ejpmr, 2018,5(8), 99-120

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

SYNTHESIS, REACTIVITY, ANTI-OXIDANT AND CYTOTOXICITY EVALUATION OF SOME COUMARIN DERIVATIVES

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Article Received on 05/06/2018 **Article Revised on 25/06/2018** **Article Accepted on 15/07/2018**

ABSTRACT

In the present work 3-amino-8-methoxy coumarin was prepared either by microwave irradiation or by convential method. It was used as a key starting material for the synthesis of triazole thione **(8)**, Schiffs' bases **(10)**, thiazolone **(11a-d)**, pyrimidinethione **(13)**, aminothiazole **(15)** and isoxazole **(18)** derivatives through its reaction with different reagents. The structures of the newly synthesized compounds were elucidated on the basis of their elemental analysis and by using different spectroscopic methods. The free radical scavenging activity of some newly synthesized derivatives was determined by measuring their interaction with the stable free radical DPPH and some compounds have shown encouraging antioxidant activity. Also, they were screened for their *in-vitro* cytotoxicity against two human cancer cell lines namely HEIA cells (human cervical carcinoma) and A-549 cells (human lung cancer cell line). The IC_{50} values (the sample concentration that produces 50% reduction in cell growth) in µg/mL showed that some of the tested compounds exhibited significant cytotoxic effect.

KEYWORDS: Coumarins, Microwave assisted, Green chemistry, Antioxidant, Cytotoxicity.

INTRODUCTION

Coumarins are one of the most important classes of heterocycles that occupy prime position in synthesis and pharmaceutical chemistry due to their diverse applications. They display a large number of biological properties such as, anti-bacterial[1,4], anti-tumoral[5,6] and anti-HIV[7,10] as well as their utility as useful synthons. uht,Thus, they have attracted researches to work on this moiety. Some derivatives of coumarin have been proven to be active as anti-inflammatory[11] and anti-depressant.[12] Moreover, coumarin derivatives were used as inhibitors of lipoxygenase (LOX) and cyclooxygenase (COX) pathways of arachidonic acid metabolism.[13] Coumarins belong to the family of lactones having a benzopyrone system that can be isolated from plants, as well as total synthesis, which can be carried out in the laboratory (Fig. 1).

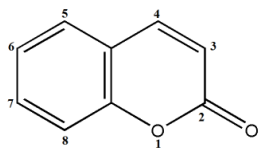
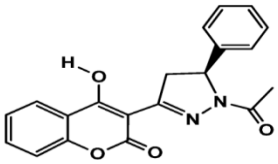


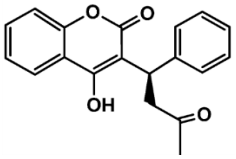
Fig. 1.

Coumarins are also used as optical brighteners[14], dispersed fluorescent and laser dyes.[15] Optical applications of coumarin derivatives such as laser dyes[16], non- linear optical chromophores fluorescents[17], whiteners' fluorescent probes[18], optical recording[19], and solar energy collectors[20] were widely investigated.

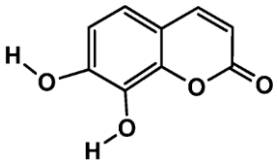
Antioxidants possess the ability to protect the cellular organelles from damage caused by free radicals induced oxidative stress. Free radicals used include hydroxyl radical, super oxide anion radical, and hydrogen peroxide. Highly reactive free radicals, which are formed by exogenous chemicals stress or in the food system, are capable of oxidizing biomolecules resulting in cancer, coronary heart disease, and hypertension.[21] Generally, most of the free radicals generated from metabolism are scavenged by endogenous defense system, such as catalase, superoxide dismutase, and peroxidase glutathione system.[22] Despite numerous attempts to search for more effective antitumor agents, coumarins still remain as one of the most versatile class of compounds against cancer cell lines. Dihydropyrazole-substituted benzopyran-2-one was identified as a novel class of MEK1 Kinase inhibitor[23], also, warfarin reduces efficiently metastases from intestinal carcinomas[24], and is also used as an adjunct in the surgical treatment of malignant tumors.[25] In addition, daphentin was found to inhibit tyrosin kinase, epidermal growth factor receptor, serine/threonine- specific protein kinase, and protein kinase C *in-vitro*[26], **(Fig. 2)**.



Dihydropyrazole-substituted benzopyran-2-one



Warfarin



Daphentin

Fig. 2.

The preparation of 3-amino-8-methoxy coumarin (**2)** which was used as a starting material of compounds (**3-20)** is discussed in this study and the chemical structures of the proposed products were conducted and confirmed. The *in-vitro* antioxidant as well as cytotoxic activity of some of the synthesized compounds are investigated and the reaction consequence of the newly prepared derivatives is outlined in **Schemes 1&2**. The objective of this investigation is to prepare some new 3-substituted coumarin derivatives and evaluate their antioxidant as well as cytotoxic activity. Because there is a need for novel therapeutic agents, and the structural variety of coumarins leads to compounds displaying multiple pharmacological and biological properties[27,30], we report herein a synthesis of substituted coumarin analogue compounds; study their reactivity towards some electrophilic as well as nucleophilic reagents. Also, invistigating the potential role of some of the synthesized coumarins as antioxidants and cytotoxic agents.

RESULTS AND DISCUSSION

Chemistry

In continuation of our work to synthesize poly functionalized biologically active heterocyclic compounds[31,33], we investigated the use of 3-amino-8-methoxy coumarin **(2)** to synthesize pyrazole, triazole, thiazolidinone, 2-thioxo pyrimidine, 2-amino thiazole, and 3-imino isoxazole derivatives incorporating coumarin moiety. Thus, 3-amino-8-methoxy coumarin **(2)** is the key starting material for the new derivatives synthesized in this work. The data for the minimized geometry and the 3D-geometrical structure of 3-amino-8-methoxy coumarin **(Fig.3)** show that the aromatic charges have been affected by the presence of the ring substituents at both the α-pyrone and the homocyclic ring as shown in **Table 1**.

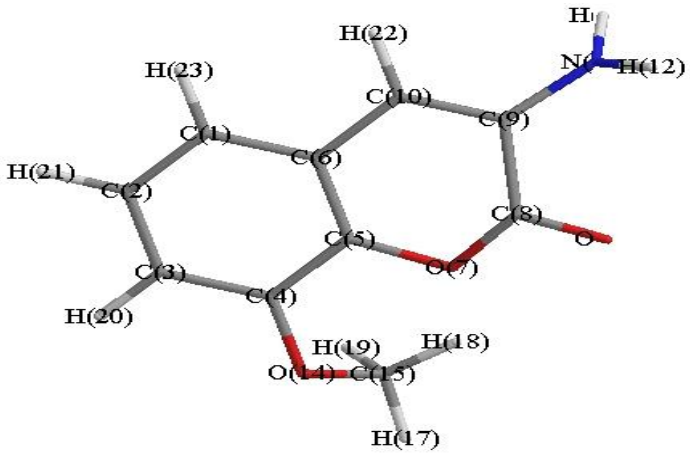


Fig. 3: The 3D structure of 3-amino-8-methoxy coumarin.

Table 1: The atomic charges of 3-amino-8-methoxy coumarin.

Atom	Charge	Atom	Charge
C(1)	-0.11972	H(13)	0.105839
C(2)	-0.0750142	O(14)	-0.238429
C(3)	-0.151828	C(15)	0.0770208
C(4)	0.225202	O(16)	-0.677439
C(5)	0.184082	H(17)	0.0285909
C(6)	-0.0259666	H(18)	0.0356774
O(7)	0.0450762	H(19)	0.0225088
C(8)	0.541649	H(20)	0.0301319
C(9)	0.079538	H(21)	0.0263765
C(10)	-0.137641	H(22)	0.0224557
N(11)	-0.126729	H(23)	0.0244871
H(12)	0.104132		

The data obtained show that the highest atomaic charge in 3-amino-8-methoxy coumarin molecule is located at [O(16) -0.677439], while the next highest charge values at [C(8) 0.541649] and [O(14) -0.238429]. These data values show clearly that oxygen atom [O(16)] is the most reactive center. The determined 3D geometrical structure **(Fig.3)** and stereochemistry of [C(9)-C(10):(E)], indicate that this molecule is planer. Also, it is clear from the above data that there is a strong permanent dipole caused by the greater electronegativity of the oxygen atom [O(16)] of the carbonyl group [C(8)]. To a lesser extent, the more electronegative nitrogen atom [C(9)-N(11)] also causes inductive polarization mainly of the σ bond. Thus, 3-amino-8-methoxy coumarin is a molecule with essentially bonds in which both inductive and mesomeric effect work in opposite directions in a dipole, the negative end of which is on the oxygen [O(16)] (c.f. **Fig. 4**).

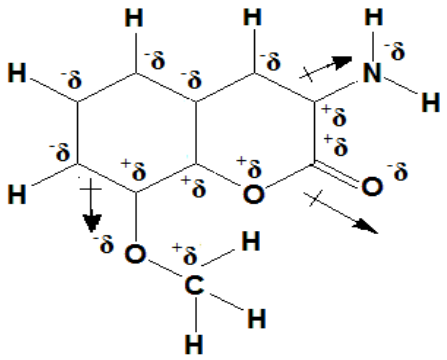


Fig. 4: Charges on the atoms.

The formal positive charge on the oxygen atom [O(7)] of the hetero ring [O(7) 0.0450762], however must interact quite strongly with the aromatic orbital system and reduces its stabilizing effect. Furthermore, the positive charge is delocalized on the ring carbons both mesomerically and inductively. The positive fractional charges have been located mainly on C4, C5, O7, C8 and C9.

The negative ends of the dipole moment in the hetero ring are at the [N(11)] and the [O(16) (of the carbonyl group)]. This polarization is the consequence of the inductive pull of the more electronegative heteroatoms, this inductive or σ-moment is operating towards the heteroatoms [N(11), O(16)] but superimposed on it the mesomeric moment, mainly involving the π-electron system and operating in the opposite direction. Thus, it is important to realize fully that the excess negative charge placed on the carbons [C(6)] and [C(10)] (in the hetero ring) for the excess negative charge refers to the π-electrons, and not the electronic system as a whole.

The desired coumarin derivative (**2**) was synthesized using both microwave irradiation method as a green approach, as well as the conventional method. Microwave heating offers several advantages over conventional technique. These include the dramatic reduction of time and efficiency in internal heating of the reactants, and this leads to induce the completion of the chemical transformations to few minutes or even seconds, whereas, under conventional conditions, several hours or days may be required. Microwave-assisted synthesis increases the product yields and enhances product purities via reducing undesired side-reactions. Moreover, it is synonymous with green chemistry and is especially adapted to organic synthesis resulting in very efficient and clean procedures.[34,38]

A possible mechanism for the formation of **2** may be explained as follows in **Fig.5.**

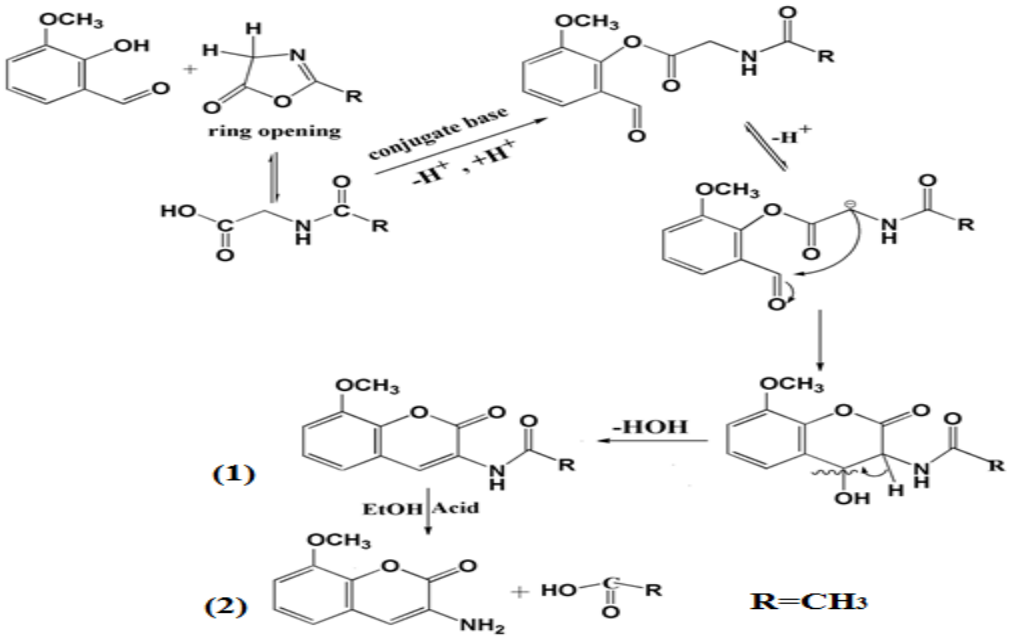


Fig. 5.

A variety of 3-subsistuted coumarin derivatives **(3-20)** have been synthesized using the classical approaches in solution, whereas the starting material **1** has been synthesized in short time (5 min) under solvent-free conditions and in an excellent yield (≈86%) whereas traditional method was found to be expensive and not time-consuming (6h). The structures of the obtained derivatives **(1-20)** were confirmed on the basis of their elemental analysis and spectral data (MS, IR, NMR). Thus, the analytical data for **1** revealed a molecular formula $C_{12}H_{11}O_4N$, m/z 233/M $^{+\bullet}$; IR showed stretching absorption at υ 1732 cm^{-1} assigned for the presence of δ-lactone, another stretching absorption band appeared at 1674 cm^{-1} corresponding to υ C=O. ^{1}H-NMR (DMSO-d_6) spectrum of **1** showed a singlet at δ 3.34 ppm corresponding to the three protons of the methoxy group attached to C-8 of the coumarin ring. Another singlet at δ 7.16 ppm, corresponding to the proton at C-4 of the coumarin ring. Also signals appeared at 7.35-7.46 ppm as a multiplet corresponding to the three aromatic protons. The three protons of the $COCH_3$ group appeared at δ 2.91 ppm as a singlet, while the NH proton appeared as a singlet at δ9.39 ppm (D_2O exchangeable). ^{13}C-NMR (DMSO-d_6) showed signal at δ 157 ppm assigned for C-2 coumarin and the carbonyl carbon of the acetamido group appeared at δ 188.1 ppm. Compound **2** was obtained by refluxing **1** with concentrated HCl-ethanol mixture. The IR spectrum of 3-amino-8-methoxy coumarin (**2)** revealed a band at υ 3407 cm^{-1} due to the amino group (c.f. **Table 5; Fig. 5**). The ^{1}H-NMR spectrum (DMSO-d_6) showed signals at δ 9.92 ppm due to the protons of the amino group, another signal at δ 7.12 due to the proton at C-4 of the coumarin ring and a multiplet at δ 7.19-7.29 for the three aromatic protons (c.f. **Fig. 7**). ^{13}C-NMR (DMSO-d_6) showed signals at δ 159.8 ppm for the coumarin C-2 (c.f. **Fig. 8**). Moreover, the mass spectrum and elemental analysis data also supported the structure assigned for the compound **2** as the analytical data revealed a molecular formula $C_{10}H_9O_3N$. MS:m/z 191/M $^{+\bullet}$. Now, we started a series of reactions to explore the reactivity of 3-amino-8-methoxy coumarin **(2)** as a nucleophile, towards a verity of electrophiles. Thus, interaction of 3-aminocoumarin derivative (**2)** with nitrous acid at 0°C gave the corresponding coumarin-3-yl-diazonium chloride (**3)** which was coupled with active methylene compounds namely, ethyl acetoacetate, malononitrile and/or ethyl cyanoacetate to give a novel series of 8-methoxy coumarin-3-yl-diazenyl substituents (**4**,**5)** and (**7)** derivatives respectively. The IR spectrum of **4** showed absorption band at υ 1732 cm^{-1} assigned for δ lactone, an absorption band at 1688 cm^{-1} corresponding to υ C=O of the ester. The ^{1}H-NMR spectrum (DMSO-d_6) of compound **4** showed signals at δ 1.31 as a triplet for the methyl ester protons, δ 2.19 ppm a singlet for the three protons of the acetyl group, a singlet at δ 3.44 ppm for the methine proton, at δ 3.39 ppm a singlet for the three protons of the methoxy group and at δ 4.31 ppm a quartet for the methylene group of the ester. It also showed a singlet at δ 7.15 ppm for the proton at C-4 of the coumarin ring and a multiplet at δ 7.49-7.81 ppm for the three aromatic protons. The structure assigned for compound **5** was in agreement with the analytical data and the MS spectrum which revealed a molecular formula $C_{13}H_8O_3N_4$ with m/z 268/M $^{+\bullet}$. The IR spectrum of **7** revealed absorption bands at 2222, 1732, 1689 cm^{-1} corresponding to υ C≡N, δ-lactone and C=O respectively. The ^{1}H-NMR spectrum (DMSO-d_6) showed of **7** a triplet at δ 1.32 ppm for the three protons of the methyl ester, at δ 3.37 ppm a singlet for the three protons of the methoxy group, a quartet at 4.31 ppm for the methylene protons of the ester, at δ 7.07 ppm a singlet for the proton at C-4 of the coumarin ring and a multiplet at δ 7.48-7.69 ppm for the three aromatic protons. Hydrazinolysis of the 2-[2-(methoxy coumarin-3-yl) hydrazono] malononitrile derivative (**5)** in boiling ethanol yielded the corresponding phenol via opening of the α-prone ring and formation of the 3,5-di amino pyrazole derivative (**6)**. The IR spectrum of **6** showed a strong absorption band at 3377 cm^{-1} for NH_2 and 1679cm^{-1} for the secondary amide group CONH. The analytical data for 6 revealed molecular formula $C_{13}H_{16}O_3N_8$, (332); MS:m/z M+2 $^{+\ddagger}$ (c.f. **Fig 9**).

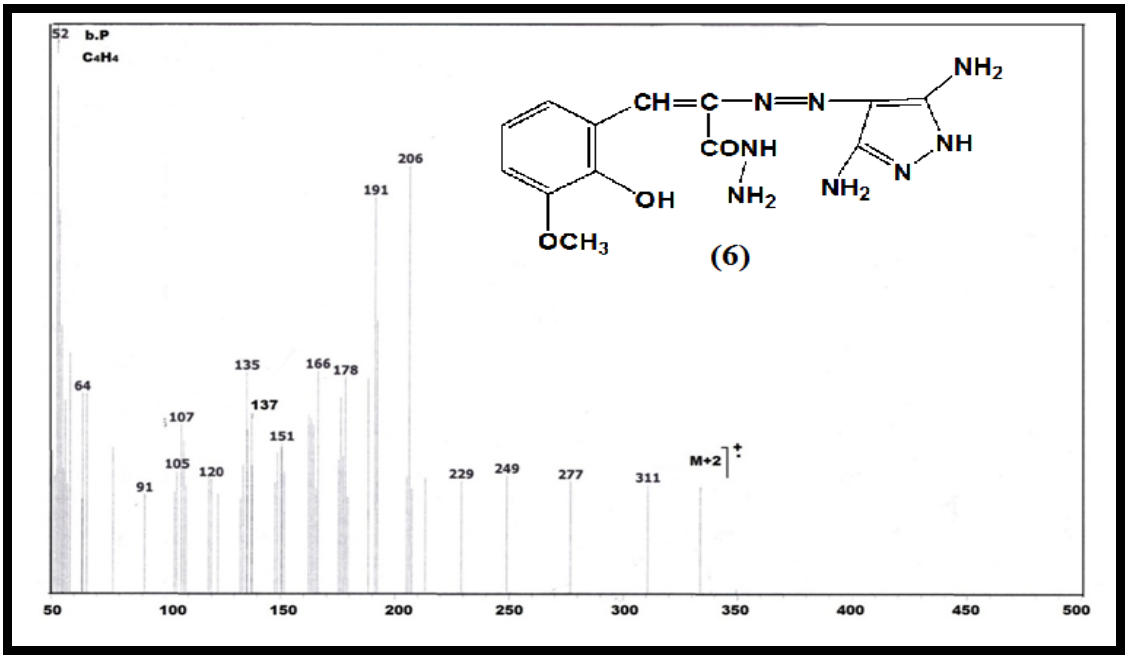


Fig. 9: Mass spectrum of compound 6.

On the other hand, the reaction of ethyl-2-cyano-2(8-methoxy coumarin-3-yl) diazenyl acetate (**7)** with thiosemicarbazide in boiling ethanol containing sodium acetate gave the corresponding coumarin-3-diazenyl-1,2,4-triazolyl-5-thioxo-3-acetonitrile derivative (**8)**. The IR spectrum of **8** showed the δ-lactone absorption band at 1731 cm^{-1}, 2221 cm^{-1} for υ C≡N and at 3225 cm^{-1} for υ NH group. ^{1}H-NMR spectrum (DMSO-d_6) of compound **8** showed a singlet at δ 2.95 ppm for the methine proton and another singlet δ 7.13 ppm for the proton at C-4 of the coumarin ring and a multiplet at δ 7.37-7.83 ppm for the three aromatic protons. In a similar manner, coupling the coumarin-3-yl-diazonium chloride derivative **(3)** with 2-phenyl indole yielded 8-methoxy-3-yl-[(2-phenyl-1H-indol-3-yl) diazenyl] coumarin (**9)**. IR spectrum of **9** revealed a band at 3307 cm^{-1} for the NH group. In addition ^{1}H-NMR spectrum (DMSO-d_6) showed signals at δ 7.14 ppm a singlet for the CH proton at C-4 of the coumarin ring, a multiplet δ 7.60-8.13 ppm for the twelve aromatic protons and a singlet at δ 10.11 ppm for the NH proton of the indol moiety (D_2O-exchangeable). ^{13}C-NMR (DMSO-d_6) showed signal at δ 56.7 ppm assigned for the methoxy carbon and the coumarin C-2 appeared at δ 158.6 ppm. Moreover, mass spectrum and elemental analysis data also supported the structure assigned for **9** as the molecular formula $C_{24}H_{17}O_3N_3$ MS:m/z 395 [M $^{+}$]$^{\cdot}$.(c.f. **Scheme 1**).

Next, we moved to studying the reactivity of compound **2** towards aromatic aldehydes for the formation of Schiffs' bases **(Scheme 2)**. Thus, the reaction of **2** with aromatic aldehydes namely 2-chloro-, 2-nitro-benzaldehyde, terphthaldehyde, and furfural in boiling glacial acetic acid-ethanol mixture yielded the azomethine derivatives **(10a-d)** respectively. The structure of compounds **(10a-d)** was based on analytical and spectral data. Thus, the IR spectrum of **10a-d** revealed stretching absorption in the region 1734-1723 cm^{-1}, 1626-1620 cm^{-1}, 1524-1514 cm^{-1} for υ δ-lactone, υ N=CH and υ C=C respectively. The ^{1}H-NMR (DMSO-d_6) for compound **10a** (as an example) showed the presence of a singlet at δ 3.37 ppm, corresponding to –OCH_3 group, coumarin H-4 at δ 7.09 ppm, a multiplet at δ 7.34-7.86 ppm for the seven aromatic protons, and a singlet at δ 8.11 ppm corresponding to the azomethine proton. Moreover, the ^{13}C-NMR spectrum showed the presence of azomethine (HC=N) at δ 164.3 ppm, the O-C (of the methoxy) at δ 156.3 ppm, the C=O of α-pyrone ring at δ 158.3 ppm and the C-Cl at δ 137.7 ppm along with the signals for coumarin and phenyl carbons. Chemically the reaction of the Schiffs' bases **(10a-d)** with mercapto acetic acid in boiling ethanol afforded the corresponding 2-arylthiazolidin-4-one-3-yl-coumarin-3-yl derivatives **(11a-d)** respectively. The structures of **11a-d** were established on the basis of their respective analytical and spectral data. The IR spectra revealed the presence of δ- lactone and carbonyl group at the region 1737-1734 cm^{-1} and 1671-1709 cm^{-1} respectively. The ^{1}H-NMR of **11b** showed signals at δ 3.39 ppm for the –OCH_3 as a singlet, another singlet at δ 5.91 ppm for N-CH-S, at δ 7.03 ppm a singlet for the coumarin H-4 and a multiplet for the seven protons at δ 7.39-7.89 ppm. The presence of the amino group at the C-3 in coumarin moiety showed interesting reactivity towards active methylene compounds, such as ethyl acetoacetate and/or chloroacetyl chloride. Thus, the reaction of compound **2** with ethyl acetoacetate in boiling ethanol and/or with chloroacetyl chloride in boiling chloroform gave N-(8-methoxy coumarin-3-yl)-3-oxobutamide**(12)**and/or 2-chloro-N-(8-methoxy coumarin-3-yl) acetamide **(14)** respectively, the structures of which were based on analytical and spectral data. Compound **12** underwent heterocyclization reaction through its reaction with

thiourea in boiling methanol containing 10% of aqueous sodium hydroxide to give 8-methoxy coumarin-3-yl-(6-methyl-2-thioxo-2,3-dihydropyrimidin-4-yl amine) **(13)**. Similary, compound **14** underwent cyclization to the corresponding 8-methoxy coumarin-3-yl-(2-amino thiazol-4-yl) amine **(15)**. Furthermore, the amino moiety present in compound **2** showed high reactivity towards electrophilic displacement. Thus, compound **2** reacted with ethyl cyanoacetate in the presence of fused sodium acetate to give the corresponding 2-cyanoacetamide derivative **(16)** which was reacted with alcoholic hydroxylamine hydrochloride containing fused sodium acetate to give the corresponding hydroxyimino propanamide derivative **(17)**. Compound **17** underwent heterocyclization reaction through its heating gently with acetic anhydride to give the corresponding 3-amino isoxazolyl derivative **(18)**.

Moreover, the reaction of **2** with ammonium thiocyanate in boiling ethanol containing concentrated hydrochloric acid afforded the corresponding thiourea derivative **(19)**. The reaction of **19** with ethyl chloroacetate in boiling glacial acetic acid containing a catalytic amount of anhydrous sodium acetate afforded the corresponding chloroacetyl thiourea **(20)**,(c.f.**Scheme 2)**, and **Tables 4-7** for the physical data of compounds.

Biology
Antioxidant Activity
Radical Scavenging Activity
DPPH assay
The 2,2'-diphenyl-1-picryl hydrazyl (DPPH) radical assay was used to screen for the antioxidant activity of compounds. DPPH is a stable free radical containing an odd electron in its structure and is usually used for detection of the radical scavenging activity in chemical compounds as the DPPH is a product of the reaction between DPPH * and an antioxidant (AH):

DPPH* + AH ⟶ DPPHH + A*

The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm indicated by antioxidants.[39]

Free radical scavenging activity of the tested compounds **2, 7, 11d, 17 and 20** was studied by the DPPH (2,2-diphenyl-1-picryl hydrazyl) radical assay method.[40] Methanolic solution of the synthesized compounds (1.5 mL, 0.2 m moL^{-1}) was added to 1.5 mL (0.2 m moL^{-1}) solution of DPPH radical in methanol (final concentration of DPPH and synthesized compounds was 0.1 m mol^{-1}). The mixture was shacked vigorously allowed to stand for 30 min, absorbance of the DPPH solution at 517 nm was determined spectro photometrically before ($A_{control}$) and 15 minutes after adding the solution of the compounds (A_{test}) and the percentage of scavenging activity was calculated. Ascorbic acid was used as the reference compound. All tests and analyses were done in three replicates and the results were averaged. Results are presented in (**Table 2** and **Fig.10)**.

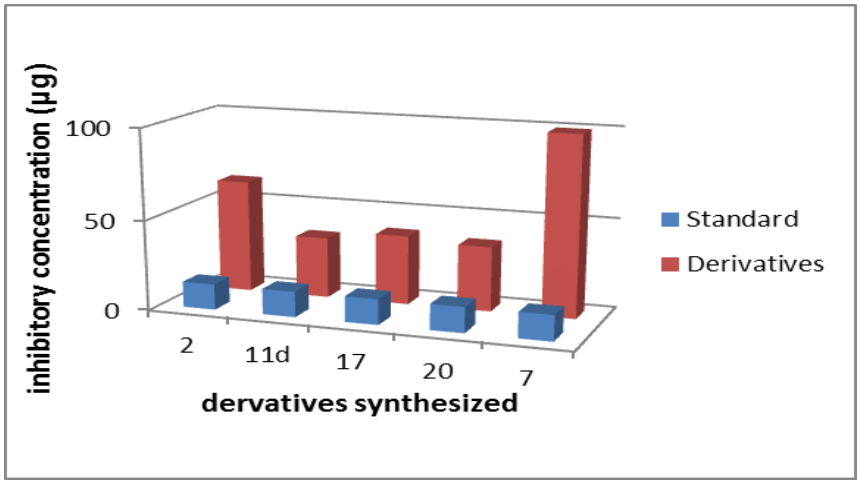


Fig. 10: Antioxidant minimum inhibitory concentration of the tested compounds.

The DPPH assay is based on assessing the substances ability to reduce the stable radical diphenyl picryl hydrazyl to diphenyl picryl hydrazine. The DPPH free radical, bearing an odd electron gives a strong absorption maximum at λ 517 nm (purple colour). When the odd electron of DPPH radical pairs with a hydrogen atom from an antioxidant substance, the reduced form DPPH-H is created and the colour is turned from purple to yellow[41], scavenging activity of ascorbic acid was also assayed for comparison. (c.f. **Fig. 11**).

The percentage of DPPH radical scavenger was calculated using the equation:

Scavenging effect (%) = [($A_{control} - A_{test}$) /$A_{control}$) × 100]

Where $A_{control}$ is the absorbance of the control sample (DPPH solution without test sample) and A_{test} is the absorbance of the test sample (DPPH solution + test compound).

From the results obtained, it was clear that compound **11d** showed an antioxidant activity with IC_{50} 33.7 µg compound **20** showed an antioxidant activity with IC_{50} 35.9 µg, and compound **17** showed an antioxidant activity with IC_{50} 38.4 µg. On the other hand compound **2** showed an antioxidant activity with IC_{50} 62.5 µg, while compound **7** showed an antioxidant activity with IC_{50} 99.6 µg; whereas the reference standard ascorbic acid antioxidant activity with IC_{50} 14.2 µg. (c.f. **Table 2**).

Table 2: The IC_{50} of the antioxidant activity of the tested compounds 2, 7, 11d, 17 and 20.

Compd. No.	IC_{50}
2	**62.5**
7	**99.6**
11d	**33.7**
17	**38.4**
20	**35.9**
Ascorbic acid	**14.2**

From the results obtained, it is clear that the compounds **11d** and **20** with IC_{50} 33.7 and 35.9µg respectively are close to the IC_{50} of the standard reference ascorbic acid with IC_{50} 14.2 µg, while compound **17** with IC_{50} 38.4 µg is moderately lesser active and compounds **2** and **7** with IC_{50} 62.5 and 99.6 µg respectively are lesser and least activity. A possible mechanism that can explain the antioxidant effect of the coumarin derivatives tested is related to the keto-enol forms present in each one. The enol form is capable to easily donate the hydrogen[42] (c.f. **Fig. 11**).

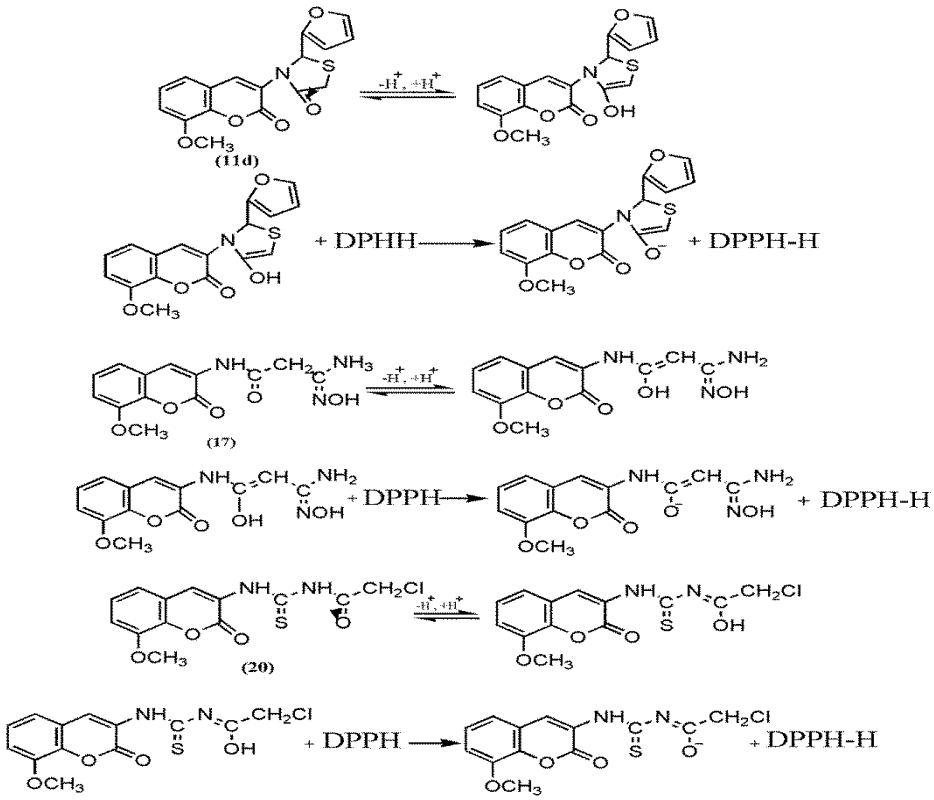


Fig. 11: Mechanism of the reaction between DPPH and antioxidants.

Compound **11d** showed activity close to ascorbic acid in DPPH assay indicating better free radical scavenging capacity. The other compounds **20** and **17** exhibited good scavenging activity compared to ascorbic acid. This presence in special aspect of the scavenging reactivity of 1,3-thiazolidin-4-one **(11d)** as it is related to the coenzyme thiamin which is believed to involve firstly the keto-enol form necessary for the DPPH scavenging; followed by the formation of thiazolium ylid, a reaction very probably involves the intermediacy of a C2 ylid and this can exert extra DPPH scavenging.

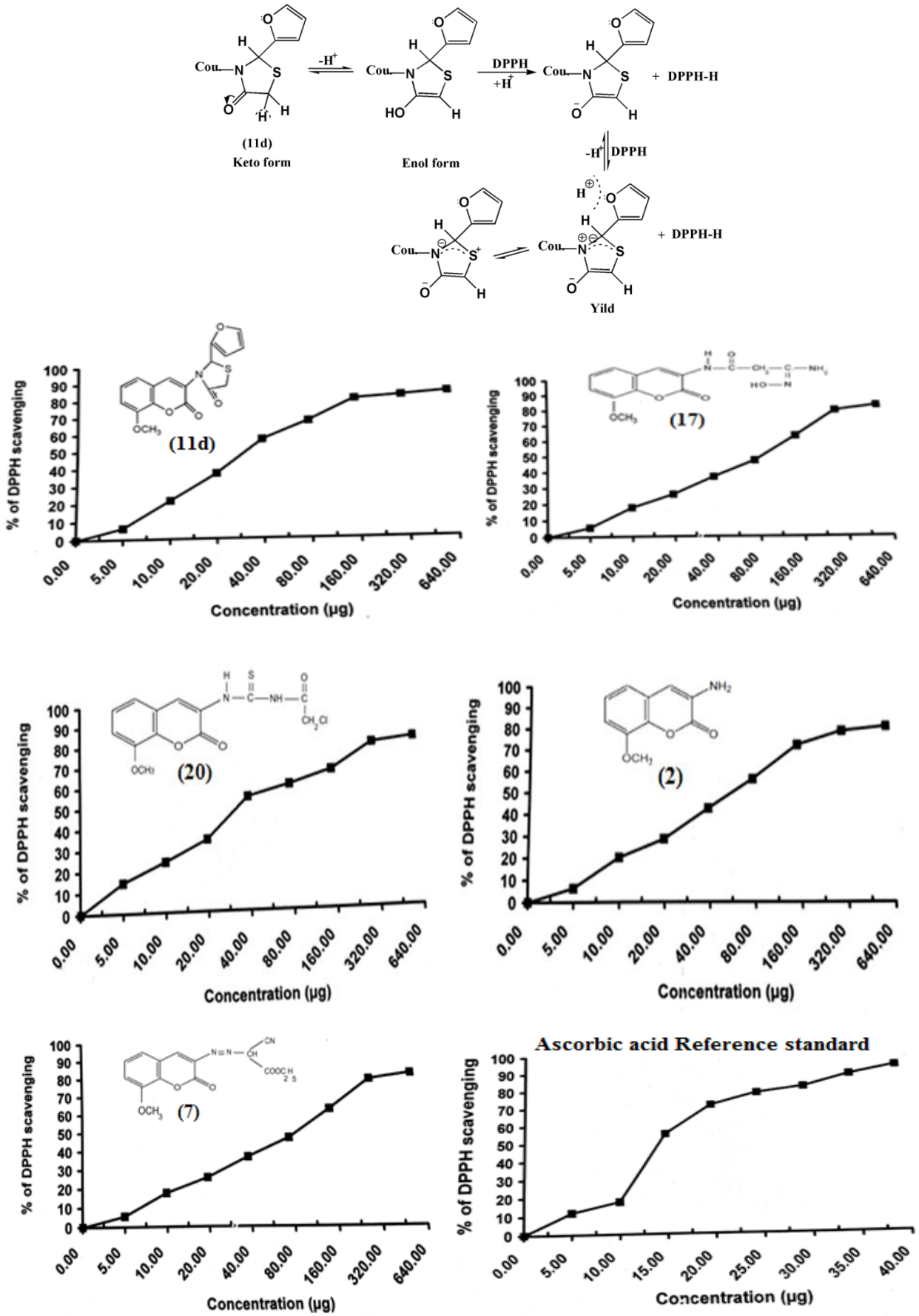


Fig. 12: Antioxidant minimum inhibitory concentration (µg) of the synthesized compounds against standard ascorbic acid.

Ascorbic acid		11d		20		17		2		7	
Conc.	DPPH%	Conc.	DPPH%	Conc.	DPPH%	Conc.	DPPH%	Conc.	DPPH%	Conc.	DPPH%
40	92.40	640	82.40	640	81.30	640	82.20	640	80.74	640	80.74
35	87.53	320	80.30	320	78.80	320	79.90	320	78.40	320	77.62
30	80.65	160	78.60	160	65.90	160	76.90	160	71.70	160	61.53
25	77.41	80	66.20	80	59.50	80	60.40	80	55.85	80	46.24
20	70.94	40	56.00	40	54.10	40	50.90	40	42.45	40	36.32
15	54.86	20	36.80	20	34.00	20	38.50	20	28.51	20	25.54
10	17.49	10	21.50	10	23.80	10	26.50	10	20.11	10	17.51
5	11.78	5	6.30	5	14.40	5	8.30	5	6.28	5	5.48
0	0	0	0	0	0	0	0	0	0	0	0

We can conclude from the above observations for the IC_{50} of the tested compounds that the antioxidant activities of 8-methoxy-3-subsistuted coumarins tested under these experimental conditions follow this order **11d** > **20** > **17** > **2** > **7**.

b. *In-vitro* Cytotoxicity

Cytotoxic drug remain the mainstay of cancer chemotherapy and are being administrated with novel ways of therapy such as inhibitors of singles and therefore it is important to discover novel cytotoxic agents with spectra of activity and toxicity that differ from those of current agents. The synthesized compounds **2**, **11d**, **17** and **20** were tested for their *in-vitro* cytotoxicity against HELA cells, cervical carcinoma cells, and A-549, lung carcinoma cells which were produced at Regional Center for Mycology & biotechnology, Al-Azhar University. All of the IC_{50} values (concentration that produces 50% reduction in cell growth) in µg/ml are listed in **Table 3**. All of the synthesized compounds showed potent inhibition with IC_{50} values in the µg/ml range and the results are presented graphically in **Figures 13, 14**.

Table 3: Cytotoxicity of the tested compounds against HELA and A-549 [IC_{50}[b](Nm)] cell lines[a]

Compd. No.	(Cytotoxicity (IC_{50} in Nm) HELA	(Cytotoxicity (IC_{50} in Nm) A-549
2	52.5 µg/ml	28.8 µg/ml
11d	191 µg/ml	110 µg/ml
17	268 µg/ml	182 µg/ml
20	113 µg/ml	61.8 µg/ml

Where: [a] The sample concentration that produces a 50% reduction in cell growth.
[b] HELA cervical carcinoma cells, A-549 lung carcinoma cells.

***In-Vitro* Cytotoxicity Study**

Mammalian cell lines: A-549 cells (human lung cancer cell line) and Hela cells (human cervical carcinoma) were obtained from VACSERA Tissue Culture Unit.

Chemicals Used: Dimethyl sulfoxide (DMSO), crystal violet and trypan blue dye were purchased from sigma(St. Louis, Mo., USA). Fetal Bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDETA were purchased from Lonza.

Crystal violet stain (1%): It composed of 0.5% (w/v) crystal violet and 50% methanol then made up to volume with ddH_2O and filtered through a Whatmann No.1 filter paper.

Cell line Propagation: The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50 µg/ml gentamycin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two times a week.

Cytotoxicity evaluation using viability assay: For the cytotoxicity assay, the cells were seeded in 96-well plate at a cell concentration of $1x10^4$ cells per well in 100µl of growth medium. Fresh medium containing different concentration of the test sample was added after 24h of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiters plates were incubated at 37°C in humidified incubator with 5% CO_2 for a period of 48h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. After incubation of the cells for at 37°C, various concentrations of sample were added, and the incubation was continued for 24 h and viable cells yield was determined by a colorimetric method. In brief, after the end of the incubation period, media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 minutes. The stain was removed and the plates were rinsed using tap water until all excess stain is removed. Glacial acetic acid (30%)

was then added to all wells and mixed thoroughly, and then the absorbance detected of the plates were measured after gently shaken on microplate reader (TECAN, Inc.), using a test wavelength of 490 nm. All results were corrected for background absorbance detected in wells without added stain. Treated samples were compared with the cell control in the absence of the tested compounds. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated. The optical density was measured with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated using the formula as follows:

Absorbance value at test compound (ODt)*x100% / Absorbance value of control (ODc)*

*Where ODt is the mean optical density of wells treated with the tested sample, and ODC is the mean optical density of untreated cell. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA). The assay was performed[43,44] to evaluate the cytotoxicity value for the synthesized coumarin derivatives **(2, 11d, 17 and 20)**. The results of the cytotoxicity (**Fig. 13,14**) indicate that compound **2.**

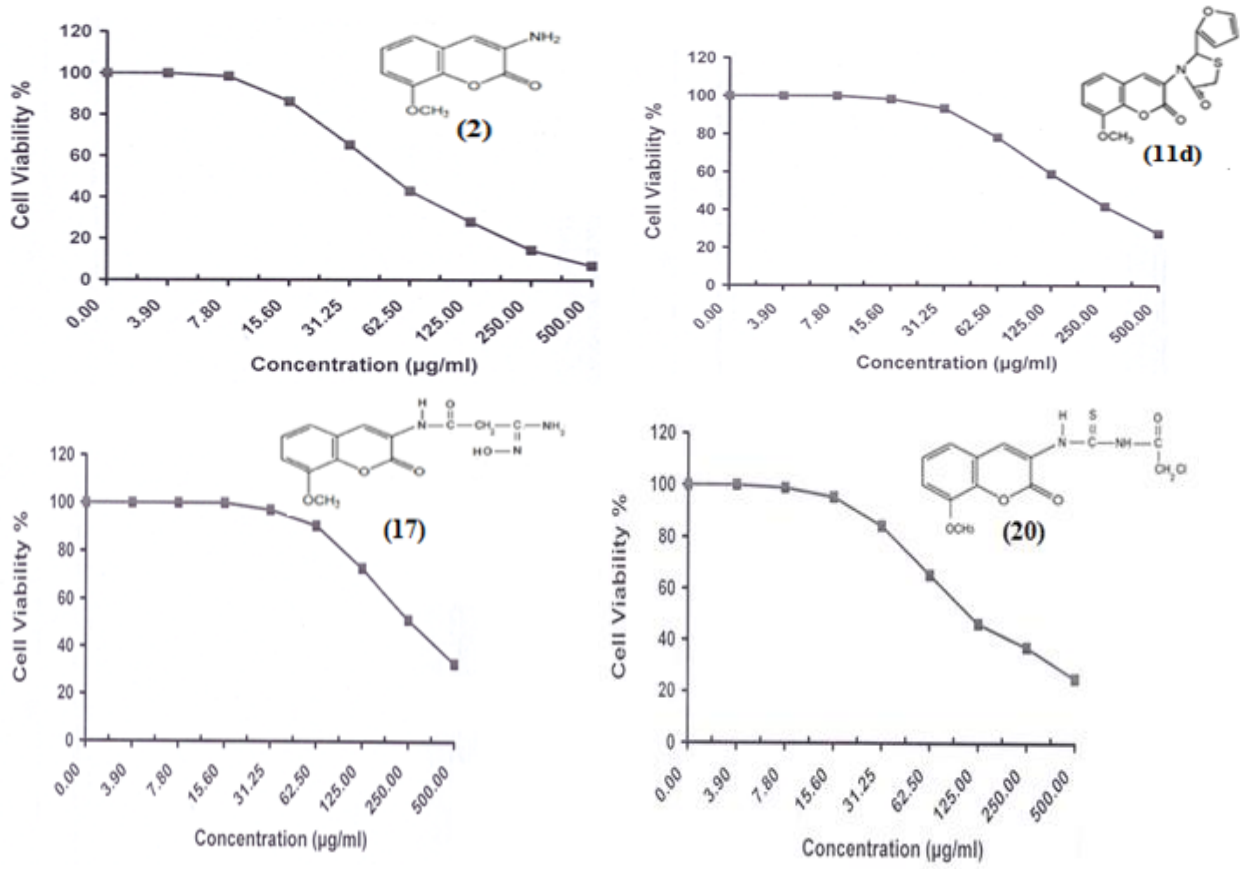


Fig. (13): Evaluation of cytotoxicity against HELA cell line.

Sample Conc. (µg/mL)	Viability %			
	2	11d	17	20
500	6.75	27.44	32.79	25.04
250	14.39	41.89	51.34	37.15
125	27.96	59.02	72.93	46.20
62.5	42.82	78.16	90.67	65.31
31.25	65.17	93.45	97.15	84.29
15.6	86.23	98.29	100	95.38
7.8	98.41	100	100	98.85
3.9	100	100	100	100
0	100	100	100	100

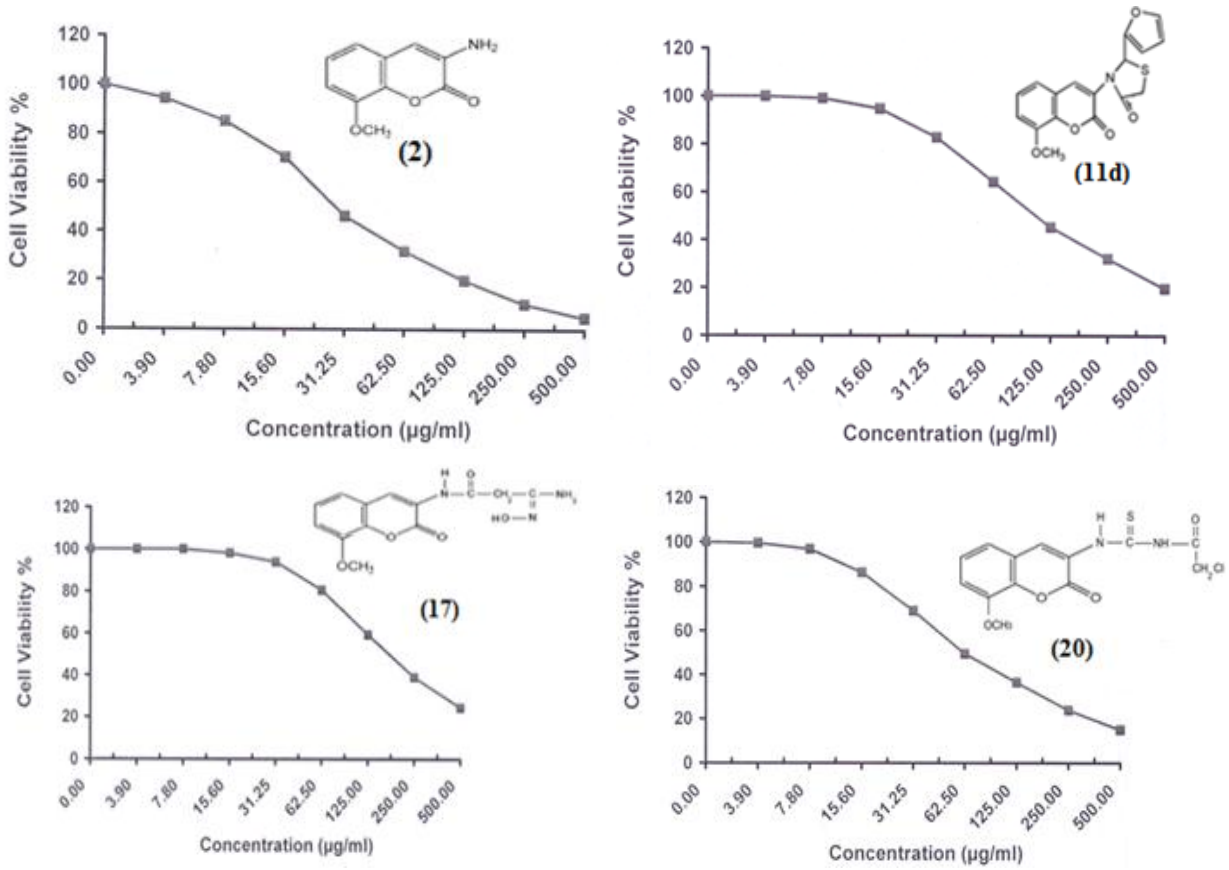


Fig. (14): Evaluation of cytotoxicity against A-549 cell line.

Sample Conc. (µg/mL)	Viability %			
	2	11d	17	20
500	4.83	19.63	24.35	14.97
250	10.42	32.15	38.94	23.85
125	19.87	45.38	59.27	36.39
62.5	31.75	64.27	80.46	49.54
31.25	46.23	82.90	93.89	68.91
15.6	70.38	94.78	98.12	86.28
7.8	85.19	99.13	100	96.70
3.9	94.20	100	100	99.42
0	100	100	100	100

The tumor HELA cell line showed normal growth in the culture system and DMSO did not seem to have any notice able effected on cellular growth. A gradual decrease in viability of cancer cells was observed with increasing concentration of the tested compounds, in a dose-dependent inhibitory effect: the median growth inhibitory concentration (IC_{50}) was for compounds **2,11d, 17, 20** was 52.5, 191, 268, 113 µg/ml respectively. It is clear from the data that the comparison of the cytotoxicity against HELA cell line **(Fig. 13)** of the tested compounds have shown that the growth inhibitory potency follows the order **2**> **20**> **11d**> **17**. Compound **2** was the best coumarin derivative exerting significant cytotoxic effect on HELA cells compared with Cisplatin (the commonly used anticancer drug). On the other hand the inhibitory activity against lung carcinoma cells (A-549 cell lines) with IC_{50} was for compounds **2, 11d, 17** and **20** as 28.8, 110, 182 and 61.8 µg/ml. Thus, it is clear that the growth inhibitory potency follows the order **2**> **20**> **11d**> **17** and it can be concluded that compound **2** was also the best coumarin derivative which exerts significant cytotoxic effect on lung carcinoma cells (A-549).

In general, the 2-amino-8-methoxy coumarin derivatives revealed the highest anticancer activity among the other tested compounds.

Table 4: Physical data of 8-metoxy-3-substituted coumarin derivatives (2-20).

Comp. No.	Mol. Formula (Mol.wt.)	M.P. °C Solvent of crys.	Yield % Colour of crys.	Analysis calcd./found %				
				C%	H%	N%	S%	Cl%
2	$C_{10}H_9NO_3$ (191)	180-182 EtOH	60 Brown	62.82 62.80	4.71 4.70	7.32 7.30	---	---
4	$C_{16}H_{16}N_2O_6$ (332)	140-142 EtOH	63 Brown	57.83 57.80	4.82 4.80	8.43 8.40	---	---
5	$C_{13}H_8N_4O_3$ (268)	200-202 P.E. (60-80°C)	70 Deep Brown	58.21 58.20	2.98 2.99	20.89 20.90	---	---
6	$C_{13}H_{16}N_8O_3$ (332)	250-252 P.E. (40-60°C)	62 Pale Brown	46.98 46.99	4.82 4.80	33.73 33.70	---	---
7	$C_{15}H_{13}N_3O_5$ (315)	228-230 EtOH	73 Brown	57.14 57.10	4.12 4.10	13.33 13.30	---	---
8	$C_{14}H_{10}N_6O_3S$ (342)	258-260 EtOH	64 Brown	49.12 49.10	2.92 2.90	24.56 24.66	9.35 9.40	---
9	$C_{24}H_{17}N_3O_3$ (395)	238-240 EtOH	77 Brown	72.91 72.90	4.30 4.32	10.63 10.60	---	---
10a	$C_{17}H_{12}NO_3Cl$ (313.5)	168-170 EtOH	70 yellow	65.07 65.10	3.83 3.80	4.46 4.50	---	11.32 11.30
10b	$C_{17}H_{12}N_2O_5$ (324)	120-122 P.E. (60-80°C)	66 brown	62.92 62.90	3.70 3.70	8.64 8.60	---	---
10c	$C_{18}H_{13}NO_4$ (307)	136-138 P.E. (60-80°C)	68 brown	70.63 70.70	4.23 4.30	4.56 4.66	---	---
10d	$C_{15}H_{11}NO_4$ (269)	166-168 P.E. (40-60°C)	71 black	66.91 66.92	4.09 4.10	5.20 5.30	---	---
11a	$C_{19}H_{14}NO_4SCl$ (387.5)	166-168 P.E. (40-60°C)	61 brown	58.84 58.86	3.61 3.60	3.61 3.64	8.26 8.30	9.16 9.20
11b	$C_{19}H_{14}N_2O_6S$ (398)	180-182 EtOH	70 black	57.28 57.30	3.51 3.52	7.04 7.00	8.04 8.00	---
11c	$C_{20}H_{15}NO_5S$ (381)	108-110 P.E. (60-80°C)	63 brown	62.99 63.00	3.93 3.94	3.67 3.77	8.39 8.40	---
11d	$C_{17}H_{13}NO_5S$ (343)	190-192 P.E. (40-60°C)	62 black	59.47 59.50	3.79 3.80	4.08 4.10	9.33 9.30	---
12	$C_{14}H_{13}NO_5$ (275)	200-202 EtOH	72 yellow	61.09 61.10	4.72 4.73	5.09 5.10	---	---
13	$C_{15}H_{13}N_3O_3S$ (315)	160-162 EtOH	66 brown	57.14 57.15	4.12 4.13	13.33 13.35	10.16 10.20	---
14	$C_{12}H_{10}NO_4Cl$ (267.5)	168-170 P.E. (40-60°C)	60 yellow	53.83 53.80	3.73 3.76	5.23 5.20	---	13.27 13.30
15	$C_{13}H_{11}N_3O_3S$ (289)	240-242 EtOH	59 brown	53.97 53.50	3.806 3.80	14.53 14.55	11.07 11.10	---
16	$C_{13}H_{10}N_2O_4$ (258)	204-206 EtOH	70 yellow	60.46 60.50	3.87 3.90	10.85 10.90	---	---
17	$C_{13}H_{13}N_3O_5$ (291)	160-162 EtOH	66 yellow	53.08 53.60	4.46 4.50	14.43 14.40	---	---
18	$C_{13}H_{11}N_3O_4$ (273)	220-222 P.E. (40-60°C)	50 brown	57.14 57.15	4.02 4.00	15.38 15.40	---	---
19	$C_{11}H_{10}N_2O_3S$ (250)	190-192 EtOH	74 brown	52.80 52.80	4.00 4.10	11.20 11.20	12.80 12.81	---
20	$C_{13}H_{11}N_2O_4SCl$ 326.5)	218-220 EtOH	51 black	47.77 47.80	3.36 3.40	8.57 8.60	9.80 9.80	10.87 10.90

Table 5: FT-IR (cm^{-1}) bands of the newly synthesized derivatives 2-20.

Sample No.	NH_2	NH	δ-lactone	C≡N	C=O	C=C	C=S	C-O-C	N=CH
2	**3381**	**---**	**1711**	**---**	**---**	**1606**	**---**	**1114**	**---**
4	**---**	**---**	**1732**	**---**	**1688**	**1517**	**---**	**1132**	**---**
5	**---**	**3224**	**1731**	**2221**	**---**	**1516**	**---**	**1134**	**---**
6[*1]	**3377**	**3226**	**---**	**---**	**1679**	**1516**	**---**	**1133**	**---**
7	**---**	**---**	**1732**	**2222**	**1689**	**1514**	**---**	**1134**	**---**
8	**---**	**3225**	**1731**	**2221**	**---**	**1515**	**1246**	**1136**	**---**
9	**---**	**3307**	**1733**	**---**	**---**	**1514**	**---**	**1134**	**---**
10a	**---**	**---**	**1734**	**---**	**---**	**1516**	**---**	**---**	**1620**
10b	**---**	**---**	**1723**	**---**	**---**	**1514**	**---**	**---**	**1625**
10c[*2]	**---**	**---**	**1727**	**---**	**---**	**1524**	**---**	**---**	**1625**
10d	**---**	**---**	**1723**	**---**	**---**	**1515**	**---**	**1136**	**1620**
11a	**---**	**---**	**1734**	**---**	**1669**	**1515**	**---**	**---**	**---**
11b	**---**	**---**	**1737**	**---**	**1671**	**1514**	**---**	**---**	**---**
11c[*3]	**---**	**---**	**1737**	**---**	**1709**	**1511**	**---**	**---**	**---**
11d	**---**	**---**	**1734**	**---**	**1706**	**1514**	**---**	**---**	**---**
12	**---**	**3190**	**1734**	**---**	**1687 1679**	**1515**	**---**	**---**	**---**
13[*4]	**---**	**3210**	**1731**	**---**	**---**	**1511**	**1241**	**---**	**---**
14	**---**	**3232**	**1734**	**---**	**1677**	**1514**	**---**	**---**	**---**
15	**---**	**3370**	**1731**	**---**	**---**	**1512**	**---**	**---**	**1616**
16	**---**	**3222**	**1734**	**2222**	**---**	**1512**	**---**	**---**	**---**
17[*5]	**3220**	**3231**	**1733**	**---**	**1661**	**1514**	**---**	**---**	**1616**
18	**3311**	**---**	**1731**	**---**	**---**	**1511**	**---**	**---**	**1631**
19[*6]	**3301**	**3221**	**1733**	**---**	**---**	**1512**	**1241**	**---**	**---**
20[*7]	**---**	**3232**	**1734**	**---**	**1676**	**1514**	**1242**	**---**	**---**

Where *1 υ OH at 4457 cm^{-1}
*2 υ CHO at 1719 cm^{-1}
*3 υ CHO at 1721 cm^{-1}
*4 υ SH (w) at 2662 cm^{-1}
*5 υ OH at 4442 cm^{-1}
*6 υ SH (w) at 2664 cm^{-1}
*7 υ SH (w) at 2662 cm^{-1}

Our observations on the IR data of coumarin derivatives **(2-5)** and **(7-20)** imply that the carbonyl group frequency bands are located in the region 1734-1711 cm^{-1}, while for the compound **6** the δ-lactone frequency disappeared and instead a frequency for the carbonyl group at 1679 cm^{-1} appeared, as well as frequency bands for OH, NH_2 and NH at 4442, 3377 and 3226 cm^{-1} respectively.

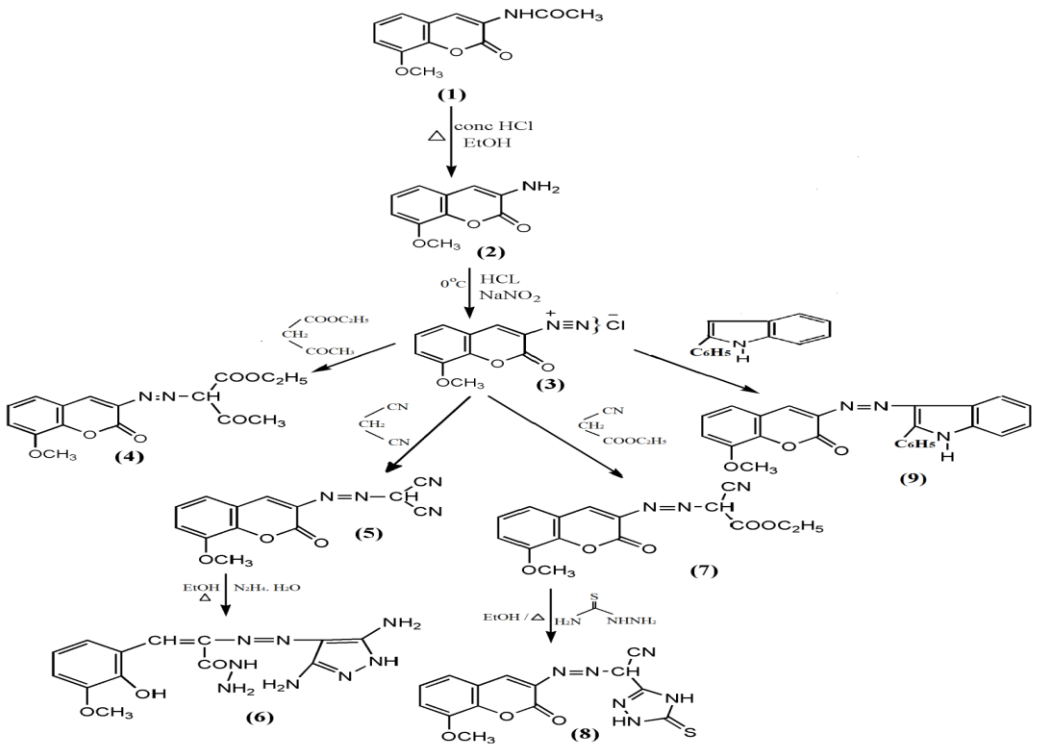
Table 6: ^{1}H-NMR (δ ppm) signals of the newly synthesized derivatives 2-20.

Comp No.	$CHCH_3$	CH-X	$-OCH_3$	CH_2-X	H C-4 coumarin	Ar-H	NH	NH_2	OH
2	---	---	3.30(s,3H)	---	7.12(s,1H)	7.17,7.81 (m,3H)	---	9,92(s,2H), (D_2O) exchangeable	---
4	1.31(t,3H) 2.19(s,3H)	3.44(s,1H) (X=CO)	3.39(s,3H)	4.31(q,2H) (X=CH_3)	7.15(s,1H)	7.49-7.81 (m,3H)	---	---	---
5	---	---	3.37(s,3H)	---	7.15(s,1H)	7.24-7.38 (m,3H)	9.19(s,1H), (D_2O exchangeable)	---	---
6	---	7.12(s,1H)(X==C)	3.47(s,3H)	---	7.43(s,1H)	7.71-7.97 (m,3H)	9.93-9.98 (2xs,2x1H)	5.01(s,2H) 10.03 (s,2H)	10.91 (s,1H)
7	1.32(t,3H)	3.14(s,1H)	3.37(s,3H)	4.31(q,2H) (X=CH_3)	7.07(s,1H)	7.48-7.69 (m,3H)	---	---	---
8	---	2.95(s,1H)	3.37(s,3H)	---	7.13(s,1H)	7.37-7.83 (m,3H)	9.92(s,1H), 10.01(s,1H) (D_2O exchangeable)	---	---
9	---	---	3.39(s,3H)	---	7.14(s,1H)	7.60-8.13 (m,12H)	10.11(s,1H) (D_2O exchangeable)	---	---
10a	---	8.11(s,1H)(X==N)	3.37(s,3H)	---	7.09(s,1H)	7.34-7.86 (m,7H)	---	---	---
10b	---	8.12(s,1H)(X==N)	3.39(s,3H)	---	7.07(s,1H)	7.44-7.89 (m,7H)	---	---	---
10c	---	8.12(s,1H)(X==N) 9.91(s,1H)(X==O)	3.39(s,3H)	---	7.04(s,1H)	7.41-7.91 (m,7H)	---	---	---
10d	---	8.11(s,1H)(X==N)	3.39(s,3H)	---	7.01(s,1H)	7.32-7.59 (m,7H)	---	---	---
11a	---	5.91(s,1H)(X=S)	3.37(s,3H)	3.70(s,2H) (X=CO)	7.09(s,1H)	7.33-7.89 (m,7H)	---		---
11b	---	5.91(s,1H)(X=S)	3.39(s,3H)	3.71(s,2H) (X=CO)	7.03(s,1H)	7.39-7.89 (m,7H)	---	---	---
11c	---	5.91(s,1H)(X=S), 9.1(s,1H)(X==O)	3.39(s,3H)	3.73(s,2H) (X=CO)	7.04(s,1H)	7.43-7.93 (m,7H)	---	---	---
11d	---	5.91(s,1H)(X=S)	3.39(s,3H)	3.81(s,2H) (X=CO)	7.09(s,1H)	7.35-7.82 (m,6H)	---	---	---
12	2.07(s,3H)	---	3.37(s,3H)	3.18(s,2H) (X=CO)	7.01(s,1H)	7.01-7.43 (m,3H)	---	---	---

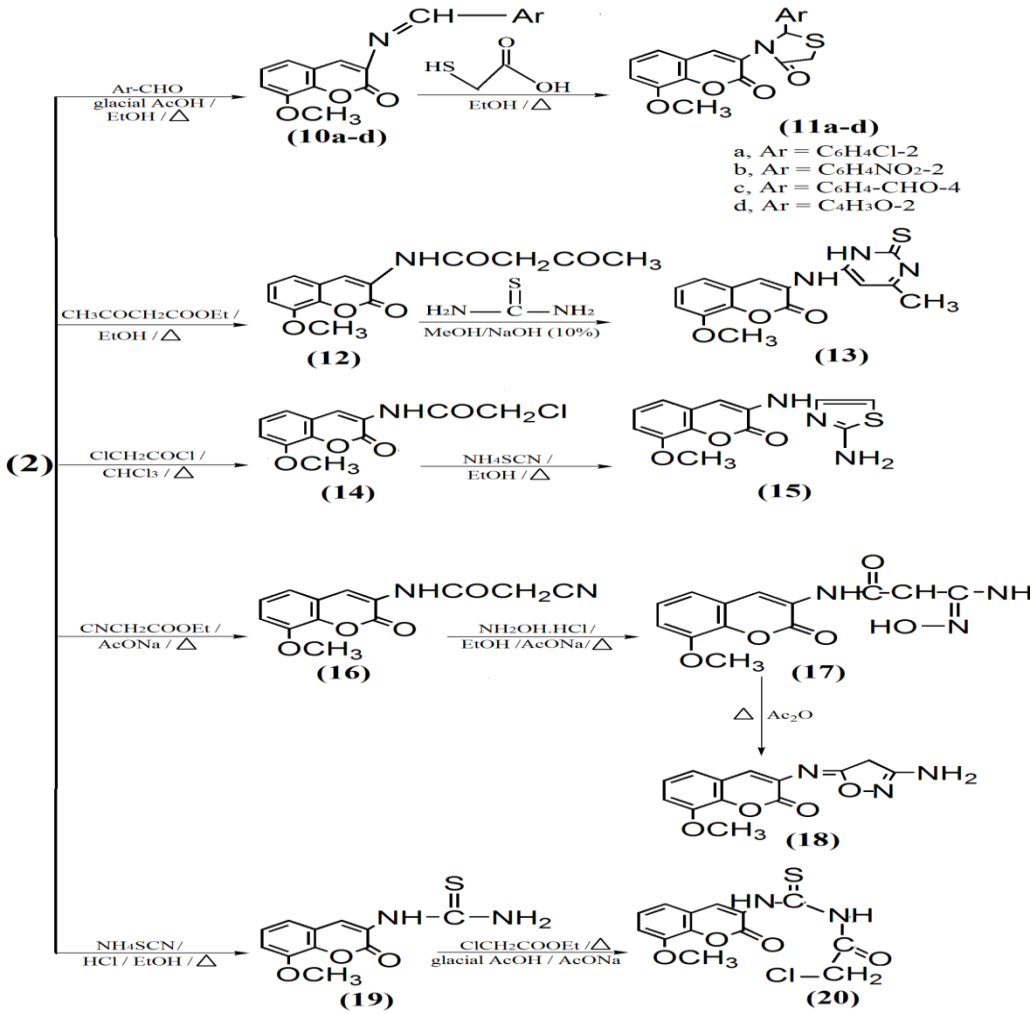
13	**1.12(s,3H)**	**4.31(s.1H) pyrimidine**	**3.37(s,3H)**	**---**	**7.10 (s,1H)**	**7.31-7.49 (m,3H)**	**8.31,8.34(2xs, 2x1H),(D_2O exchangeable)**	**---**	**---**
14	**---**	**---**	**3.37(s,3H)**	**4.31(s,2H) (X=$COCH_2$ Cl)**	**7.09(s,1H)**	**7.27-7.51 (m,3H)**	**8.39(s,1H), D_2O (exchangeable**	**---**	**---**
15	**---**	**5.5(s,1H)(X=S), 5.76(S,1H)**	**3.37(s,3H)**	**---**	**7.01(s,1H)**	**7.31-7.59 (m,3H)**	**8.31(s,1H), (D_2O exchangeable)**	**---**	**---**
16	**---**	**---**	**3.37(s,3H)**	**3.81(s,2H) (X=CN)**	**7.10(s,1H)**	**7.31-7.56 (m,3H)**	**4.71(s,1H), (D_2O exchangeable)**	**8.26(s,2H), (D_2O exchangeable**	**---**
17	**---**	**---**	**3.37(s,3H)**	**2.68(s,2H) (X=CN)**	**7.09(s,1H)**	**7.31-7.49 (m,3H)**	**8.71(s,1H), 8.83(s,2H) (D_2O exchangeable)**	**9.81(s,1H)**	**---**
18	**---**	**---**	**3.37(s,3H)**	**2.31(s,2H)**	**7.10(s,1H)**	**7.31-7.46 (m,3H)**	**8.23(s,2H), (D_2O exchangeable)**	**---**	**---**
19	**---**	**---**	**3.37(s,3H)**	**---**	**7.01(s,1H)**	**7.17-7.41 (m,3H)**	**8.21(s,1H), 8.43(s,2H)D_2O exchangeable)**	**---**	**---**
20	**---**	**---**	**3.37(s,3H)**	**4.31(s,2H) (X=Cl)**	**7.11(s,1H)**	**7.21-7.41 (m,3H)**	**8.31(s,1H), 8.43(s,1H)D_2O exchangeable)**	**---**	**---**

Table 7: ^{13}C-NMR spectra of compounds 2-20.

Comp. No.	^{13}C-NMR (1100 MHZ, DMSO-d6) δ:ppm
2	55.8 (H_3C-O), 113.4 (CH-4), 113.9 (CH-8), 120.2 (CH-6), 123.2 (CH-5), 126.4 (CH-7), 137.9 (C-3), 140.6 (C-O), 148.5 (C-9), 159.8 (C=O)
4	15.3 (H_3C-CH_2), 26.3 (H_3C-CO), 56.9 (H_3C-O), 62.2 (H_2C-O), 77.8 (CH-CO), 114.9 (CH-4), 118.1 (C-N), 119.9 (CH-6), 124.7 (C-5), 127.6 (CH-7), 142.9 (C-10), 149.7 (C-O), 159.9 (C=O), 162.8 (C=O), 167.9 (C=O)
5	56.7 (H_3C-O), 74.3 (N-CH), 113.7, 113.8 (2xCN), 114.7 (CH-4), 114.9 (CH-8), 119.9 (CH-6), 124.2 (C-8), 127.5 (CH-7), 138.9 (C-3), 142.7 (C-10), 159 (C=O)
6	56.8 (H_3C-O), 79.2 (N-C), 115.3 56.7 (CH-4), 117.6 (C-8), 118.9 (CH=C), 121.3 (CH=C-N), 122.1 (CH-3), 123.1 (CH-4), 148.3 (C-OH), 152.3 (C-6), 154.1, 154.2 (2xC-NH_2),167.8 (C=O)
7	15.4 (H_3C-CH_2), 52.7 (HC-C-C), 56.8 (H_3C-O), 61.7 (H_2C-O), 114.7 (CH-8), 115.9 (C≡N), 118.1 (C-N), 119.9 (CH-6), 124.7 (C-5), 127.9 (CH-7), 142.7 (C-O), 158.9 (C=O), 159.3 (C-O), 167.1 (O-C=O)
8	50.1 (N-CH), 56.7 (H3C-O), 113.9 (CH-4), 115.9 (C≡N), 116.9 (C-N 3), 120.3 (CH-6), 124.7 (C-5), 127.9 (CH-7), 152.7 (C-10), 156.3 (C-3`), 158.7 (C=O), 159.1 (C-O), 163.9 (C=S)
9	56.7 (H_3C-O), 114.9 (CH-4), 115.6 (CH-8), 119.7 (CH-6), 124.1 (C-5), 127.1 (CH-7), 128.9 (C-3), 142.3 (C-10), 156,8 (C-10), 158.6 (C=O),102.3 (N-C-3`), 111.9 (CH-8`), 119.9 (CH-5`), 120.9 (CH-7`), 123.7 (CH-6`), 123.9 (C-2`), 27.8 (C-4`), 136.7 (C-9`), 133.9 (C-1˝), 117.9 (CH-4˝), 127.9 (CH-2˝), 130.1 (CH-3˝)
10a	56.8 (H_3C-O), 114.7 (CH-4), 114.9 (CH-6), 119.9 (CH-6), 124.4 (C-5), 127.8 (CH-7), 132.8 (C-3), 142.8 (C-10), 156.3 (C-O), 158.3q (C=O), 164.3 (N=CH), 128.1 (CH-3`), 130.1 (CH-5`), 130.9 (CH-2`), 133.1 (CH-4`), 135.4 (C-1`), 137.7 (C-Cl)
10b	149.8 (C-N), 164.3 (N=CH) 
10c	141.2 (C-4`-CH), 166.4 (N=CH), 192.1 (C 
10d	164.3 (N-CH), 110.5 (CH-3`), 110.9 (CH-4`), 149.6 (C-5`), 150.3 (C-2`)
11a	33.9 (CH2-S), 56.1 (N-CH-S), 56.7 (O-CH3), 103.7(C-2`), 114.9 (C-8), 116.1 (C-4), 121.3 (CH-6), 124.3 (C-5), 127.6 (CH-7), 128.6 (CH-4`), 129.6 (CH-5`), 129.9 (CH-6`), 131.1 (CH-3`), 134.4 (C-3), 135.1 (C-Cl), 141.6 (C-10), 149.6 (C-9), 160.8 (C=O), 166.6 (C=O)
11b	125.8 (CH-6`), 129.0 (CH-5`), 130.6 (CH-3`), 134.6 (C-2`), 135.6 (CH-4`), 150.6 (C-NO_2)
11c	60.9 (N-CH-S), 129.4 (CH-3`), 129.9 (CH-2`), 130.7 (CH-5`), 133.7 (CH-6`), 140.4 (C-4`), 192.1(HC=O)
11d	60.6 (N-CH-S), 108.1 (CH-4`), 111.1 (CH-3`), 143.1 (CH-5`), 152.6 (C-2`)
12	30.8 (CH_3-C=O), 52.1 (CO-CH_2-CO), 56.8 (H_3C-O), 114.9 (CH-8), 116.7 (CH-4), 119.9 (CH-6), 124.2 (C-5), 127.6 (CH-7), 132.8 (C-3), 142.9 (C-10), 158.3 (C-9), 159.9 (C=O), 168.1 (C=O), 199.9 (C=O)
13	23.8 (CH_3-C), 56.8 (H_3C-O), 66.1 (CH-pyrimidine), 114.2 (CH-4), 114.9 (CH-8), 119.9 (CH-6), 124.3 (C-5), 127.7 (CH-7), 132.9 (C-3), 142.9 (C-10), 156.7 (NH-C=), 158.2 (C-9), 159.9 (C=O), 172.1 (C=N), 179.4 (C=S)
14	43.8 (CH_2-Cl), 56.8 (H_3C-O), 114.5 (CH-4), 114.9 (CH-8), 120.7 (CH-6), 124.1 (C-5), 127.5 (CH-7), 142.9 (C-10), 149.4 (C-NH), 159.9 (C=O), 169.9 (HN-C=O)
15	56.8 (H_3C-O), 114.3 (CH-4), 114.9 (CH-8), 120.7 (CH-5`), 120.7 (CH-6), 124.3 (C-5), 127.7 (CH-7), 138.9 (C-N), 139.1 (CH-4`), 142.7 (C-10), 153.4 (CH-2`), 158.2 (C-9), 159.9 (C=O)
16	26.7 ($COCH_2CN$), 56.8 (H_3C-O), 114.9 (CH-8), 116.7 (CH-4), 117.8 (C≡N), 120.7 (CH-6), 124.3 (C-5), 127.5 (CH-7), 139.9 (C-10), 158.2 (C-9), 159.9 (C=O), 166.3 (C=O)
17	43.9 (CH_2-C=N), 56.8 (H_3C-O), 114.9 (CH-8), 116.7 (CH-4), 120.7 (CH-6), 124.3 (C-5), 127.5 (CH-7), 132 (C-3), 158.2 (C-9), 159.9 (C=O), 164.9 (C=N), 168.1 (C=O)
18	29.8 (CH_2C-NH_2), 56.8 (H_3C-O), 114.9 (CH-8), 116.7 (CH-4), 120.7 (CH-6), 124.3 (C-5), 127.5 (CH-7), 132.9 (C-3), 142.3 (C-10), 158.8 (C=O), 166.9 (N=C)
19	56.8 (H_3C-O), 115.9 (CH-4), 114.9 (CH-8), 120.4 (CH-6), 124.3 (C-5), 127.5 (CH-7), 142.3 (C-10), 158.9 (C=O), 168.2 (C=S)
20	43.9 (Cl-CH_2-CO), 56.8 (H3C-O), 114.9 (CH-4), 115.1 (CH-8), 120.7 (CH-6), 124.3 (C-5), 127.5 (CH-7), 133.7 (C-3), 141.9 (C-10), 158.1 (C-9), 159.9 (C=O), 169.9 (CH_2-C=O), 170.2 (C=S)



Scheme (1)



Scheme (2)

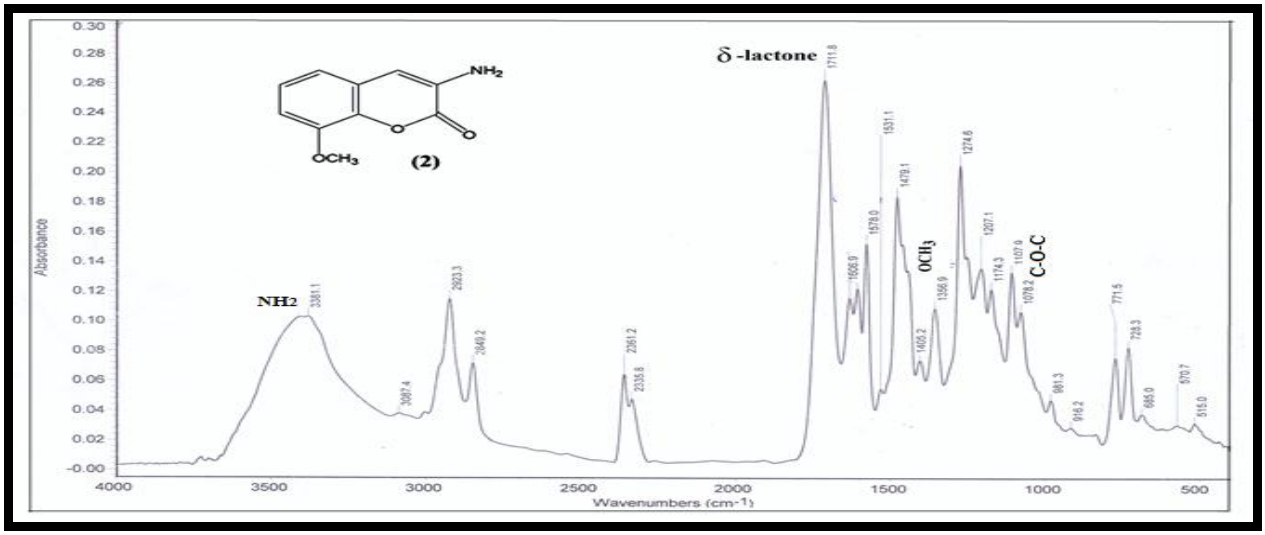


Fig. 6: The IR spectrum of compound 2.

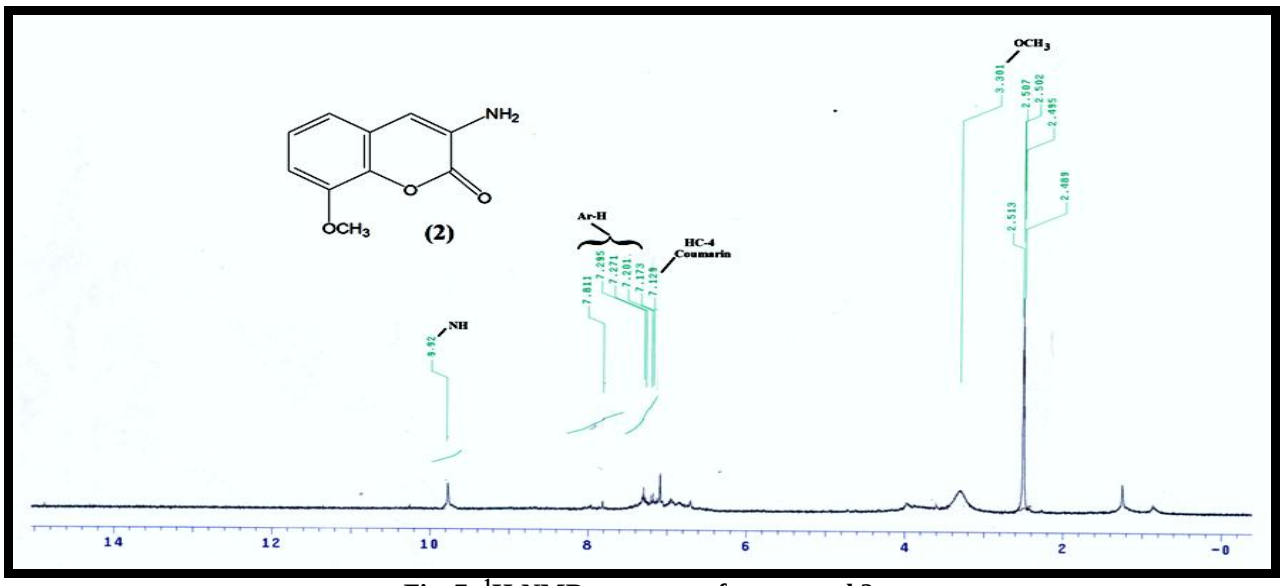


Fig. 7: ^{1}H-NMR spectrum of compound 2.

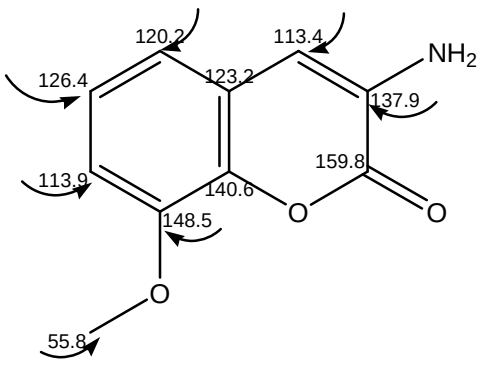


Fig. 8: ^{13}C-NMR of compound 2.

CONCLUSION

We have served a number of novel nitrogen containing functionalized coumarins as potent antioxidants as well as anticancer agents using novel synthetic approaches in high yield and purity. Some of the synthesized compounds were evaluated for their antioxidant and anticancer activity. The results showed a significant antioxidant activity of some of the investigated derivatives. Similarly, the investigation of the anticancer screening data revealed that the tested compounds show moderate activity at higher concentration.

Experimental

Melting points were determined in open capillaries and were uncorrected. The IR spectra were determined as KBr pellets on a Shimadzu model 470, spectrophotometer and are expressed in cm. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a JEOL GSX (270 MH_2) spectrophotometer; chemical shifts were expressed in δ (ppm) relative to tetramethylsilane as given. All the exchangeable protons were confirmed by addition of D_2O. Mass spectral data were obtained with a variant CP3800 model with ionization energy 70eV. Elemental analyses were performed at Micro Analysis Center, Cairo University, Egypt; found values were within ±0.4% of predicted values for all compounds. The

progress of the reactions was monitored using thin layer chromatography (TLC) sheets precoated with UV fluorescent silica gel Merck 60F254 and was visualized using UV lamp.

We report a facile synthesis of 8-methoxy-3-amino-coumarin **(2)**, starting from commercial and available materials. The starting material *o*-vanillin and the reaction sequence for the synthesis of **1-9** are outlined in **Scheme 1**, while the synthesis of compounds **10-20** are outlined in **Scheme 2**. The physical data are recorded in **Table 4** while spectral data are shown in **Table 5,6 and 7**.

Synthesis of 8-methoxy-3-acetamido coumarin (1)
Microwave method (method A)
A mixture of equimolar quantities of *o*-vanillin and 2-methyl oxazolone derivative (0.01 mol. each) was dissolved in 5 ml of acetic anhydride containing 3 gm of freshly fused sodium acetate then was irradiated for 5 min. in closed vial using Microwave system. After completing of the reaction, the obtained product was purified by crystallization form EtOH as 3-acetamido-8-methoxy coumarin **(1)**.

Conventional method (method B)
A mixture of (0.01 mol.) of *o*-vanillin and (0.01 mol.) of 2-methyl oxazol-5-one was dissolved in 5 ml of acetic anhydride followed by the addition of freshly fused sodium acetate (3 g) and the reaction mixture was heated under reflux for 6 h. It was filtered off then left to cool. After cooling, the crude product was collected and recrystallized form EtOH as 3-acetamido-8-methoxy coumarin **1**.

Synthesis of 3-amino-8-methoxy coumarin (2)
3-acetamido coumarin **(1)** (0.01 mol.) was treated with a mixture of (ethanol/conc. HCl 50:50 by volume) (50 ml) and the mixture was refluxed for 4 hrs. The solution was concentrated on water bath, diluted with water and to the clear solution was added the solid sodium bicarbonate until it was alkaline to litmus. The resultant solid was collected by filtration, washed well with water and recrystallized form EtOH as **2** (c.f. **Table 4**); MS: $C_{10}H_9NO_3$: m/z, 291(M^+).

Diazotization of 3-amino-8-methoxy coumarin (2). Formation of 8-methoxy coumarin-3-yl diazonium chloride (3)
To (0.01 mol.; 1.9 g) of **2** was added 3 ml 6N HCl solution (2ml concentrated HCl and 2ml of distilled water). This mixture was stirred while cooling in an ice-salt bath. In a test tube, $NaNO_2$ (0.01 mol., 0.69g) was dissolved in 1ml ofdistilled water, cooled and then added dropwise with stirring to the cold stirred amine hydrochloride. (The end point was determined by putting a drop of the solution on starch-KI paper).

Coupling reaction of the diazonium chloride derivative (3) with active methylene compounds and 2-phenyl indole. Formation of (4,5,7) and (9) derivatives
To the crude stirred and well cooled diazonium chloride solution **(3)** (kept at about 0-5°C), was added dropwise while stirring, a cold solution of active methylene derivatives namly, ethyl acetoacetate, malononitrile, ethyl cyanoacetate (0.01mol.) and/or 2-phenyl indole (0.01mol.), dissolved in 10 ml of absolute ethanol containing 2 ml of 5% aqueous NaOH solution. The reaction mixture was kept in ice while stirring, then left overnight in refrigerator. The product that separated was collected and washed well with cold water then diluted ethanol and recrystallized from the proper solvent to give **4**, **5, 7** and **9**. (c.f. **Table 4**). 4) MS: $C_{16}H_{16}N_2O_6$ m/z, 332(M^+)., 5) MS: $C_{13}H_8N_4O_3$ m/z, 268/$M^{+\bullet}$.

Reaction of the compound (5) with hydrazine hydrate. Formation of 2-[(1E)-3-(aminoxy-2-(3,5-diamino-1H-pyrazol-4-yl)diazenyl-3-amino-prop-1-en-1-yl]-6-methoxy phenol (6)
A mixture of **5** (0.01mol.) and hydrazine hydrate (0.01mol.) in 25 ml of absolute ethanol was refluxed for 6 hrs. After concentration and cooling, the product that separated was collected, washed well with dilute alcohol and recrystallized to give **6**. It gave a characteristic colour with aq.$FeCl_3$ solution (violet). (c.f. **Table 4**).

Reaction of the ethyl cyanoester derivative (7) with thiosemicarbazide. Formation of the 1,2,4-triazolyl-5-thioxo derivative (8)
A mixture of **7** (0.01mol.) and thiosemicarbazide (0.01mol., 0.9g dissolved in 1ml of water) and sodium acetate(0.005 mol.) in 20 ml of absolute ethanol was refluxed for 6 hr. After cooling, the product was collected, washed well with water then with dilute alcohol and recrystallized to give **8**. (c.f. **Table 4**).

Reaction of the coumarin derivatives (2) with aromatic aldehydes. Formation of the Schiffs' bases (10a-d)
A mixture of **2** (0.01mol.) in 10 ml of absolute ethanol and aromatic aldehydes namely, 2-chloro; 2-nitro benzaldehyde, terphthaldehyde and 2-furaldehyde (0.01mol.) in (10 ml) of absolute ethanol and (2 ml) of glacial acetic acid was heated under reflux for 6 hrs. After cooling the product was collected, washed well with dilute alcohol and recrystallized from the proper solvent as **10a-d**. (c.f. **Table 4**).

Reaction of the Schiffs' bases (10a-d) with mercapto acetic acid. Formation of 2-aryl-3N-thiazolidin-4-one derivatives (11a-d)
A mixture of each of the Schiffs' bases **(10a-d)** (0.01 mol.) and mercapto acetic acid (0.01mol.) in 30 ml of absolute ethanol was refluxed for 6 hrs. The product that separated after cooling was collected, washed well with water then with dilute ethanol and recrystallized from the

proper solvent as **11a-d**. (c.f. **Table 4**). 11a) MS: $C_{19}H_{14}NO_4SCl$ m/z, 377/M^+ ; 11b) MS: $C_{19}H_{14}N_2O_6S$ m/z, 398/M .

Reaction of 8-methoxy-3-amino coumarin with active methylene compounds. Formation of (12, 14 and 16)

A mixture of **2** (0.01 mol.) ethyl acetoacetate (0.01 mol.) in 3.0 ml of absolute ethanol containing three drops of piperidine/or **2** (0.01 mol.) with chloroacetyl chloride (0.01 mol.) in 20 ml of chloroform and/or **2** (0.01 mol.) with ethyl cyanoacetate (0.01 mol) in 30 ml of absolute ethanol containing (0.01 mol.) of freshly fused sodium acetate was refluxed for 6hr for each reaction. After cooling, the product for each reaction was collected, washed well with water the recrystallized from the proper solvent to give **12, 14 and 16.** (c.f. **Table 4**).

Reaction of (12) with thiourea. Formation of pyrimidin-2-thioxo-4-yl-imino-coumarin-3-yl (13)

A mixture of **12** (0.01 mol.) and thiourea (0.01 mol.) in 30 ml of absolute methanol containing 5 ml of 10% sodium hydroxide was refluxed for 6hr. After cooling, the mixture was neutralized with dilute HCl and the product was collected, washed well with dilute alcohol, the recrystallized from the proper solvent to give **13.** (c.f. **Table 4**).

Reaction of (14) with ammonium thiocyanate. Formation of 2-amino thiazolidin-4-yl-imino-coumarin-3-yl (15)

A mixture of **14** (0.01 mol.) and ammonium thiocyanate (0.01 mol.) in 30 ml of absolute ethanol was refluxed for 6hr. After cooling the product was collected, washed well with water and dilute alcohol and recrystallized from the proper solvent to give **15.** (c.f. **Table 4**). MS: $C_{13}H_{11}N_3O_3S$ m/z, 289/M .

Reaction of (16) with hydroxylamine hydrochloride. Formation of the oxime derivative (17)

A mixture of equimolar quantities of **16** and hydroxylamine hydrochloride (0.01 mol.) for each in 20 ml of absolute ethanol containing fused sodium acetate (0.01 mol.; 0.8g) was refluxed for 6hr. After cooling, the product that separated was collected, washed well with water and dilute alcohol then recrystallized from the proper solvent to give **17.** (c.f. **Table 4**).

Reaction of the oxime derivative (17) with acetic anhydride. Formation of 3-amino isoxazole derivative (18)

A suspension of the oxime derivative **17** (0.01 mol.) in 20 ml of acetic anhydride was heated on a steam bath for 2hr. The solid that separated after cooling was collected, washed well with water then dilute alcohol and recrystallized from the proper solvent to give the isoxazole derivative **18.** (c.f. **Table 4**).

Reaction of 3-amino-8-methoxy coumarin (2) with ammonium thiocyanate. Formation of 8-methoxy coumarin-3-yl-1-thiourea (19)

A mixture of **2** (0.01 mol.) and ammonium thiocyanate (0.01 mol.) in 30 ml of concentrated HCl- ethanol mixture (1:1 by volume) was refluxed for 6hr. The product that separated after cooling was collected, washed well with water, then dilute alcohol and recrystallized from the proper solvent as **19.** (c.f. **Table 4**).

Reaction of (19) with ethyl chloroacetate. Formation of (20)

A mixture of **19** (0.01 mol.), freshly fused sodium acetate (0.01 mol.) and ethyl chloroacetate (0.02 mol.) in 20 ml of glacial acetic acid was refluxed for 12 hr. The solid that separated product after cooling was collected, washed well with water dilute alcohol and recrystallized from the proper solvent to give **20.** (c.f. **Table 4**). MS: $C_{13}H_{11}N_2O_4SCl$; m/z: 250/M .

Conflict of Interest

The authors have no conflict of interest to declare regarding the publication of this article.

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