±2.7*              | 32±3.9    | 45±15   | 218±23*** | 228±24** |
| RAD + IC50 | 0.14±0.04*** | 0.15±0.05***   | 9.7±0.72***           | 12±1.8***             | 79±3.0    | 116±12** | 154±18   | 151±33  |
| RAD + ½ IC50 | 0.17±0.06*** | 0.25±0.09***   | 8.6±1.2***            | 8.7±2.1***            | 65±8.7    | 85±2.7  | 182±2.9*** | 182±16  |
| RAD + IC50 | 0.25±0.04*** | 0.39±0.06***   | 8.0±0.62***           | 8.4±1.8**             | 68±2.4    | 52±4.2  | 183±24*** | 163±17  |

Each value represents the mean ± standard deviation.

* Significant difference versus control group at p≤0.05.

### 3.3 DFT study

The optimization of the heterocyclic compounds is designed as minimized energetic geometrical structures of the synthesized compounds 5 and 6 (Fig 1). The electronic structures of three compounds 5 and 6 revealed entirely spread over all molecular structure and confirmed these energetic structures of synthesized compounds. Frontier molecular orbitals have the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) [56–59].

### Conclusions

Synthesis and anticancer evaluation of some novel 8-azacoumarin and pyrazole derivatives can be achieved. The most potent derivative 8 showed much better activity was produced via simple, two-step, and ecofriendly synthetic protocols. Qualified study was concerning the result density function theory (DFT) has thru on grinding and ultrasound-assisted tools. All azacoumarin and pyrazole structures can be elucidated by elemental and spectral data. The new derivatives of the azacoumarin and pyrazole have an effective anti-cancer effect and it
was found that using the new compounds with ionizing radiation at a dose of 8 Gy improves the effectiveness of the compound on liver cancer cells. The new azacoumarin and pyrazole derivatives was able to cause cell cycle arrest at least partly by down regulating the expression level of the cell cycle inhibitor p21 and up regulating caspase 3 Inducing cancer cell apoptosis via caspase-3 dependent pathway.

**Declarations**

**Author Contributions**

The listed authors contributed to this work as described in the following: Sameh A. Rizk and Monda M. M. Badawy and Mohamed R. Aly gave the concepts of the work, interpreted the results and prepared the manuscript, Mohamed S. Megahed carried out the synthetic work, cooperated in the preparation of the manuscript results. All authors read and approved the final manuscript.

**Acknowledgement**

The authors look forward to sincere gratitude to Ain Shams University and STDF project No. 37139

**Conflict of interest**

The authors declare that they have no competing interests.

**References**

1. Patel, N. B., Agravat, S. N., & Shaikh, F. M. (2011). Synthesis and antimicrobial activity of new pyridine derivatives-I. Medicinal Chemistry Research, 20(7), 1033-1041.
2. Patel, N. B., & Agravat, S. N. (2009). Synthesis and antimicrobial studies of new pyridine derivatives. Chemistry of heterocyclic compounds, 45(11), 1343-1353.
3. Ali, T. E. S. (2009). Synthesis of some novel pyrazolo [3, 4-b] pyridine and pyrazolo [3, 4-d] pyrimidine derivatives bearing 5, 6-diphenyl-1, 2, 4-triazine moiety as potential antimicrobial agents. European journal of medicinal chemistry, 44(11), 4385-4392.
4. Srivastava, A., & Pandeya, S. N. (2011). Indole: A versatile nucleus in pharmaceutical field. Int. J. Curr. Pharm. Res. Rev, 4, 5-8.
5. Paronikyan, E. G., Noravyan, A. S., Dzhagatspanyan, I. A., Nazaryan, I. M., & Paronikyan, R. G. (2002). Synthesis and anticonvulsant activity of isothiazolo [5, 4-b] pyrano (thiopyrano)[4, 3-d] pyridine and isothiazolo [4, 5-b]-2, 7-naphthyridine derivatives. Pharmaceutical Chemistry Journal, 36(9), 465-467.
6. Bernardino, A. M. R., de Azevedo, A. R., da Silva Pinheiro, L. C., Borges, J. C., Carvalho, V. L., Miranda, M. D., ... & de Frugulhetti, I. C. P. P. (2007). Synthesis and antiviral activity of new 4-(phenylamino)/4-[(methylpyridin-2-yl) amino]-1-phenyl-1H-pyrazolo [3, 4-b] pyridine-4-carboxylic acids derivatives. Medicinal Chemistry Research, 16(7-9), 352-369.
7. De Clercq, E. (2005). Recent highlights in the development of new antiviral drugs. Current opinion in microbiology, 8(5), 552-560.
8. Eizuru, Y. (2003). Development of new antivirals for herpesviruses. Antiviral Chemistry and Chemotherapy, 14(6), 299-308.
9. Tucker, T. J., Sisko, J. T., Tynebor, R. M., Williams, T. M., Felock, P. J., Flynn, J. A., ... & Yan, Y. (2008). Discovery of 3-{5-[(6-amino-1 H-pyrazolo [3, 4-b] pyridine-3-yl) methoxy]-2-chlorophenoxy}-5-chlorobenzonitrile (MK-4965): a potent, orally bioavailable HIV-1 non-nucleoside reverse transcriptase inhibitor with improved potency against key mutant viruses. Journal of medicinal chemistry, 51(20), 6503-6511.

10. Mamolo, M. G., Zampieri, D., Falagiani, V., Vio, L., Fermeglia, M., Ferrone, M., ... & Scialino, G. (2004). Antifungal and antimycobacterial activity of new N1-[1-aryl-2-(1H-imidazol-1-yl and 1H-1, 2, 4-triazol-1-yl)-ethylidene]-pyridine-2-carboxamidrazone derivatives: a combined experimental and computational approach. Arkivoc, 231, 250.

11. de Mello, H., Echevarria, A., Bernardino, A. M., Canto-Cavalheiro, M., & Leon, L. L. (2004). Antileishmanial pyrazolopyridine derivatives: synthesis and structure− activity relationship analysis. Journal of medicinal chemistry, 47(22), 5427-5432.

12. Gangjee, A., Adair, O., & Queener, S. F. (1999). Pneumocystis carinii and Toxoplasma gondii Dihydrofolate Reductase Inhibitors and Antitumor Agents: Synthesis and Biological Activities of 2, 4-Diamino-5-methyl-6-[(monosubstituted anilino) methyl]-pyrido [2, 3-d] pyrimidines. Journal of medicinal chemistry, 42(13), 2447-2455.

13. Al-Bayati RI, Hussain Al-Amiery AA, Al-Majedy YK. Design, synthesis and bioassay of novel coumarins. J. AfricanPure and Applied Chemistry. 2010;4:74- 86.

14. Behrami A, Krasniqi I. Antibacterial activity of coumarine derivatives synthesized from8-amino-4,7-dihydroxymphen-2-one and comparison with standard drug, J of Chemical and Pharm Res. 2012;4:2495-2508.

15. Togna AR, Firuzi O, Latina V, Parmar VS,Prasad AK, Salemme A, Togna Gl. L. Saso 4-Methylcoumarin derivatives with anti-inflammatory effects in activated microglial cells. Biol Pharm Bull. 2014;37:60-66.

16. Sivakumar KK, Rajasekaran A, Senthilkumar P, Wattamwar PP. Conventional and microwave-assisted synthesis of pyrazolone Mannich bases possessing anti-inflammatory, analgesic, ulcerogenic and antimicrobial properties. Bioorganic and Medicinal Chemistry Letters. 2014;24:2940-2944.

17. Cao D, Liu Y, Yan W, Wang C, et al. Design, synthesis, and evaluation of invitro and in vivo anticancer activity of 4-substituted coumarins: a completely unique class of potent tubulin polymerization inhibitors J. Med. Chem. 2016;59:5721–5739.

18. Weigt S, Huebler N, Strecker R, Braunbeck T, Broschard T, Reprod H. Developmental effects of coumarin and the anticoagulant coumarin derivative warfarin on zebrafish (Danio rerio) embryos. Reproductive Toxicol. 2012;33:133–141.

19. Witaicenis A, Seito LN, Chaqas A Silveira, de almeida LD, Jr. Luchini AC, et al. Antioxidant and intestinal anti-inflammatory effects of plant derived coumarin derivatives (Phytomedicine 15). 2014;21:240-246.

20. Kirkiacharian S, Thuy DT, Sicsic S, Bakhchinian R, Kurkjian R, Tonnaire T. Structure–activity relationships of some 3-substituted-4-hydroxycoumarins as HIV-1 protease inhibitors. J. Il Farmaco. 2002;57:703–708.

21. Nida N, Farshori MR, Banday AA, AsadUK, Rauf A. 7-Hydroxy-coumarin derivatives: Synthesis, characterization and preliminary antimicrobial activities. Med. Chem. Res. 2011;20:535–541.

22. Fioravanti S, Pellacani L, Tardella PA, Vergari MC. Facile and highly stereo selective one-pot synthesis of either(E) or (Z)-Nitro Alkenes. Org. Lett.2008;10:1449–1451.
23. Amin KM, Eissa AM, Abou-Seri SM, Awadallah FM, Hassan GS. Synthesis and biological evaluation of novel coumarinpyrazoline hybrids endowed with phenylsulfonyl moiety as antitumor agents. Eur. J. Med. Chem. 2013;60:187-198.

24. Nasr T, Bondock S, Youns M. Anticancer activity of new coumarin substituted hydrazide-hydrazone derivatives. Eur. J. Med. Chem. 2014;76:539-548.

25. Kim, R., Tanabe, K., Uchida, Y., Emi, M., Inoue, H., & Toge, T. (2002). Current status of the molecular mechanisms of anticancer drug-induced apoptosis. Cancer chemotherapy and pharmacology, 50(5), 343-352.

26. Vermes, I., Haanen, C., & Reutelingsperger, C. (2000). Flow cytometry of apoptotic cell death. Journal of immunological methods, 243(1), 167-190.

27. Thornberry, N. A. (1998). Caspases: key mediators of apoptosis. Chemistry & biology, 5(5), R97-R103.

28. Gartel, A. L., & Radhakrishnan, S. K. (2005). Lost in transcription: p21 repression, mechanisms, and consequences. Cancer research, 65(10), 3980-3985.

29. Hanahan, D. & Weinberg R. A. (2000). The hallmarks of cancer. Cell 100: 57–70.

30. Scharf, G., Prustomersky, S., Knasmüller, S., Schulte-Hermann, R., & Huber, W. W. (2003). Enhancement of glutathione and γ-glutamylcysteine synthetase, the rate limiting enzyme of glutathione synthesis, by chemoprotective plant-derived food and beverage components in the human hepatoma cell line HepG2. Nutrition and cancer, 45(1), 74-83.

31. Martin, M. A., Ramos, S., Mateos, R., Granado Serrano, A. B., Izquierdo-Pulido, M., Bravo, L., & Goya, L. (2008). Protection of human HepG2 cells against oxidative stress by cocoa phenolic extract. Journal of agricultural and food chemistry, 56(17), 7765-7772.

32. Song D., (2020). Effects of nalmefene combined with hyperbaric oxygen therapy on serum levels of S100β protein, hypoxi-inducible factor-1α, malondialdehyde and lactate in acute severe craniocerebral injury[J]. Anhui Medical and Pharmaceutical Journal ,28(1):47-50.

33. Ji Z S,Wu Q,Liu X Y., (2019). The application of epalrestat in the protection of MDA,SOD,TAC and endothelial function in early diabetic retinopathy[J]. Chinese Journal of Health Care and Medicine, 21(6):514-517.

34. Wilson, A. P. (2000). Cytotoxicity and viability assays. Animal cell culture: a practical approach, 3, 175-219.

35. Livak, K. J., Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. methods. 25(4), 402-408.

36. Kaur, M., Kohli, S., Sandhu, S., Bansal, Y., & Bansal, G. (2015). Coumarin: a promising scaffold for anticancer agents. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 15(8), 1032-1048.

37. Parveen, A., Akash, M. S. H., Rehman, K., & Kyunn, W. W. (2016). Dual role of p21 in the progression of cancer and its treatment. Critical Reviews™ in Eukaryotic Gene Expression, 26(1).

38. Zhang, Y., Fujita, N., & Tsuruo, T. (1999). Caspase-mediated cleavage of p21 Waf1/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. Oncogene, 18(5), 1131-1138.

39. Khalilzadeh, B., Shadjou, N., Charoudeh, H. N., & Rashidi, M. R. (2017). Recent advances in electrochemical and electrochemiluminescence based determination of the activity of caspase-3. Microchimica Acta, 184(10), 3651-3662.
40. Martin, M. A., Ramos, S., Mateos, R., Granado Serrano, A. B., Izquierdo-Pulido, M., Bravo, L., & Goya, L. (2008). Protection of human HepG2 cells against oxidative stress by cocoa phenolic extract. Journal of agricultural and food chemistry, 56(17), 7765-7772.

41. Song, D. (2020). Effects of nalmefene combined with hyperbaric oxygen therapy on serum levels of S100β protein, hypoxi-inducible factor-1α, malondialdehyde and lactate in acute severe craniocerebral injury[J]. Anhui Medical and Pharmaceutical Journal, 28(1): 47-50.

42. Ji, Z., Wu, Q., Liu, X. Y. (2019). The application of epalrestat in the protection of MDA, SOD, TAC and endothelial function in early diabetic retinopathy[J]. Chinese Journal of Health Care and Medicine, 21(6): 514-517.

43. Rizk, S. A.; Shaban, S. A Facile One-pot Synthesis and Anticancer Evaluation of Interesting Pyrazole and Pyrimidinethione via Heterocyclic Interconversion (2019). J. Het. Chem., 56, 2379.

44. El-Hashash, M. A.; Rizk, S. A. (2016) Regioselective Diastereomeric Michael Adducts as Building Blocks in Heterocyclic Synthesis. J. Het. Chem, 53,1236-1240.

45. El-Hashash, M.; Rizk, S.; A burzeza, M. Utility of p-Acetamidobenzoyl Prop-2-enolic Acid in the Synthesis of New α-Amino Acids and Using Them as Building Blocks in Heterocyclic Synthesis (2011) Egypt J Chem, 54, 299.

46. Rizk, S. A.; Abdelwahab, S. S.; Elrazaz, E. (2019) Synthesis and QSAR Study of Some Novel Heterocyclic Derivatives as In Vitro Cytotoxic Agents J. Het. Chem., 56, 443.

47. Rizk, S.; El-Hashash, M.; Mostafa, K. (2008) Utility of β-aroyl acrylic acid in heterocyclic synthesis Egypt J. Chem., 51(5), 611.

48. Rizk, S. A.; El-Hashash, M.; Elbadawy A.A. (2017) Ultrasonic and Grinding Aptitudes of One-Pot Synthesis of 5-(4-Chlorophenyl)-7-(3,4-Dimethyl Phenyl)-2-oxo-2H-Pyrano[2,3-b]Pyridine Derivatives as Antibacterial Agents J. Het. Chem., 54, 2003.

49. Rizk, S.A. Abdelwahab, S.S. Sallam, H.A. (2018). Regioselective Reactions, Spectroscopic Characterization, and Cytotoxic Evaluation of Spiro-pyrrolidine Thiophene. J. Het. Chem. 55 1604.

50. El-Hashash, M.; Rizk, S. A.; El-Badawy A. (2018) Ultrasonic Aptitude of Regioselective Reaction of 6-bromo-spiro-3,1-benzoxazinone2,1'-isobenzofuran-3',4-dione Towards Some Electrophilic and Nucleophilic Reagents. J. Het. Chem, 55, 2090-2098.

51. Fahmy, A.F.M., Rizk, S.A., Hemdan, M.M., El-Sayed, A.A., Hassaballah, A.I. (2018) Efficient Green Synthesis and Computational Chemical Study of Some Interesting Heterocyclic Derivatives as Insecticidal Agents. J. Het. Chem., 55, 2545-2555.

52. Rizk S.A.; El-Sayed G.A.; El-Hashash, M. (2018) One-pot synthesis, spectroscopic characterization and DFT study of novel 8-azacoumarin derivatives as eco-friendly insecticidal agents. J. Iranian Chem. Soc. 15, 2093–2105.

53. Attia, S.K.; El-Gendy, A.T.; Rizk, S.A. (2019) Efficient green synthesis of antioxidant azacoumarin dye bearing spiro-pyrrolidine for enhancing electro-optical properties of perovskite solar cells. J. Mol. Struct. 1184, 583.

54. El-Hashash, M.A.; Rizk, S.A.; El-Naggar, A.M.; El-Bana, M.G. (2017) Regiospecific Isomerization of 2-Benzoxazinon-2-yl Benzoic Acid Toward Some Nitrogen Nucleophiles as Environmental Insecticide J. Het. Chem. 54, 3716-3724.

55. El-Hashash, M.A.; Darwish, K.M.; Rizk, S.A.; El-Bassiouny, F.A. (2011) The uses of 2-ethoxy-(4H)-3,1-benzoxazin-4-one in the synthesis of some quinazolinone derivatives of antimicrobial activity.
Pharmaceuticals, 4(7), 1032-1051.

56. Elgendy, A.T, Youssef, A, Rizk, S. A, (2020). Which Energetically Favorable Sustainable Synthesis of 4-amino-8-azacoumarin ester or 4-hydroxy-3-cyano derivative Based on New Exact Kinetic Arrhenius and DFT Stimulation. J. Iranian Chem. Soc. 17, 1001.

57. El-Hashash, M.A.; Rizk, S.A.; El-Bassiouny, F.A.; Guirguis, D.B.; Khairy, S.M.; Guirguis, (2017). Facile Synthesis and Structural Characterization of Some Phthalazin-1(2H)-one Derivatives as Antimicrobial Nucleosides and Reactive Dye. Egypt J. Chem. 60, 407.

58. Elkholy, A.E., Rizk, S.A., Rashad, A.M. (2019) Enhancing lubricating oil properties using novel quinazolinone derivatives: DFT study and molecular dynamics simulation. J. Mol. Struct., 1175, 788.

59. Heakal, F. E.; Attia, S.K.; Rizk, S.A.; Abou Essa, M.A.; Elkholy, A.E. (2017) Synthesis, characterization and computational chemical study of novel pyrazole derivatives as anticorrosion and antiscalant agents. J. Mol. Struct. 1147, 714.