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## AN EFFICIENT SYNTHESIS OF SOME NEW COUMARIN HYBRIDS ENDOWED WITH EXPECTED BIOLOGICAL ACTIVITY

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**Abstract** – Reactions of 5-bromo-2-hydroxybenzaldehyde with cyanoacetic acid hydrazide derivatives in refluxing ethanol afforded some new chromene-based polyfunctionally substituted heteroaromatics. The antimicrobial activity of some of the obtained products was evaluated. The structures of the newly synthesized compounds were elucidated based on their elemental analyses and spectral data.

### INTRODUCTION

Coumarins are among the most important part of heterocyclic compounds and their derivatives have a wide range of biological activities due to their interactive nature with various proteins. Coumarins are bioactive substances that have considerable biological activities, such as anti-inflammatory, antioxidant anti-HIV, anticancer, antibacterial and anticoagulant activities.<sup>1-12</sup> Also, pyranocoumarin derivatives are applied as photoactive drugs and as anti-HIV, anticancer, anti-inflammatory and antibacterial agents.<sup>13-20</sup> Moreover, coumarin derivatives also played a vital role in organic material chemistry like molecular devices, fluorescent dyes, pigments and electrophotographic.<sup>21,22</sup>

Heterocyclic compounds containing nitrogen and oxygen atoms play an important role in pharmaceutical, agrochemical, and material chemistry.<sup>23-31</sup> For example, 2-oxoindoline, thiophene, pyran, pyridine and benzimidazole as well as coumarin occupy an important position in medicinal chemistry due to their various biological activities. Obviously, combining one or more of these heterocyclic compounds with coumarin may lead to a novel candidate that has more bioactivity, lower toxicity and multiple mechanisms of action. According to the above facts, we are interested to design and synthesize a new

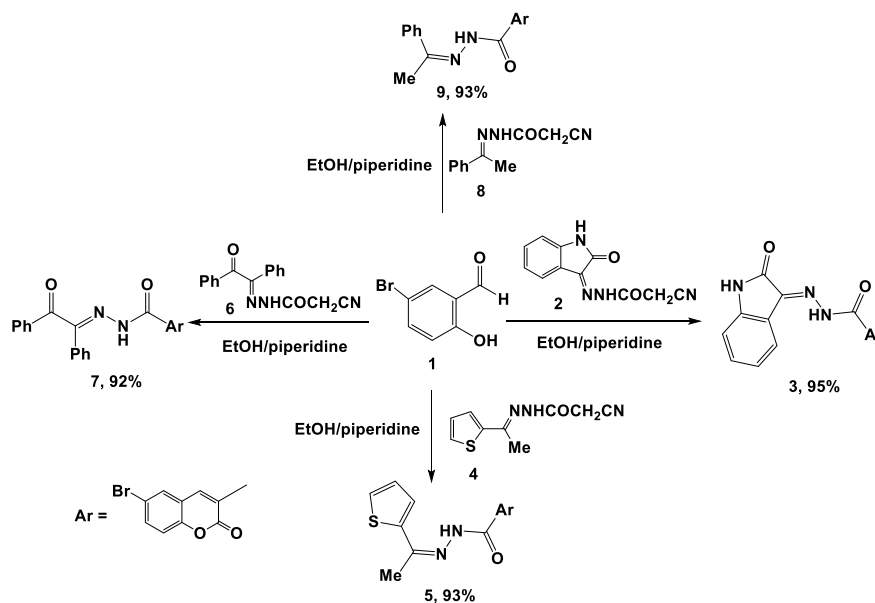
series of heterocyclic compounds by merging two or three heterocyclic moieties in a single molecular framework with evaluation of their biological activities to fulfill the goal of our study. In continuation of our main objectives related to the synthesis and biological properties of new heterocyclic products containing coumarin,<sup>32-38</sup> we report herein the synthesis and characterization of several new coumarin derivatives.

## RESULTS AND DISCUSSION

Treatment of 5-bromo-2-hydroxybenzaldehyde (**1**) with cyanoacetic acid hydrazide derivatives **2**, **4**, **6** and **8** in refluxing ethanol containing a catalytic amount of piperidine afforded the chromene derivatives **3**, **5**, **7** and **9**, respectively in excellent yields (92-95%). The newly synthesized compounds were confirmed based on their elemental analyses and spectral data (IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR and MS) (Scheme 1). The IR spectrum of 6-bromo-2-oxo-*N'*-(2-oxoindolin-3-ylidene)-2*H*-chromene-3-carbohydrazide (**3**) revealed absorption bands at 3414 and 1715 cm<sup>-1</sup> corresponding to NH and C=O functions, respectively. Its <sup>1</sup>H NMR spectrum revealed three singlet signals at δ 8.82, 8.24 and 8.07 ppm corresponding to chromene C=CH, NH and NH-indoline protons, respectively, in addition to aromatics protons at δ 7.19-7.49 ppm. Its mass spectrum showed a molecular ion peak at *m/z* 413 (M<sup>+</sup>+2, 100) corresponding to a molecular formula C<sub>18</sub>H<sub>10</sub>BrN<sub>3</sub>O<sub>4</sub>.

In similar way, the IR spectrum of 6-bromo-2-oxo-*N'*-(1-(thiophen-2-yl)ethylidene)-2*H*-chromene-3-carbohydrazide (**5**) revealed absorption bands at 3330, 3048 and 1684 cm<sup>-1</sup> corresponding to NH, C-H aromatic and C=O, respectively. Its <sup>1</sup>H NMR spectrum revealed three singlet signals at δ 8.81, 8.02 and 1.41 ppm due to chromene C=CH, NH protons and methyl protons, respectively, in addition to aromatic protons at δ 7.25-7.96 ppm. Its mass spectrum showed a peak at 390 (M<sup>+</sup>, 100) corresponding to its molecular ion. Also, the IR spectrum of 6-bromo-2-oxo-*N'*-(2-oxo-1,2-diphenylethylidene)-2*H*-chromene-3-carbohydrazide (**7**) revealed absorption bands at 3257, 3048 and 1660 cm<sup>-1</sup> corresponding to NH, C-H aromatic and C=O, respectively. Its <sup>1</sup>H NMR spectrum showed two singlet signals at δ 8.63 and 7.96 ppm due to chromene C=CH, and NH protons, respectively, in addition to aromatic protons at δ 7.25-7.71 ppm. Its mass spectrum revealed a peak at 474 (M<sup>+</sup>, 50.9) corresponding to its molecular ion.

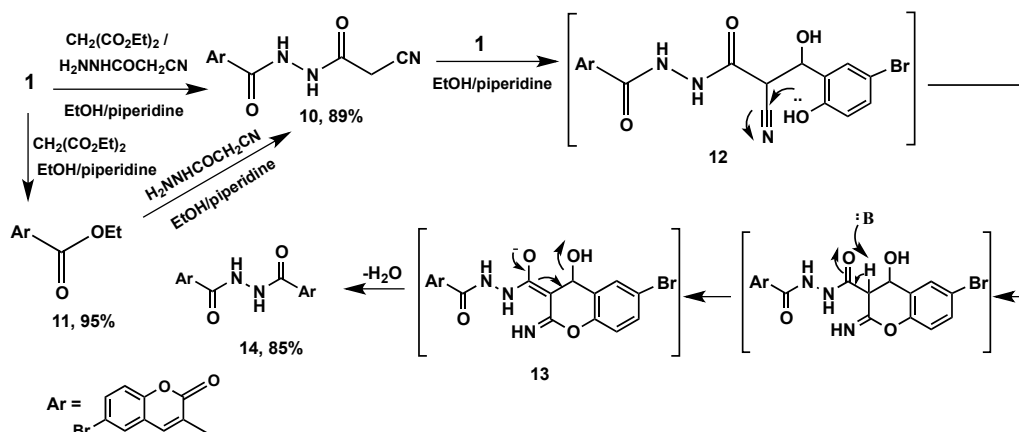
On the other hand, the IR spectrum of 6-bromo-2-oxo-*N'*-(1-phenylethylidene)-2*H*-chromene-3-carbohydrazide (**9**) revealed absorption bands 3335, 3038 and 1690 cm<sup>-1</sup> corresponding to NH, C-H aromatic and C=O, respectively. Its <sup>1</sup>H NMR spectrum showed three singlet signals at δ 8.36, 7.95 and 1.37 ppm due to chromene C=CH, NH and methyl protons, respectively, in addition to aromatic protons at δ 7.25-7.71 ppm. Its mass spectrum revealed a peak at 384 (M<sup>+</sup>, 100) corresponding to its molecular ion. (Scheme 1).



**Scheme 1.** Formation of 2*H*-chromene-3-carbohydrazide **3**, **5**, **7** and **9** derivatives

One pot reaction of **1** with diethyl malonate and cyanoacetic acid hydrazide in refluxing ethanol containing a catalytic amount of piperidine afforded the corresponding 6-bromo-*N'*-(2-cyanoacetyl)-2-oxo-2*H*-chromene-3-carbohydrazide (**10**). The analytical and spectral data of the latter product were consistent with the proposed structure. Its IR spectrum showed absorption bands at 3367, 3275, 2201, 1719 and 1654  $\text{cm}^{-1}$  due to 2NH, CN and two C=O functions, respectively. Its  $^1\text{H}$  NMR spectrum displayed, besides an aromatic multiplet at  $\delta$  7.25-7.71 ppm, four singlet signals at  $\delta$  3.91, 7.96, 8.07 and 8.91 ppm due to methylene, 2NH and chromene C=CH protons, respectively. Compound **10** was alternatively obtained *via* two-step route: by reaction of **1** with diethyl malonate to afford ethyl 6-bromo-2-oxo-2*H*-chromene-3-carboxylate (**11**) followed by reaction of **11** with cyanoacetic acid hydrazide (Scheme 2).

Reaction of compound **10** with 5-bromo-2-hydroxybenzaldehyde (**1**) resulted in the formation of the diacylhydrazide derivative **14**. Mechanistically, formation of **14** proceeded *via* the initial addition of the active methylene nucleophilic carbon ( $-\text{CH}_2\text{CN}$ ) of **10** at the activated carbonyl carbon of **1** to give Michael adduct **12** which underwent an intramolecular cyclization via attack of the OH group to the nitrile function to form the imino-cyclic derivative (**13**), which upon oxidation and isomerization gave 6-bromo-*N'*-(6-bromo-2-oxo-2*H*-chromene-3-carbonyl)-2-oxo-2*H*-chromene-3-carbohydrazide (**14**). Structure **14** (Scheme 2) was assigned as the correct structure based on its mass spectroscopy which showed a peak at  $m/z$  534 corresponding to the molecular ion ( $\text{M}^{+2}$ ) of  $\text{C}_{20}\text{H}_{10}\text{Br}_2\text{N}_2\text{O}_6$ . Its IR spectrum was free of absorption band due to CN function, as well as the absence of methylene group at  $\delta$  3.91 ppm in its  $^1\text{H}$  NMR spectrum.



**Scheme 2.** Formation of 2*H*-chromene-3-carbohydrazide **10**, **14** and its 3-carboxylate **11** derivatives

We extended our study to synthesize some tri- and tetra-fused-heterocyclic derivatives. Thus, the reaction of **1** with ethyl benzoylacetate in refluxing ethanol in the presence of piperidine yielded compound **15** in excellent yield. The structure of **15** was confirmed based on its elemental analyses and spectral data (IR,  $^1\text{H}$  NMR and MS). The IR spectrum of **15** revealed absorption bands at 3050 and 1763  $\text{cm}^{-1}$  corresponding to C-H aromatic and C=O function, respectively. Its  $^1\text{H}$  NMR spectrum revealed one singlet signal at  $\delta$  8.51 ppm due to chromene C=CH proton. Its mass spectrum showed a molecular ion peak at  $m/z$  328 ( $\text{M}^+$ , 72) corresponding to a molecular formula  $\text{C}_{16}\text{H}_9\text{BrO}_3$ .

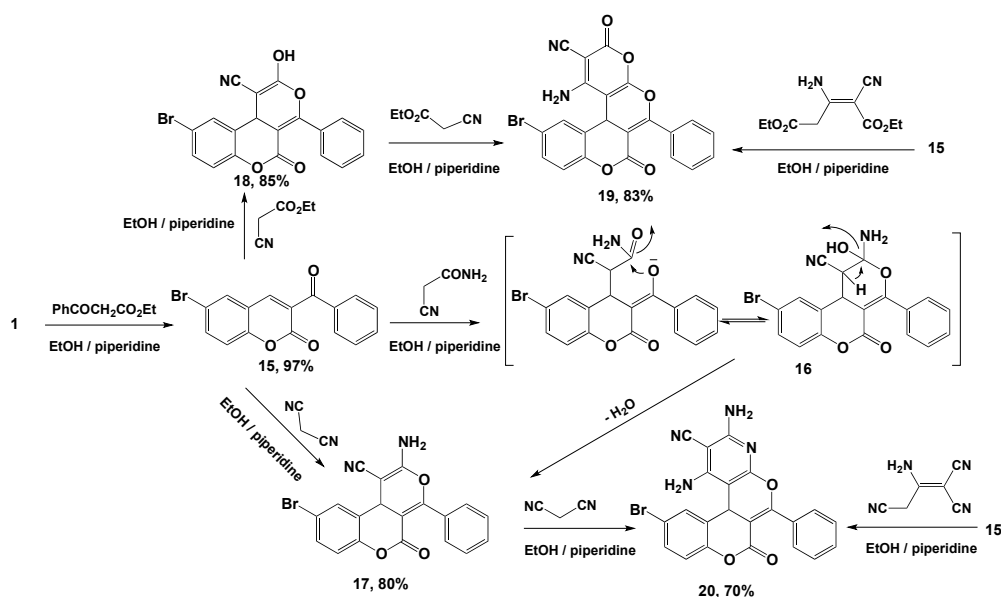
The reaction of **15** with cyanoacetamide afforded 2-amino-9-bromo-5-oxo-4-phenyl-5,10*b*-dihydro-pyrano[3,4-*c*]chromene-1-carbonitrile (**17**) *via* intermediate **16**. The structure of the formed product was unambiguously confirmed from its elemental analyses and spectral data. The IR spectrum of **17** showed absorption bands at 3347, 2210 and 1652  $\text{cm}^{-1}$  due to  $\text{NH}_2$ , CN and CO, respectively. Its  $^1\text{H}$  NMR spectrum showed a singlet signal at  $\delta$  4.23 ppm due to 4*H*-pyran proton. Its mass spectrum showed a molecular ion peak at  $m/z$  394 ( $\text{M}^+$ , 92). Alternatively, reaction of **15** with malononitrile under typical reaction condition afforded a product identical in all aspects (TLC, mp and spectra) with compound **17** (Scheme 3).

The tricyclic compound; 9-bromo-2-hydroxy-5-oxo-4-phenyl-5,10*b*-dihydropyrano[3,4-*c*]chromene-1-carbonitrile (**18**) was synthesized through the reaction of **15** with ethyl cyanoacetate. The structure of the cyclocondensation product **18** was established based on its elemental analyses and spectral data. The IR spectrum of **18** showed absorption bands at 3556, 2250 and 1658  $\text{cm}^{-1}$  due to OH, CN and CO, respectively. Its  $^1\text{H}$  NMR spectrum showed two singlet signals at  $\delta$  16.11 and 4.21 ppm due to OH and 4-pyran protons, respectively. Its mass spectrum showed peak at  $m/z$  395 corresponding to the molecular ion ( $\text{M}^+$ ).

In addition, the tetracyclic compound; 1-amino-11-bromo-3,7-dioxo-6-phenyl-7,12-

dihydro-3*H*-pyrano[3',2':5,6]pyrano[3,4-*c*]chromene-2-carbonitrile (**19**) and 1,3-diamino-11-bromo-7-oxo-6-phenyl-7,12*b*-dihydrochromeno[4',3':4,5]pyrano[2,3-*b*]pyridine-2-carbonitrile (**20**) were synthesized through the reaction of compounds **18** and **17** with ethyl cyanoacetate and malononitrile, respectively, in refluxing ethanol. An alternative support for products **19** and **20**, the coumarin derivative **15** was treated with diethyl 3-amino-2-cyanopent-2-enedioate and with 2-aminoprop-1-ene-1,1,3-tricarbonitrile, respectively, in refluxing ethanol to afford products identical (TLC, mp and spectra) with compounds **19** and **20**, respectively.

The structures of **19** and **20** were confirmed from their elemental analyses and spectral data (IR, <sup>1</sup>H NMR and MS). The IR spectrum of **19** displayed absorption bands at 3250, 2200 and 1630 cm<sup>-1</sup> due to NH<sub>2</sub>, CN and CO, respectively. Its <sup>1</sup>H-NMR spectrum exhibited two singlet signals at δ 3.7 and 8.21 ppm due to 4-pyran and NH<sub>2</sub> protons, respectively. Also, its mass spectrum indicated a molecular ion peak at *m/z* 462 (M<sup>+</sup>). Similarly, the IR spectrum of **20** revealed absorption bands at 3254, 3208, 2246 and 1658 cm<sup>-1</sup> due to two NH<sub>2</sub>, CN and CO groups, respectively. Its <sup>1</sup>H NMR spectrum showed three singlet signals at δ 7.99, 6.12 and 4.23 ppm due to 2NH<sub>2</sub> and 4-pyran protons, respectively. Also, its mass spectrum indicated a molecular ion peak at *m/z* 460 (M<sup>+</sup>) (Scheme 3).

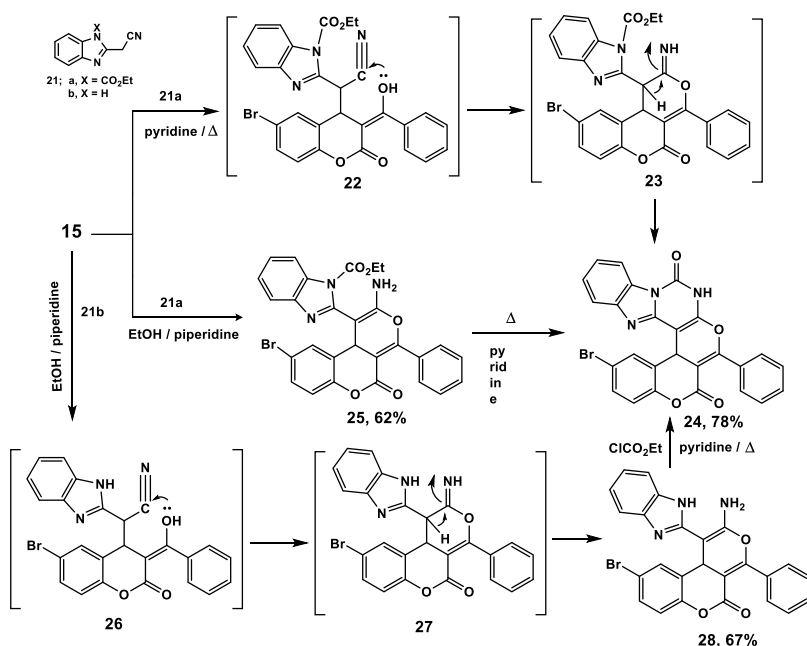


**Scheme 3.** Formation of 2*H*-chromene-based tri- and tetra-fused-heterocycles **17**–**20** derivatives

Next, ethyl 2-cyanomethylbenzimidazole-1-carboxylate (**21a**) was also found to be a fruitful substrate for one-step synthesis of the novel polyheterocyclic structure **24** via its reaction with compound **15**. Thus, reaction of **21a** with **15** in refluxing pyridine afforded directly a single isolable product named as 2-bromo-7-phenylbenzimidazo[1,2-*c*]chromeno[4',3':4,5]pyrano-[3,2-*e*]pyrimidine-6,10(9*H*,16*cH*)-dione (**24**) in a high yield. Formation of **24** is assumed to proceed via the non-isolable intermediates **22** and **23**

which underwent spontaneous intramolecular cyclization under the reaction conditions to give the final product **24**. The structure of the latter product was established based on its elemental analyses and spectral data. Its IR spectrum showed absorption bands at 3332, 1735 and 1658  $\text{cm}^{-1}$  assignable to one NH, and two carbonyl functions, respectively.

It is noteworthy that compound **15** reacted with **21a** in refluxing ethanol containing catalytic amount of piperidine and furnished ethyl 2-(2-amino-9-bromo-5-oxo-4-phenyl-5,10*b*-dihydropyrano[3,4-*c*]chromen-1-yl)-1*H*-benzimidazole-1-carboxylate (**25**), which underwent a readily intramolecular cyclization under reflux in pyridine affording a product identical in all aspects (TLC, mp and spectra) with **24**.  $^1\text{H}$  NMR spectrum of **25** revealed, besides an aromatic multiplet at  $\delta$  7.19-7.86, a broad singlet signal at  $\delta$  8.52 ppm assignable to  $\text{NH}_2$ . Moreover, structure of the product **24** was confirmed by an unambiguous alternative synthesis via two-step reaction sequence as shown in scheme 4. Firstly, reaction of 2-(1*H*-benzimidazol-2-yl)acetonitrile (**21b**) with **15** furnished 2-amino-1-(1*H*-benzimidazol-2-yl)-9-bromo-4-phenylpyrano[3,4-*c*]chromen-5-(10*bH*)-one (**28**). The structure of the latter product was substantiated from its elemental analyses and spectral data. Its IR spectrum showed absorption bands at 3364, 3281 and 1728  $\text{cm}^{-1}$  assignable to NH,  $\text{NH}_2$  and CO groups, respectively.  $^1\text{H}$  NMR spectra of **28** revealed an aromatic multiplet in the region  $\delta$  7.18-7.89 in addition to broad singlet signals at  $\delta$  8.64 and 9.94 ppm assignable to  $\text{NH}_2$  and NH protons, respectively. The absence of any CH-proton signals in the  $^1\text{H}$  NMR spectrum of compound **28** excluded the presence of the intermediates **26** and **27** indicating that they exist mostly in the pyrano[3,4-*c*]chromene form. Second, treatment of **28** with ethyl chloroformate in pyridine under similar reaction condition afforded a product identical (TLC, mp and spectra) with compound **24**.



**Scheme 4.** Formation of 2*H*-chromene-based fused-poly-heterocyclic derivative **24**

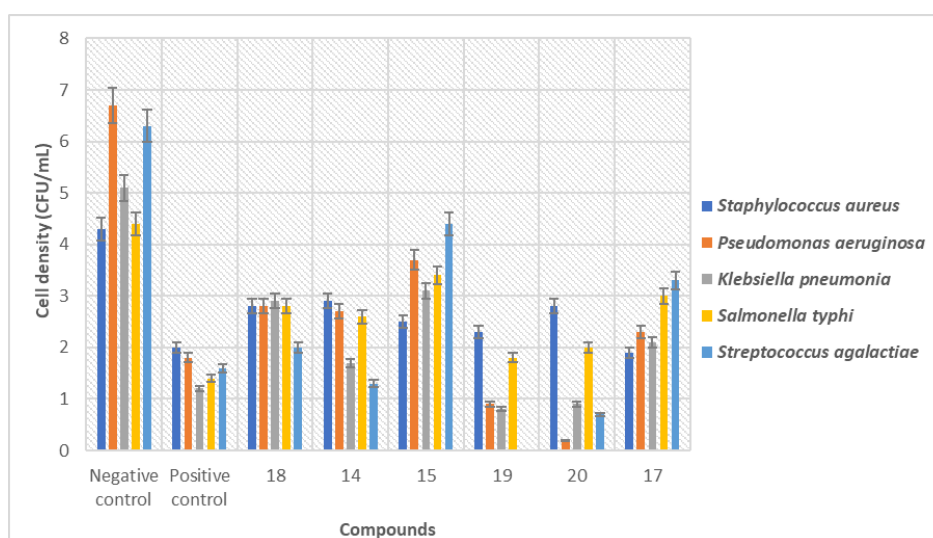
## BIOLOGICAL ACTIVITY

In the last years, the public health has been threatened due to increasing of bacterial resistance to the existing drugs which resulted from the extensive use of antibiotics.<sup>39,40</sup> Therefore, the discovery of new bioactive compounds with antibacterial potential is an urgent need. Synthetic coumarin derivatives have drawn much attention as structurally interesting compounds for synthesizing antimicrobial agents which possess a wide spectrum of biological activities.<sup>41-44</sup> In this study, the inhibitory effect of some new synthesized coumarin derivatives on the growth and secondary metabolites of some common human pathogenic bacteria was evaluated.

As a topic of concern, the antimicrobial drugs progressively loss their effectiveness and the band of resistant pathogens constantly increasing. So, the continual search for effective antimicrobial agents that act against pathogens becomes crucial. Coumarin derivatives are structurally diverse and they can serve as potential agents for novel antimicrobial drugs.<sup>44,45</sup>

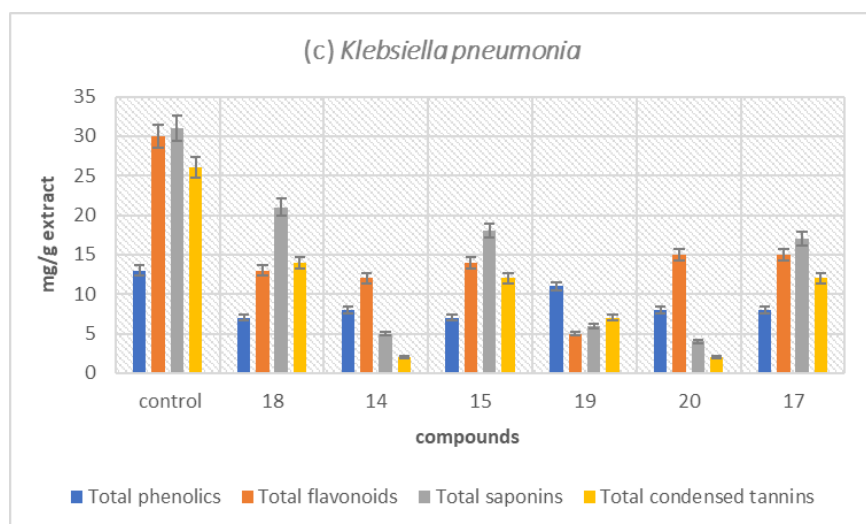
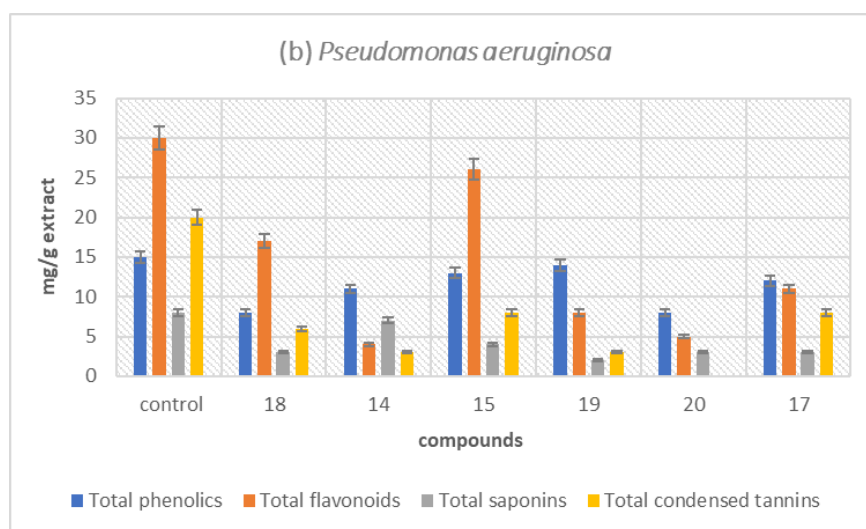
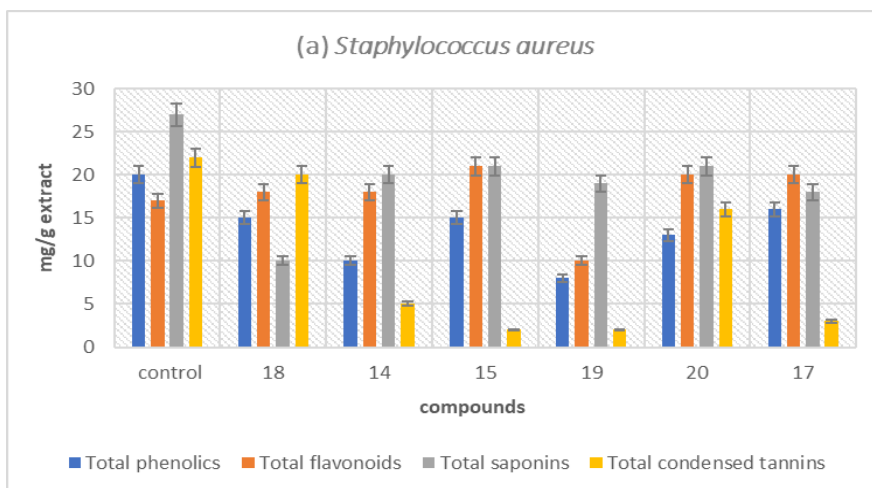
It was reported that, coumarins from natural sources have been extensively studied for their antibacterial activities, whereas, few studies have been focused on antimicrobial characteristics of synthetic coumarin derivatives.<sup>44,46,47</sup> In this study, six new synthesized coumarin derivatives were tested for their antibacterial activity against five common pathogenic bacteria. The study was focused on the inhibition of bacterial growth through the suppression of secondary metabolites production.

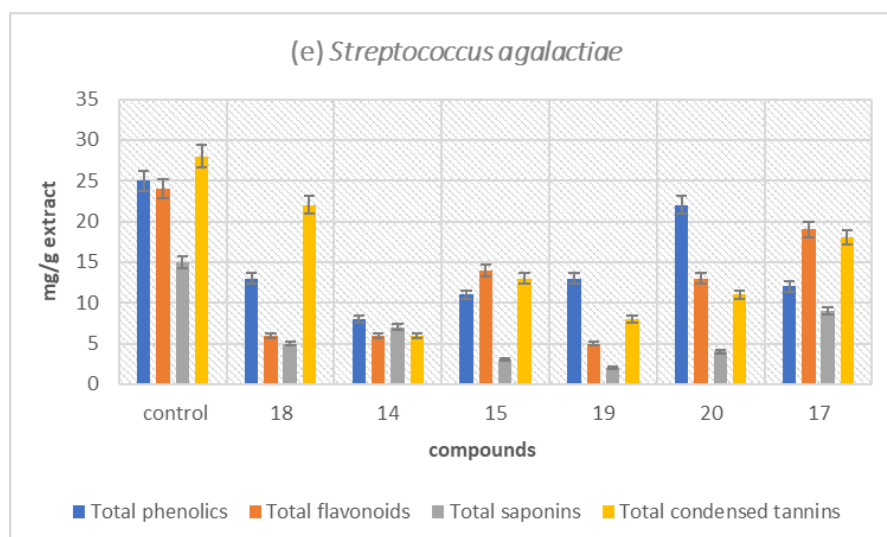
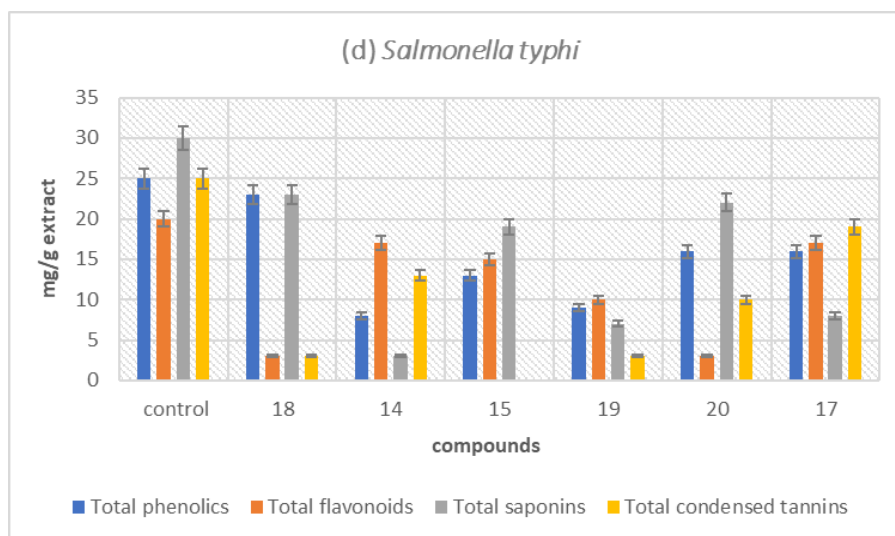
Analysis of the results showed that, all compounds significantly ( $P < 0.05$ ) inhibit the growth of the tested bacterial species compared with the control (Figure 1). The broad-spectrum antibacterial potency of the present synthesized coumarin compounds may be attributed to the presence of bromo and phenyl groups connected to the chromene ring that can control microbial growth via the cell membrane inactivation and metabolite suppression.



**Figure 1.** The effect of compounds on the growth of the tested bacteria (values are means $\pm$  standard deviations)

Antimicrobial drugs affect and alter the metabolites of bacteria resulting in the death or stasis of the cells.<sup>48</sup> In this work, it was found that, all the tested compounds meaningfully suppressed the production of all the screened bioactive secondary metabolites from all the tested bacterial species in parallel with the control (Figure 2a-e).





**Figure 2a-e.** The effect of compounds on the secondary metabolites of the tested bacteria (values are means± standard deviations)

In the field of drug discovery, the determination of the minimum inhibitory concentration (MIC) of a new candidate against pathogenic bacteria is the first step for larger evaluations of antimicrobial agents.<sup>49</sup> The MIC confirms the efficiency of the antimicrobial agent to increase the success of treatment.<sup>50</sup> The MIC of all the tested compounds against each species of the tested pathogenic bacteria were determined and recorded in Table 1.

**Table 1.** The minimum inhibitory concentrations (MIC) in ( $\mu\text{g/mL}$ ) of compounds against the tested bacterial species

Bacterial species					
	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>K. pneumonia</i>	<i>S. typhi</i>	<i>S. agalactiae</i>
Compound	MIC ( $\mu\text{g/mL}$ )				
<b>14</b>	1000	800	1000	400	100
<b>15</b>	400	1000	400	800	400
<b>17</b>	400	100	100	400	400
<b>18</b>	400	100	800	400	100
<b>19</b>	100	50	50	100	25
<b>20</b>	100	400	50	100	100

Our findings are in agreement with previous studies reported by Rama Ganesh et al. who synthesized some coumarin derivatives that were effective against *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumonia* and *Escherichia coli*.<sup>51</sup> Behrami et al. synthesized some novel coumarin derivatives with antibacterial activity against three strains such as *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus*.<sup>52</sup> On the other hand, it was also found that, the synthesized coumarin derivatives by Vaso et al., exhibited antibacterial activity against the Gram-positive bacterial strains; *Staphylococcus aureus* and *Bacillus aureus* and the Gram-negative bacterial strain; *Escherichia coli*.<sup>53</sup> Govori et al. stated the synthesis of new coumarins that revealed antibacterial potential against *Staphylococcus aureus*, *Escherichia coli*, *Hafnia alvei*, *Pseudomonas aeruginosa* and *Enterobacter cloacae*.<sup>54</sup>

Therefore, we suggest that the present compounds can successfully be used as antibacterial agents against the pathogenic species *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi* and *Streptococcus agalactiae*.

## CONCLUSION

Interestingly, we reported a facile and efficient route to several polyheterocyclic structures employing readily accessible starting substrates. The synthesized compounds were tested for their antimicrobial activity and they exhibited significant effects against all the tested bacterial species. Compounds **19** and **20** were the most active compounds, while **17** and **18** were moderately effective and **15** had the weakest effect. Therefore, we suggest that compounds **19** and **20** can be used as a source of potential antibacterial drugs candidate.

## EXPERIMENTAL

All melting points are uncorrected. IR spectra were recorded using KBr pellets and a Perkin-Elmer 2000 FT-IR instrument at Aswan University.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker spectrometer (400 MHz for  $^1\text{H}$  NMR and 100 MHz for  $^{13}\text{C}$  NMR). Chemical shifts ( $\delta$ ) were expressed in parts per million and internally referenced (2.49 ppm for DMSO- $d_6$  for  $^1\text{H}$  NMR and 39.5 ppm for DMSO- $d_6$  for  $^{13}\text{C}$  NMR). Mass spectra were measured using VG Autospec MS 9 (AEI) spectrometer, with the EI (70 eV) model. The microanalysis was performed at Microanalytical Center, Cairo University Egypt.

**Synthesis of 6-bromo-2-oxo- $N'$ -(2-oxoindolin-3-ylidene)-2H-chromene-3-carbohydrazide (3), 6-bromo-2-oxo- $N'$ -(1-(thiophen-2-yl)ethylidene)-2H-chromene-3-carbohydrazide (5), 6-bromo-2-oxo- $N'$ -(2-oxo-1,2-diphenylethylidene)-2H-chromene-3-carbohydrazide (7) and 6-bromo-2-oxo- $N'$ -(1-phenylethylidene)-2H-chromene-3-carbohydrazide (9) (General Procedures).**

A solution of 5-bromo-2-hydroxybenzaldehyde (**1**) (2 mmol) in absolute EtOH (30 mL) was treated with 2-cyano- $N'$ -(2-oxoindolin-3-ylidene)acetohydrazide (**2**), 2-cyano- $N'$ -(1-(thiophen-2-yl)ethylidene)acetohydrazide (**4**), 2-cyano- $N'$ -(2-oxo-1,2-diphenylethylidene)acetohydrazide (**6**) and 2-cyano- $N'$ -(1-phenylethylidene)acetohydrazide (**8**) (2 mmol) in the presence of piperidine as a basic catalyst. The reaction mixture was refluxed for 4-8 h (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from dioxane to afford (92-95%, yield) **3**, **5**, **7** and **9**, respectively.

**6-Bromo-2-oxo- $N'$ -(2-oxoindolin-3-ylidene)-2H-chromene-3-carbohydrazide (3).**

Pale brown solid (95%, yield), mp 260-261 °C; IR: ( $\nu_{\text{max}}$  / $\text{cm}^{-1}$ ): 3414 (NH) and 1715 (CO);  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ),  $\delta$  8.82 (s, 1H), 8.24 (s, 1H,  $D_2O$ -exchangeable), 8.07 (s, 1H,  $D_2O$ -exchangeable) and 7.19-7.49 (m, 7H) ppm;  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$ : 115.8, 118.2, 119, 119.5, 119.9, 120.3, 125.1, 124.8, 129.6, 130.5, 132.2, 134.4, 135.1, 141.8, 153.7, 161.3, 168.9, 169.4 ppm; MS,  $m/z$  (%) 411 ( $M^+$ , 99.8), 412 ( $M^++1$ , 24.6), 413 ( $M^++2$ , 100), 391 (29.07), 367 (46.3), 399 (11), 289 (6.6), 84 (23.8), 77 (9.1), 55 (12.6); (Anal. Calcd for  $\text{C}_{18}\text{H}_{10}\text{BrN}_3\text{O}_4$ : C, 52.45; H, 2.45; Br, 19.39; N, 10.19, found: C, 52.43; H, 2.42; Br, 19.37; N, 10.15%).

**6-Bromo-2-oxo- $N'$ -(1-(thiophen-2-yl)ethylidene)-2H-chromene-3-carbohydrazide (5).**

Yellow solid (93%, yield), mp 220-222 °C; IR: ( $\nu_{\text{max}}$  / $\text{cm}^{-1}$ ): 3330 (NH), 3048 (C-H aromatic) and 1684 (CO);  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ),  $\delta$  1.41 (s, 3H), 7.25-7.96 (m, 6H), 8.02 (s, 1H,  $D_2O$ -exchangeable) and 8.81 (s, 1H) ppm,  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$ : 21.3, 115.2, 119.1, 119.8, 120.3, 124.6, 125.1, 126.9, 127.6, 128.1, 130.8, 135.1, 152.8, 156.3, 160.8, 169.1 ppm; MS,  $m/z$  (%) 390 ( $M^+$ , 100), 391 ( $M^++1$ , 18.4), 392 ( $M^++2$ , 96), 330 (22.2), 251 (7.3), 223 (12.6), 89 (16.5), 71 (6.3), 66 (13.6); (Anal. Calcd for  $\text{C}_{16}\text{H}_{11}\text{BrN}_2\text{O}_3\text{S}$ : C, 49.12; H, 2.83; Br, 20.42; N, 7.16, found: C, 49.10; H, 2.80; Br, 20.40; N, 7.14%).

**6-Bromo-2-oxo-*N'*-(2-oxo-1,2-diphenylethylidene)-2*H*-chromene-3-carbohydrazide (7).**

Yellow solid (92%, yield), mp 235-237 °C; IR: ( $\nu_{\max}$  /cm<sup>-1</sup>): 3257 (NH), 3048 (C-H aromatic) and 1660 (CO); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>),  $\delta$  8.63 (s, 1H, ), 7.96 (s, 1H, *D*<sub>2</sub>O-exchangable) and 7.25-7.71 (m, 13H) ppm; <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$ : 194.1, 168.9, 160.5, 152.7, 142.5, 138.6, 135.1, 134.6, 133.8, 131.6, 130.9, 129.6, 129.3, 128.9, 128.4, 125.3, 120.1, 119.8, 119, 114.7 ppm; MS, *m/z* (%) 474 (M<sup>+</sup>, 50.9), 475 (M<sup>+</sup>+1, 25.5), 476 (M<sup>+</sup>+2, 50.5), 448 (19.4), 250 (72.5), 89 (26.7), 77 (23.9), 63 (29.4); (Anal. Calcd for C<sub>24</sub>H<sub>15</sub>BrN<sub>2</sub>O<sub>4</sub>: C, 60.65; H, 3.18; Br, 16.81; N, 5.89, found: C, 60.61; H, 3.15; Br, 16.80; N, 5.9%).

**6-Bromo-2-oxo-*N'*-(1-phenylethylidene)-2*H*-chromene-3-carbohydrazide (9).**

Pale yellow solid (93%, yield), mp 250-252 °C; IR: ( $\nu_{\max}$  /cm<sup>-1</sup>): 3335 (NH), 3038 (C-H aromatic) and 1690 (CO); <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>),  $\delta$  8.36 (s, 1H), 7.95 (s, 1H, *D*<sub>2</sub>O-exchangable), 7.25-7.71 (m, 8H) and 1.37 (s, 3H) ppm; <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$ : 169.3, 160.1, 152.6, 148.2, 138.1, 135.1, 131.8, 130.4, 129.3, 128.7, 125.2, 120.4, 119.3, 118.5, 114.9, 23.1 ppm; MS, *m/z* (%) 384 (M<sup>+</sup>, 100), 385 (M<sup>+</sup>+1, 36.1), 386 (M<sup>+</sup>+2, 99.9), 370 (14.7), 252 (4.3), 223 (5.5), 89 (8.95), 77 (15.5); (Anal. Calcd for C<sub>18</sub>H<sub>13</sub>BrN<sub>2</sub>O<sub>3</sub>: C, 56.12; H, 3.40; Br, 20.74; N, 7.27, found: C, 56.10; H, 3.38; Br, 20.71; N, 7.26%).

**6-Bromo-*N'*-(2-cyanoacetyl)-2-oxo-2*H*-chromene-3-carbohydrazide (10).**

**Route A:** A solution of (1) (2 mmol) in absolute EtOH (30 mL) was treated with 2-cyanoacetohydrazide (2 mmol) and diethyl malonate (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4-6 h (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from EtOH to give (89%, yield) of pale brown solid of 10, mp 190-192 °C; IR: ( $\nu_{\max}$  /cm<sup>-1</sup>): 3367, 3275 (2NH), 2201 (CN) and 1719, 1654 (2CO), <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>),  $\delta$  8.91 (s, 1H), 8.07 (s, 1H, *D*<sub>2</sub>O-exchangable), 7.96 (s, 1H, *D*<sub>2</sub>O-exchangable), 7.25-7.71 (m, 3H) and 3.91 (s, 2H) ppm; <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$ : 171.2, 166.4, 160.1, 152.7, 135.1, 130.8, 126.3, 125.2, 120.2, 119.7, 118.3, 115.4, 27.3 ppm; MS, *m/z* (%) 348 (M<sup>+</sup>, 100), 349 (M<sup>+</sup>+1, 32.5), 347 (M<sup>+</sup>-1, 35.5), 346 (M<sup>+</sup>-2, 96.6), 291 (4.4), 84 (3.9), 77 (3.4); (Anal. Calcd for C<sub>13</sub>H<sub>8</sub>BrN<sub>3</sub>O<sub>4</sub>: C, 44.60; H, 2.30; Br, 22.82; N, 12.00, found: C, 44.57; H, 2.28; Br, 22.80; N, 12.00%).

**Route B:** A solution of ethyl 6-bromo-2-oxo-2*H*-chromene-3-carboxylate (11) (2 mmol) in absolute EtOH (30 mL) was treated with 2-cyanoacetohydrazide (0.2 g, 2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4-6 h (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from EtOH to afford a product identical (TLC, mp and spectra) with compound (10).

**Ethyl 6-bromo-2-oxo-2*H*-chromene-3-carboxylate (11).**

A solution of (1) (2 mmol) in absolute EtOH (30 mL) was treated with diethyl malonate (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4 h (TLC control), after

evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from dioxane to give (95%, yield) of pale yellow solid, mp 200-201 °C; IR: ( $\nu_{\max}$  / $\text{cm}^{-1}$ ): 3050 (C-H aromatic) and 1763, 1698 (2CO);  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-}d_6$ )  $\delta$ : 166.4, 157.1, 152.7, 134.6, 131.2, 125.3, 120.2, 119.8, 118.7, 114.1, 62.3, 14.9 ppm; MS,  $m/z$  (%) 296 ( $\text{M}^+$ , 99), 297 ( $\text{M}^++1$ , 17.2), 298 ( $\text{M}^++2$ , 100), 253 (44.7), 224 (13.9), 88 (30.9), 62 (32.6); (Anal. Calcd for  $\text{C}_{12}\text{H}_9\text{BrO}_4$ : C, 48.51; H, 3.05; Br, 26.89, found: C, 48.49; H, 3.01; Br, 26.85%).

#### **6-Bromo-*N'*-(6-bromo-2-oxo-2*H*-chromene-3-carbonyl)-2-oxo-2*H*-chromene-3-carbohydrazide (14).**

A solution of **1** (2 mmol) in absolute EtOH (30 mL) was treated with **10** (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4-6 h (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from EtOH to give (85%, yield) of red solid, mp 290-291 °C; IR: ( $\nu_{\max}$  / $\text{cm}^{-1}$ ): 3390, 3255 (2NH) and 1713, 1653 (2CO),  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ),  $\delta$  9.31 (s, 1H,  $\text{D}_2\text{O-exchangable}$ ), 9.11 (s, 1H,  $\text{D}_2\text{O-exchangable}$ ) and 7.25-8.06 (m, 8H) ppm;  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-}d_6$ )  $\delta$ : 166.5, 160.4, 152.6, 135.1, 131.6, 124.8, 120.3, 119.5, 118.1, 115.2 ppm; MS,  $m/z$  (%) 532 ( $\text{M}^+$ , 47.55), 534 ( $\text{M}^++2$ , 85), 536 ( $\text{M}^++4$ , 47.45), 487 (17.92), 348 (96.2), 267 (28), 251 (79.12), 211 (23.68), 114 (19.88), 77 (36.37), 57 (39.68); (Anal. Calcd for  $\text{C}_{20}\text{H}_{10}\text{Br}_2\text{N}_2\text{O}_6$ : C, 44.97; H, 1.89; Br, 29.92; N, 5.24, found: C, 44.95; H, 1.87; Br, 29.90; N, 5.25%).

#### **3-Benzoyl-6-bromo-2*H*-chromen-2-one (15).**

A solution of **1** (5 mmol) in absolute EtOH (50 mL) was treated with ethyl 3-oxo-3-phenylpropanoate (5 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4-6 h (TLC control). After evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from EtOH to give (97%, yield) of colorless needle, mp 170-172 °C; IR: ( $\nu_{\max}$  / $\text{cm}^{-1}$ ): 3050 (C-H aromatic) and 1763 (CO),  $^1\text{H-NMR}$  (400 MHz,  $\text{DMSO-}d_6$ ),  $\delta$  8.51 (s, 1H) and 7.25-8.06 (m, 8H) ppm;  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-}d_6$ )  $\delta$ : 192.2, 160.7, 152.4, 140.2, 138.4, 135.1, 134.8, 130.5, 129.7, 128.4, 128.1, 125.2, 120.3, 118.6 ppm; MS,  $m/z$  (%) 328 ( $\text{M}^+$ , 72.05), 329 ( $\text{M}^++1$ , 17.6), 330 ( $\text{M}^++2$ , 71.25), 251 (52.6), 105 (50), 88 (12.22), 77 (45.5), 62 (10.51), 51 (26.5); (Anal. Calcd for  $\text{C}_{16}\text{H}_9\text{BrO}_3$ : C, 58.38; H, 2.76; Br, 24.28, found: C, 58.35; H, 2.74; Br, 24.26%).

#### **2-Amino-9-bromo-5-oxo-4-phenyl-5,10*b*-dihydropyrano[3,4-*c*]chromene-1-carbonitrile (17).**

**Route A:** A solution of **15** (2 mmol) in absolute EtOH (30 mL) was treated with 2-cyanoacetamide (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4-6 h (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from dioxane to give (80%, yield) of yellow crystal, mp >300 °C; IR: ( $\nu_{\max}$  / $\text{cm}^{-1}$ ): 3347 ( $\text{NH}_2$ ), 2210 (CN) and 1652 (CO),  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ),  $\delta$  4.23 (s, 1H) and 7-8.19 (m, 10H) ppm;  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO-}d_6$ )  $\delta$ : 163.2, 160.1, 156.7, 150.2, 135.6, 133.7, 130.6, 129.5, 129.1, 128.2, 127.9, 124.3, 120.2, 119.3, 104.7, 59.2, 38.3 ppm; MS,  $m/z$  (%) 394 ( $\text{M}^+$ , 92), 395 ( $\text{M}^++1$ , 13), 381

(20), 380 (100), 378 (97.77), 77 (5.3); (Anal. Calcd for C<sub>19</sub>H<sub>11</sub>BrN<sub>2</sub>O<sub>3</sub>: C, 57.74; H, 2.81; Br, 20.22; N, 7.09, found: C, 57.72; H, 2.80; Br, 20.21; N, 7.07%).

**Route B:** A solution of **15** (2 mmol) in absolute EtOH (30 mL) was treated with malononitrile (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4-6 h (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from dioxane to afford a product identical (TLC, mp and spectra) with compound (**17**).

**9-Bromo-2-hydroxy-5-oxo-4-phenyl-5,10b-dihydropyrano[3,4-c]chromene-1-carbonitrile (18).**

A solution of **15** (2 mmol) in absolute EtOH (30 mL) was treated with ethyl 2-cyanoacetate (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4-6 h (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from EtOH to give (85%, yield) of colorless crystals, mp 260-262 °C; IR: ( $\nu_{\max}$  /cm<sup>-1</sup>): 3556 (OH), 2250 (CN) and 1658 (CO), <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>),  $\delta$  16.11 (s, 1H), 7.3-7.78 (m, 8H) and 4.21 (s, 1H) ppm; <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$ : 207.1, 162.3, 160.5, 150.1, 136.7, 135.2, 130.7, 129.4, 128.5, 128, 127.6, 124.1, 120.3, 116.8, 104.9, 51.4, 37.2 ppm; MS, *m/z* (%) 395 (M<sup>+</sup>, 51.22), 396 (M<sup>+</sup>+1, 12.02), 397 (M<sup>+</sup>+2, 50.28), 381 (45.51), 201 (30.92), 77 (10.66), 51 (18.76); (Anal. Calcd for C<sub>19</sub>H<sub>10</sub>BrNO<sub>4</sub>: C, 57.60; H, 2.54; Br, 20.17; N, 3.54, found: C, 57.56; H, 2.53; Br, 20.16; N, 3.50%).

**1-Amino-11-bromo-3,7-dioxo-6-phenyl-7,12b-dihydro-3H-pyrano[3',2':5,6]pyrano[3,4-c]chromene-2-carbonitrile (19).**

**Route A:** A solution of **18** (2 mmol) in absolute EtOH (30 mL) was treated with ethyl 2-cyanoacetate (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from dioxane to give (83%, yield) of yellow crystal of **19**, mp >300 °C, IR: ( $\nu_{\max}$  /cm<sup>-1</sup>): 3250 (NH<sub>2</sub>), 2200 (CN) and 1630 (CO), <sup>1</sup>H-NMR (400 MHz, DMSO-*d*<sub>6</sub>),  $\delta$  8.21 (s, 1H), 3.7 (s, 1H) and 7.0-7.71 (m, 9H) ppm; <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$ : 182.4, 162.6, 160.9, 153.1, 150.3, 149.9, 137.2, 134.8, 131.3, 129.4, 128.9, 128, 127.8, 124.2, 120.2, 115.3, 105.7, 90.8, 63.9, 37.2 ppm; MS, *m/z* (%) 462 (M<sup>+</sup>, 27.80), 464 (M<sup>+</sup>+2, 25.59), 448 (19.36), 266 (33.33), 251 (72.5), 223 (19.14), 196 (5.68), 89 (26.68), 77 (23.86); (Anal. Calcd for C<sub>22</sub>H<sub>11</sub>BrN<sub>2</sub>O<sub>5</sub>: C, 57.04; H, 2.39; Br, 17.25; N, 6.05, found: C, 57.01; H, 2.36; Br, 17.22; N, 6.03%).

**Route B:** A solution of **15** (2 mmol) in absolute EtOH (30 mL) was treated with diethyl 3-amino-2-cyanopent-2-enedioate (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4-6 h (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from dioxane to afford a product identical (TLC, mp and spectra) with compound (**19**).

**1,3-Diamino-11-bromo-7-oxo-6-phenyl-7,12b-dihydrochromeno[4',3':4,5]pyrano[2,3-b]pyridine-2-carbonitrile (20).**

A solution of **18** (2 mmol) in absolute EtOH (30 mL) was treated with malononitrile (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4-6 h (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from dioxane to give (70%, yield) of brown crystals **20**, mp >300 °C; IR: ( $\nu_{\max}$  /cm<sup>-1</sup>): 3254, 3208 (2NH<sub>2</sub>), 2246 (CN) and 1658 (CO), <sup>1</sup>H-NMR (400 MHz, DMSO-*d*<sub>6</sub>),  $\delta$  7.99 (s, 2H), 6.12 (s, 2H), 4.23 (s, 1H) and 7.04-7.78 (m, 8H) ppm, MS, *m/z* (%) 460 (M<sup>+</sup>, 38.5), 461 (M<sup>+</sup>+1, 16), 462 (M<sup>+</sup>+2, 36.70), 378 (45.9), 278 (44.8), 105 (38.36), 77 (42.94); (Anal. Calcd for C<sub>22</sub>H<sub>13</sub>BrN<sub>4</sub>O<sub>3</sub>: C, 57.28; H, 2.84; Br, 17.32; N, 12.15, found: C, 57.25; H, 2.81; Br, 17.30; N, 12.13%).

**Route B:** A solution of **15** (2 mmol) in absolute EtOH (30 mL) was treated with 2-aminoprop-1-ene-1,1,3-tricarbonitrile (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from dioxane afforded a product identical (TLC, mp and spectra) with compound (**20**).

**2-Bromo-7-phenylbenzimidazo[1,2-c]chromeno[4',3':4,5]pyrano[3,2-e]pyrimidine-6,10(9H,16cH)-dione (24).**

A solution of **15** and/or **28** (2 mmol) in pyridine (30 mL) was treated with ethyl 2-(cyanomethyl)-1*H*-benzimidazole-1-carboxylate (**21a**) (2 mmol) or ethyl chloroformate (2 mL), respectively. The reaction mixture was refluxed (TLC control) for 4-6 h, then left to cool, diluted with acidic ice water, the solid product was collected by filtration and finally recrystallized from dioxane to give (78%, yield) of dark brown solid, mp > 350 °C; IR: ( $\nu_{\max}$  /cm<sup>-1</sup>): 3332 (NH), 1735 and 1658 (2CO), <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>),  $\delta$  10.23 (s, 1H, *D*<sub>2</sub>*O*-exchangeable), 7.16-7.83 (m, 12H) and 4.11 (s, 1H) ppm; (Anal. Calcd for C<sub>26</sub>H<sub>14</sub>BrN<sub>3</sub>O<sub>4</sub>: C, 60.95; H, 2.75; Br, 15.60; N, 8.20, found: C, 60.89; H, 2.70; Br, 15.56; N, 8.18%).

**Synthesis of ethyl 2-(2-amino-9-bromo-5-oxo-4-phenyl-5,10b-dihydropyrano[3,4-c]chromen-1-yl)-1*H*-benzimidazole-1-carboxylate (25) and 2-amino-1-(1*H*-benzimidazol-2-yl)-9-bromo-4-phenylpyrano[3,4-c]chromen-5-(10b*H*)-one (28) (General Procedures)**

A solution of **15** (2 mmol) in absolute EtOH (30 mL) was treated with ethyl 2-(cyanomethyl)-1*H*-benzimidazole-1-carboxylate and/or 2-(1*H*-benzimidazol-2-yl)acetonitrile (2 mmol) in the presence of piperidine as a catalyst. The reaction mixture was refluxed for 4-6 h (TLC control), after evaporation of the solvent, the solid product was collected by filtration and finally recrystallized from DMF/EtOH.

**Ethyl 2-(2-amino-9-bromo-5-oxo-4-phenyl-5,10*b*-dihydropyrano[3,4-*c*]chromen-1-yl)-1*H*-benzimidazole-1-carboxylate (25).**

Yellow solid, (62%, yield), mp 310-312 °C; IR: ( $\nu_{\max}$  /cm<sup>-1</sup>): 3210 (NH<sub>2</sub>) 1738 and 1723 (2CO), <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>),  $\delta$  8.52 (brs, 2H, *D*<sub>2</sub>O-exchangable), 7.19-7.86 (m, 12H), 4.17 (s, 1H), 4.21-4.23 (q, 2H) and 1.27-1.29 (t, 3H) ppm; (Anal. Calcd for C<sub>28</sub>H<sub>20</sub>BrN<sub>3</sub>O<sub>5</sub>: C, 60.23; H, 3.61; Br, 14.31; N, 7.53, found: C, 60.17; H, 3.54; Br, 14.27; N, 7.51%).

**2-Amino-1-(1*H*-benzimidazol-2-yl)-9-bromo-4-phenylpyrano[3,4-*c*]chromen-5-(10*bH*)-one (28).**

Pale brown solid, (67%, yield), mp 280-281 °C; IR: ( $\nu_{\max}$  /cm<sup>-1</sup>): 3364, 3281 (NH and NH<sub>2</sub>) and 1725 (CO), <sup>1</sup>H-NMR (400 MHz, DMSO-*d*<sub>6</sub>),  $\delta$  9.94 (brs, 1H, *D*<sub>2</sub>O-exchangable), 8.64 (brs, 2H, *D*<sub>2</sub>O-exchangable), 7.18-7.89 (m, 12H) and 3.99 (s, 1H) ppm; <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>)  $\delta$ : 162.4, 157.2, 157.1, 149.8, 142.6, 139.2, 134.3, 133.7, 130.4, 129.4, 128.8, 127.8, 124, 123.4, 120.1, 115.5, 105.6, 87.2, 32.3 ppm; MS, *m/z* (%) 485 (M<sup>+</sup>, 40.67), 486 (M<sup>+</sup>+1, 19.8), 487 (M<sup>+</sup>+2, 39.83), 407 (17.24), 332 (100), 304 (16.04), 84 (12.94), 57 (16.31); (Anal. Calcd for C<sub>25</sub>H<sub>16</sub>BrN<sub>3</sub>O<sub>3</sub>: C, 61.74; H, 3.32; Br, 16.43; N, 8.64, found: C, 61.73; H, 3.30; Br, 16.41; N, 8.61%).

**BIOLOGICAL PART****Tested pathogenic bacteria:**

Stock cultures of five common pathogenic bacteria i.e. *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa*, *Klebsiella pneumonia* (ATCC 13882), *Salmonella typhi* (ATCC 14028) and *Streptococcus agalactiae* were kindly obtained from the Bacteriology Lab, Department of Botany, Faculty of Science, Aswan University by Dr. Noura Sh. A. Hagaggi (Co-author).

**Effect of compounds on the growth and secondary metabolites of the tested bacterial species:**

In 250 mL capacity conical flasks, 50 mL of Mueller-Hinton broth was supplemented with 0.5 g of each compound, inoculated with 0.5 mL of bacterial suspension (1×10<sup>7</sup> CFU/mL) and incubated for 48 h at 37 °C under 150 rpm. 50 mL of Mueller-Hinton broth inoculated with 0.5 mL of bacterial suspension was used as negative control. 50 mL of Mueller-Hinton broth supplemented with standard ampicillin solution (30 µg/mL) inoculated with 0.5 mL of bacterial suspension was used as positive control. The growth was estimated spectrophotometrically by measuring the optical density (OD) at 600 nm. Cultures were centrifuged and the supernatants were extracted with equal volumes of EtOAc. Total phenolics, flavonoids, saponins and tannins were quantified in each extract.

**Estimation of total phenolics:**

The total phenolics content was estimated by Folin-Ciocalteu method using gallic acid standard curve.<sup>55</sup> Briefly, in test tubes, 1 mL of Folin-Ciocalteu reagent was mixed with 1 mL of the extract and 1 mL of 10% Na<sub>2</sub>CO<sub>3</sub> was then added. Tubes were incubated for 1 h at room temperature. After that, the

absorbance was recorded at 700 nm and the total phenolics amount was calculated.

#### **Estimation of total flavonoids:**

Briefly, 1 mL of the extract was mixed with 0.3 mL of NaNO<sub>2</sub> (5%). After 6 min, 0.3 mL of AlCl<sub>3</sub> (10%) was added and the mixture was incubated for 6 min. Then, 0.4 mL of NaOH (1 M) was added and the mixture was kept for 12 min, with shaking. The absorbance was measured at 510 nm and the content of total flavonoids was calculated using quercetin standard curve.<sup>56</sup>

#### **Estimation of total saponins:**

The total saponins was quantified according to the method of Hiai *et al.*,<sup>57</sup> 2.5 mL of vanillin reagent prepared in sulfuric acid (2%, w/v) and 1 mL of each extract or saponin standard were mixed. The mixture was vortexed and incubated for 1 h at 60 °C. After that, the mixture was placed in an ice bath for 10 min. Absorbance was recorded at 473 nm and the content of saponins was calculated as mg saponins equivalent/g extract.

#### **Estimation of total condensed tannins:**

The content of total tannins was determined using vanillin assay,<sup>58</sup> 2 mL vanillin (4%, w/v) in MeOH was mixed with 1 mL of the extract, vortexed, then 0.75 mL concentrated HCl was added and leftward at the room temperature for 20 min. Reads were recorded at 550 nm. Total tannins content was calculated using catechol standard curve.

#### **Determination of the minimum inhibitory concentration (MIC):**

One milliliter of sterilized Mueller-Hinton broth supplemented with different concentrations (µg/mL) of each tested compound: 0 (control), 10, 25, 50, 100, 200, 400, 800 and 1000 was inoculated with a loopful of each species suspension (approximately 1×10<sup>7</sup> CFU/mL). Cultures were incubated overnight at 37 °C. After that, the growth was measured spectrophotometrically at 600 nm. The lowest concentration of each compound that inhibited the visible growth was considered as the minimum inhibitory concentration (MIC).<sup>59</sup>

#### **Statistical Analysis:**

The obtained data were analyzed by one-way analysis of variance (ANOVA) using Minitab 12 Statistical Software,<sup>60</sup> *P*- values <0.05 were considered significant.

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