Synthesis, characterization, antibacterial, antioxidant and anti-inflammatory activities of new 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivatives

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ABSTRACT

This study aimed for the synthesis of 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** suitable for use as antibacterial, anti-oxidant and anti-inflammatory activities via a versatile, readily accessible 3-((2E)-3(aryl)prop-2-enoyl)-2H-chromen-2-one **3** which was prepared by refluxing 3-acetyl-4-hydroxycoumarin with aromatic aldehydes in chloroform in the presence of a catalytic amount of piperidine**.** Then the direct reaction of the obtained chalcones **3** with malonitrile in the presence of ammonium acetate in one step gave products **5** The structures of the new compounds **3-4** were confirmed by elemental analysis, IR, and multinuclear/multidimensional NMR spectroscopy (^{1}H, ^{13}C-NMR, NOESY, HMBC) which allowed assignment of the complete network of proton and carbon atoms. Most of the new compounds **4** exhibit moderate antibacterial activity against Gram Negative bacterial strains (E. coli, K. pneumonia, S. typhi and S. flexneri). In addition, the compounds **4** were investigated for anti-oxidant activities by super oxide radical, DPPH (2,2-Diphenyl-1-picrylhydrazyl), and hydroxyl radical scavenging assays, where most of the compounds displayed significant antioxidant activities. Furthermore, these 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** were evaluated for anti-inflammatory activity by indirect haemolytic and lipoxygenase inhibition assays where compounds revealed good activity.

Keywords

4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles; Antibacterial; Antioxidant; Anti-inflammatory; Synthesis

1. INTRODUCTION

The synthesis of 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivatives **4** has attracted considerable attention of organic and medicinal chemists due to its wide usage in the field of drugs and pharmaceuticals with diverse biological activities like anticoagulant [1], antihypertensive [2], anti-inflammatory [3], antipsychotic [4], antidiabetic [5], antihelminthic [6] and antimicrobial activities [7, 8]. Nowadays various bacteria are found to resist classical antimicrobial agents [9]. Also, free radicals are highly reactive species generated endogenously with the involvement of oxygen in normal metabolic conditions and lack of antioxidant capacity of an organism may result in cancer development [10]. Besides, pathogen invasion, injuries and manifestation to chemicals results in inflammation which may leads to cancer, allergy, Alzheimer's disease, rheumatoid arthritis and diabetes mellitus [11].

Based on the literature survey on the pharmacological properties [12], we aimed to synthesize and characterize new 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles incorporating coumarin moiety. The chemical structures of the synthesized compounds **4** were determined by spectroscopic techniques. Their antibacterial, antioxidant activities and anti inflammaratory anti-inflammatory activity by indirect haemolytic and lipoxygenase were investigated.

2. EXPERIMENTAL
1. General

Reactions were monitored by thin layer chromatography (TLC) using precoated sheets of silica gel G/UV-254 of 0.25 mm thickness (Merck 60F254). The melting points were measured in Selaco melting point apparatus in an open capillary and are uncorrected. Elemental analysis (C, H, N) was performed using the CE-400 CHN elemental analyzer. Infrared spectra were recorded on Shimadzu FT-IR model 8300 spectrophotometer using KBr method. ^{1}H NMR spectra were recorded using Brucker 400 NMR spectrometer operating at 400 MHz using TMS as the internal standard and CDCl$_3$ as a solvent. Mass spectra were obtained using Agilant mass spectrometer in ESI mode. All chemicals were obtained from Aldrich, Fluka and Merck Chemicals.

2. Acetyl-4-hydroxycoumarin 2

To a solution of 4-hydroxy-2H-chromen-2-one (3.0 g, 1.86mmol) in acetic acid (16 ml) phosphorus oxychloride (5.6 ml) was added. The mixture was heated at reflux for 30 min. After cooling to room temperature, the precipitate which separated out was collected by filtration and recrystallized from ethanol to give 3-acetyl-4-hydroxy-*2H*-chromen-2-one as white needles. Yield 2.7 g (90%);Mp = 135 °C. IR spectrum, v cm^{-1}: 3185 (OH); 1705 (CO);1700 (O–CO lactone). ^{1}H NMR spectrum (CDCl$_3$) δ:2.72 (3H, s, CH$_3$); 7.98 (1H, s, H-5); 7.95 (1H, dd, ^3J 7.8, 8.35, ^4J 6.8, 1.2 Hz, H-8); 7.1–7.4 (2H, m, H-6 + H-7); 17.7 (1H, s, OH). ^{13}C{^{1}H} NMR spectrum (CDCl$_3$): δ: 29.9 (CH$_3$); 178.5 (CO);159.8 (C-4); 154.6 (C-2); 101.3 (C-3); 115.0–136.0 (C$_{arom}$).

3. General procedure for the preparation of the coumarinic chalcones 3a–e

3-Acetyl-4-hydroxy-*2H*-chromen-2-one (0.031 mol) and the substituted aromatic aldehyde (0.03 mol) were dissolved in 30 mL of chloroform. A catalytic amount of piperidine (0.02 mol) was added and the reaction mixture was refluxed for 1.5 h. The chloroform was removed under vacuum and the residue was washed with methanol

3-((2E)-3(Phenyl)prop-2-enoyl)-2H-chromen-2-one: 3a

Yield 85 Yiled 85%; mp 265 ºC. IR spectrum, v cm^{-1}: 1490 (C=O), 1728 (O-C-O), 1529 (C=C). ^{1}H NMR spectrum (CDCl$_3$) δ ppm: 6.5-8.3 (m,11H,H$_{arom}$,H$_{eth}$); 18.25 (s,1H,OH). ^{13}C NMR spectrum (CDCl$_3$): δ ppm 116. 9 (C$_3$); 135.8 (C$_2$); 147.1(C$_4$); 134.7 (C$_{ethyl}$); 180.6 (CO); 135.9(C$_{éthyl\ 1}$); 131.4(C$_{éthyl\ 2}$); 126. 9 (C5); 125. 4 (C6); 128. 3 (C7); 128. 3 (C8); 135. 1 (C9); 126. 3 (C10); 128. 6 (C11); 127. 9 (C12); 128. 7 (C13); 126. 3 (C14).

3-((2E)-3(4-Fluorophenyl)prop-2-enoyl)-2H-chromen-2-one: 3b

Yiled 80%; mp 255ºC. IR spectrum, v cm^{-1}: 1494 (C=O), 1716 (O-C-O), 1531 (C=C). ^{1}H NMR spectrum (CDCl$_3$, 400MHz) δ ppm: 6.51-8,21(m, 10H, H$_{arom+ethyl}$); 18. 4 (s, 1H, OH). ^{13}C NMR spectrum (CDCl$_3$) δ ppm: 100.9 (C$_3$); 154.8 (C$_2$); 166.0 (C$_4$); 181.5 (CO); 136.2(C$_{éthyl\ 1}$); 131.5(C$_{éthyl\ 2}$);126. 8 (C5); 125. 3 (C6); 128. 4 (C7); 129. 3 (C8); 135. 2 (C9); 126. 5 (C10); 128. 7 (C11); 127. 8 (C12); 128. 6 (C13); 126. 4 (C14). NMR ^{19}F (CDCl$_3$, 400 MHz, t$_{amb}$) δ ppm: -107,8

3-((2E)-3(2, 5-Dimethylphenyl) prop-2-enoyl)-2H-chromen-2-one: 3c

Yield 75 %; mp 224ºC. IR spectrum, v cm^{-1}: 1490 (C=O), 1720 (O-C-O), 1527 (C=C). ^{1}H NMR spectrum (CDCl$_3$,400 MHz) δ ppm: 2,41 (s,1H,CH$_3$),3,21 (s,3H,CH$_3$), 6,82-8,21 (m,9H,H$_{arom+éthy}$);13.81 (s,1H,OH). ^{13}C NMR spectrum (CDCl$_3$): δ ppm: 91. 4 (C$_3$); 135.8 (C$_2$); 161.4 (C$_4$); 139;1 (C$_{ethyl}$), 134.1 (C$_{éthy2}$); 126. 7 (C5); 125. 4 (C6); 128. 3 (C7); 128. 9 (C8); 135. 4 (C9); 126. 4 (C10); 127. 8 (C11); 127. 7 (C12); 128. 5 (C13); 126. 3(C14); 176.2 (CO).

3-((2E)-3(4-Methoxyphenyl) prop-2-enoyl)-2H-chromen-2-one: 3d

Yield 75 %; mp 194ºC. IR spectrum, v cm^{-1}: 1494 (C=O), 1708 (O-C-O), 1595 (C=C). ^{1}H NMR spectrum (CDCl$_3$): δ ppm 3.84(s,3H,OCH$_3$); 6.92 (d,1H,CH); 8.25 (d,1H,CH); 7.25-8.08 (m,8H,H$_{arom}$); 18.05(s,1H,OH). ^{13}C NMR spectrum (CDCl$_3$): δ ppm 55.4 (OCH$_3$); 114.4 (C$_3$); 126. 8 (C5); 125. 2 (C6); 127. 9 (C7); 128. 8 (C8); 134. 8 (C9); 126. 3 (C10); 127. 7 (C11); 127. 5 (C12); 128. 4 (C13); 126. 2(C14); 135.6 (C$_2$); 147.5 (C$_4$); 162.4 (CO).

3-((2E)-3(4-Methoxyphenyl) prop-2-enoyl)-2H-chromen-2-one: 3e

Yield 75 %; mp 194ºC. IR spectrum, ν cm^{-1}: 1494 (C=O), 1708 (O-C-O), 1595 (C=C). ^{1}H NMR spectrum (CDCl$_3$): δ ppm 3.84(s,3H,OCH$_3$); 6.92 (d,1H,CH); 8.25 (d,1H,CH); 7.25-8.08 (m,8H,H$_{arom}$); 18.05(s,1H,OH). ^{13}C NMR spectrum (CDCl$_3$): δ ppm 55.4 (OCH$_3$); 114.4 (C$_3$); 126. 8 (C$_5$); 125. 2 (C$_6$); 127. 9 (C$_7$); 128. 8 (C$_8$); 134. 8 (C$_9$); 126. 3 (C$_{10}$); 127. 7 (C$_{11}$); 127. 5 (C$_{12}$); 128. 4 (C$_{13}$); 126. 2(C$_{14}$); 135.6 (C$_2$); 147.5 (C$_4$); 162.4 (CO).

3-((2E)-3(4-tollyl)prop-2-enoyl)-2H-chromen-2-one: 3f

Yield 72 %; mp 185ºC. IR spectrum, ν cm^{-1}: 1490 (C=O), 1715 (O-C-O), 1540 (C=C). ^{1}H NMR spectrum (CDCl$_3$) δ ppm: 2.1(s,3H,CH$_3$),7.1-8.6 (m,1OH, H$_{arom+ethyl}$); 18.4 (s, 1H,OH). ^{13}C NMR spectrum (CDCl$_3$): δ ppm:22.6(CH$_3$), 98.7 (C$_3$); 151.8 (C$_2$); 178.7 (C$_4$); 182.5 (CO); 151.9(C$_{ethyl\,1}$); 122.5(C$_{ethyl\,2}$); 126. 7 (C$_5$); 125. 3 (C$_6$); 127. 8 (C$_7$); 128. 7 (C$_8$); 135. 2 (C$_9$); 126. 4 (C$_{10}$); 127. 6 (C$_{11}$); 127. 4 (C$_{12}$); 128. 5 (C$_{13}$); 126. 3(C$_{14}$).

4. General procedures for the preparation of 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivative 4

To a mixture of coumarinic chalcones **3a–e** (1g, 5 mmol) in ethyl alcohol (20mL), malonitrile (0.33g, 5 mmol) and ammonium acetate (0.75g, 10 mmol) were added. The reaction mixture was refluxed for 7-9h. The obtained solid was filtered off, washed with absolute ethylalcohol and recrystallized from methyl alcohol to give the desired compounds

4-phenyl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles 4a

Yield 86 %; mp 265 ºC. IR spectrum, ν cm^{-1}: 3264 (OH, br s), 3043 (NH), 2364 (CN), 1697 (C=O, a-pyrone), 1612 (C=O).1643 (C=N). ^{1}H NMR spectrum (CDCl$_3$) δ ppm: 6.3-8.6 (m,10H,H$_{arom}$,H$_{2'}$); 4.81 (s,2H,NH$_2$) ,18.25 (s,1H,OH). ^{13}C NMR spectrum (CDCl$_3$): δ ppm 56.1(OCH$_3$), 97.6 (C$_3$); 109.5 (C$_{4'}$); 122.6(C$_{2'}$); 151.9 (C$_{3'}$); 153.7 (C$_{5'}$), 162.1 (C$_2$), 168.2 (C$_4$), 114.1-129.5(C$_{arom}$+ C$_{coumarinic}$), 160.4 (C$_{1'}$). Anal. Calcd for C22H15O3N3: C, 71.536%; H, 4.093%, N, 11.376%, Found: C, 71.4%; H,4.093%,N, 11.3 %,

4-fluorophenyl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles 4b

Yield 80 %; mp 265 ºC. IR spectrum, ν cm^{-1}: 3430 (OH, br s), 3187 (NH), 1675 (C=O, a-pyrone), 1603 (C=N).. ^{1}H NMR spectrum (CDCl$_3$) δ ppm: 6.2-8.3 (m,9H,H$_{arom}$,H$_{2'}$); 4.81 (s,2H,NH$_2$),18.25 (s,1H,OH). ^{13}C NMR spectrum (CDCl$_3$): δ ppm 56.1(OCH$_3$), 97.6 (C$_3$); 109.5 (C$_{4'}$); 122.6(C$_{2'}$); 151.9 (C$_{3'}$); 153.7 (C$_{5'}$), 162.1 (C$_2$), 168.2 (C$_4$), 114.1-129.5(C$_{arom}$+ C$_{coumarinic}$), 160.4 (C$_{1'}$). Anal. Calcd for C21H12O3N3F: C, 67.560%; H, 3.240%, N, 11.255%, F, 5.089% Found: C, 67.4%; H, 3.10%, N, 11.2%

4-2.5-phenyl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles 4c

Yield 85 %; mp 265 ºC. IR spectrum, ν cm^{-1}: 3262 (OH, br s), 3098 (NH), 2354 (CN), 1696 (C=O, a-pyrone), 1658 (C=O). ^{1}H NMR spectrum (CDCl$_3$) δ ppm: 6.4-8.2 (m,8H,H$_{arom}$,H$_{2'}$); 4.81 (s,2H,NH$_2$), 2.1(s,CH$_3$), 2.3(s,CH$_3$) 18.25 (s,1H,OH). ^{13}C NMR spectrum (CDCl$_3$): δ ppm 56.1(OCH$_3$), 97.6 (C$_3$); 109.5 (C$_{4'}$); 122.6(C$_{2'}$); 151.9 (C$_{3'}$); 153.7 (C$_{5'}$), 162.1 (C$_2$), 168.2 (C$_4$), 114.1-129.5(C$_{arom}$+ C$_{coumarinic}$), 160.4 (C$_{1'}$). Anal. Calcd for C23H17O3N3: C, 72.052%; H, 4.469%, N, 10.960%, Found: C, 72.1%; H, 4.4%, N, 10.9%

4-Anisyl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles 4d

Yield 80 %; mp 265 ºC. IR spectrum, ν cm^{-1}: 3316 (OH, br s), 3208, 3057 (NH2) , 2206 (CN), 1680 (C=O, α-pyrone), 1643 (C=N). ^{1}H NMR spectrum (CDCl$_3$) δ ppm: 3.75(s, 3H, OCH$_3$), 6.4-8.2 (m,12H,H$_{arom}$,H$_{2'}$); 4.81 (s,2H,NH$_2$), 18.25 (s,1H,OH). ^{13}C NMR spectrum (CDCl$_3$): δ ppm 56.1(OCH$_3$), 97.6 (C$_3$); 109.5 (C$_{4'}$); 122.6(C$_{2'}$); 151.9 (C$_{3'}$); 153.7 (C$_{5'}$), 162.1 (C$_2$), 168.2 (C$_4$), 114.1-129.5(C$_{arom}$+ C$_{coumarinic}$), 160.4 (C$_{1'}$). Anal. Calcd for C21H13O3N3: C, 70.980% ; H, 3.687%, N, 11.825%, Found: C, 70.9%; H, 3.6%, N, 11.8%,

4-nitrophenyl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles 4e

Yield 85 %; mp 265 ºC. IR spectrum, ν cm^{-1}: 3392 (OH, br s), 3118, 3099 (NH2), 2210 (CN), 1722 (C=O, α-pyrone), 1653 (C=N). ^{1}H NMR spectrum (CDCl$_3$) δ ppm: 6.5-8.3 (m,9H,H$_{arom}$,H$_{2'}$); 18.25 (s,1H,OH), 4.81 (s,2H,NH$_2$). ^{13}C NMR spectrum (CDCl$_3$): δ ppm 56.1(OCH$_3$), 97.6 (C$_3$); 109.5 (C$_{4'}$); 122.6(C$_{2'}$); 151.9 (C$_{3'}$); 153.7 (C$_{5'}$), 162.1 (C$_2$), 168.2 (C$_4$), 114.1-129.5(C$_{arom}$+ C$_{coumarinic}$), 160.4 (C$_{1'}$). Anal. Calcd for C22H15O4N3: C, 68.566% ; H, 3.923, N, 10.904%, Found: C, , 68.5%; H, 3.9%, N, 10.9%

4-tollyl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles 4f

Yield 75 %; mp 265 ºC. IR spectrum, ν cm^{-1}: 3449 (OH, br s), 3057(NH), 2739 (CN), 1739 (C=O, a-pyrone), 1692 (C=O). ^{1}H NMR spectrum (CDCl$_3$) δ ppm: 6.4-8.2 (m,12H,H$_{arom}$,H$_{2'}$); à,2.1(s,CH$_3$), 18.25 (s,1H,OH). ^{13}C NMR spectrum (CDCl$_3$): δ ppm 56.1(OCH$_3$), 97.6 (C$_3$); 109.5 (C$_{4'}$); 122.6(C$_{2'}$); 151.9 (C$_{3'}$); 153.7 (C$_{5'}$), 162.1 (C$_2$), 168.2 (C$_4$), 114.1-129.5(C$_{arom}$+ C$_{coumarinic}$), 160.4 (C$_{1'}$). Anal. Calcd for C23H17O3N3: C, 72.052%; H, 4.469%, N, 10.960%, Found: C, 72.1%; H, 4.4%, N, 10.9%.

All 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivative 4 derivatives were evaluated for their antibacterial activity against four human pathogenic bacterial strains. These include *Escherichia coli (MTCC 40)*, *Klebsiella pneumoniae (MTCC 661)*, *Salmonella typhi (MTCC 733)* and *Shigella flexneri (MTCC 1457)*.

The nutrient agar was prepared and sterilized by autoclave. The sterile nutrient agar is cooled to 40^0C, poured to the sterile petriplate and allowed to solidify. The nutrient agar was then inoculated with 10^6 cfu/mL of respective microorganism using sterile spreader. Wells are made using sterile cork borer (8.5 mm) and compounds to be tested are added to each well with different concentrations ranging from 10 to 1000 *µ*g/mL. These were made from the stock solution of 4 mg/mL in dimethyl sulfoxide (DMSO). A 100 *µ*L volume of each dilution was introduced into wells (in duplicates) in the agar plates already seeded with bacterial suspension. These plates were incubated at 37°C for 24 h and observed for inhibition zones. Gentamicin was used as positive control.

Antioxidant activity

DPPH radical scavenging assay: The free radical scavenging activity for DPPH radicals wasperformed as described previously [22]. In brief, reaction mixture containing 200 µL of 0.1 mM DPPH–ethanol solution, 90 µL of 50 mM Tris–HCl buffer (pH 7.4) and 10 µL of deionised water (as control) and various concentrations of compounds **4-a-f** (3.0 – 16.0 µM), and ascorbic acid was used as a control. Reaction mixture was incubated for 30 min at room temperature and absorbance was read at 540 nm. The percentage radical scavenging activity was calculated according to the following formula: Inhibition (%) = [(Absorbance control-Absorbance sample)/Absorbance Control] X 100

Hydroxyl radical scavenging assay: The hydroxyl radical (˙OH) scavenging activity of newly synthesized compounds was determined previously [23]. We used Fe (III) - ascorbate – ethylenediaminetetraacetic acid – hydrogen peroxide system (Fenton's reaction) to generate hydroxyl radical. In brief, reaction mixture containing 0.01 mL of $FeCl_3$ (10 mM), 0.1 mL of EDTA (1 mM),0.36 mL of deoxyribose (10 mM), 0.1 ml of H_2O_2 (10 mM), 1 mL of the compounds **4a-f**(concentrations ranging from 3.0 – 16 .0 µM), 0.33 mL of phosphate buffer (50 mM, pH 7.4) and 0.1 mL ascorbic acid (1 mM) was added. The mixture was incubated at 37^0C for 1 h and 1 mL of the incubated mixture was mixed with 1 mL of 10% trichloro acetic acid (TCA) and 1mL of thiobarbituric acid (TBA) (1% in 0.025 M NaOH), the resulting mixture was incubated in water bath at 90^0C for 20 min. The absorbance was measured at 532 nm. Ascorbic acid was used as a positive control. The percentage of hydroxyl radical scavenging activity was calculated using the formula: Inhibition (%) = [(Absorbance control-Absorbance sample)/Absorbance Control] X 100

Superoxide anion radical scavenging assay: The superoxide anion radical scavenging activity of newly synthesized compounds were determined previously [24]. 1 mL of Nicotinamide adenine dinucleotide (NADH) (468 µM in 100 mM phosphate buffer of pH 7.4), 1 mLofNitro blue tetrazolium (NBT) (156 µM NBT in 100 mM phosphate buffer of pH 7.4), and different concentration of compounds **4a-f** (3.0– 16.0 µg/mL) were added to get the final volume of 3 mL. The reaction was started by the addition of 100 µL of PMS (60 µM in 100 mM phosphate buffer of pH 7.4). The mixture was incubated for 5 min at 25^0C and the absorbance was measured at 560 nm. Quercetin was used as a control. The percentage radical scavenging activity was calculated using the formula: Inhibition (%) = [(Absorbance control-Absorbance sample)/Absorbance Control] X 100 .

Anti-inflammatory activity

Lipoxygenase inhibition assay: The lipoxygenase inhibition assay was performed according to the method previously described [25]. Briefly, to a solution of 0.1 mL of 0.2 M borate buffer (pH 9.0), 0.1 mL of 1000 units lipoxydase enzyme solution, test compounds **4a- f** dissolved in DMSO (3 - 16 µM) was added, agitated and incubated at room temperature for 5 min. Later, 2.0 mL of 0.6 mM linoleic acid was added and the absorbance was measured at 234 nm. Indomethacin was used as standard. The percent (%) inhibition was calculated by the following equation:

Inhibition (%) = [(Absorbance control-Absorbance sample)/Absorbance Control] X 100

Indirect haemolytic assay: Indirect haemolytic assay was performed according to the reported method [26]. One mL of fresh human red blood cells and 1 mL of fresh Hen's egg yolk in 8 mL of phosphate buffered saline was mixed to prepare the substrate for indirect hemolytic activity. One mL of this suspension was incubated with 4-28 µg of partially purified venom for 45 min at 37^0C and 9 mL ice cold sodium perborate was used to stop the reaction. The reaction mixture was centrifuged at 2000 rpm for 20 min then the released hemoglobin was read at 540 nm. For inhibition studies 10 µg of venom sample (secretory- PLA_2 purchased from sigma) was incubated with various concentrations of compounds **4a-f** (20-100 µM in DMSO) for 30 min at room temperature and mixed with 1 mL of substrate solution and incubated at room temperature for 30 min. The reaction was stopped by adding 9 mL of ice cold sodium perborate and extent of hemolysis is measured at 540 nm. Aristolochic acid was used as reference drug. The percent (%) inhibition was calculated as follows: Inhibition (%) = [(Absorbance control-Absorbance sample)/Absorbance Control] X 100 .

3. RESULTS AND DISCUSSION

In this investigation, a series of 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** were designed, synthesized (**Schemes 1**) and biologically evaluated for their antibacterial , antioxidant activities and anti inflammaratory anti-inflammatory activity by indirect haemolytic and lipoxygenase were investigated. Thus, compound **3** was prepared by reaction of 4-hydroxycoumarine with $POCl_3$ in chloroform in the presence of acetic acid, The resulting compound **2** was then reacted with arylaldehydes in the presence of piperidine at reflux of $CHCl_3$ to give the (E) the coumarinic chalcones **3**, which precipitated from the hot MeOH solution after mixing **2** with the corresponding ArCHO . **Scheme 1**
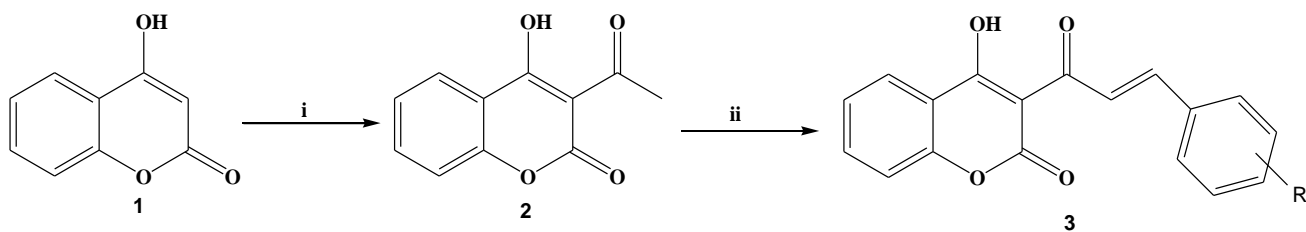
i: POCl₃, ACOH, ii: CHCl₃, piperidine, Arylaldehyde

Compounds 3	R	Yield (%)
3a	H	75
3b	F	80
3c	2,5 CH₃	76
3d	OCH₃	85
3e	NO₂	75
3f	HC₃	85

Synthesis of compounds 3

Compounds **3** were identified from analysis of their spectroscopic data. The infrared (IR) spectrum of compound **3d** showed the coumarin carbonyl groups at 1768 cm^{-1} in addition to a broad band for the C=C group at 1595 cm^{-1}. The ^{1}H NMR spectrum showed *trans* olefinic protons H$_a$ and H$_b$ as *ortho*-coupled doublets at δ 8.25 (*J* = 15.6 Hz) and 6.92 (*J* = 15.9 Hz), respectively. The remaining aromatic protons of the aromatic aldehydes and the four protons of the coumarin moiety appeared as a multiplet in the region δ 7.25–8.08. The ^{13}C{^{1}H} NMR spectrum of **3d** in DMSO-*d$_6$* showed two downfield signals at δ 147.4ppm (C$_4$) and δ 162.4 ppm (lactone C=O) as well as an upfield signal to at δ 55.4 ppm (OCH₃).

The condensation of compounds **3** with malontrile in the presence of ammonium acetate at reflux of EtOH in one step afforded 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** in good yields . **Scheme 2**
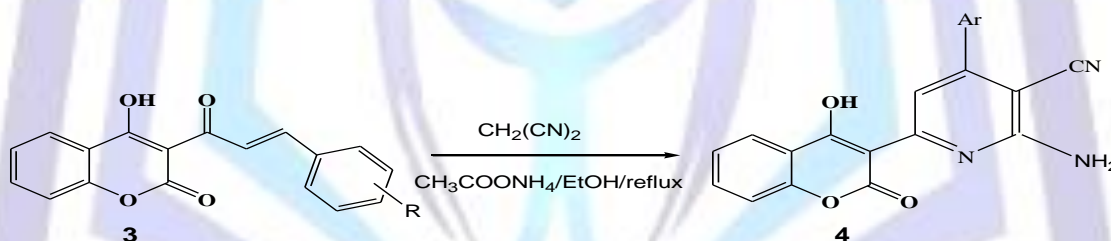
Compounds 4	R	Yield (%)
4a	H	80
4b	F	75
4c	2,5 CH₃	85
4d	OCH₃	75
4e	NO₂	70
4f	HC₃	85

Scheme 2: Synthesis of 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles 4

All new 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles 4 were characterized by IR, 1H, 13C-NMR spectra as well as by NOESY and HMBC 2D-NMR experiments to elucidate their structures and completely assign the structural network of both protons and carbons. The spectral data were in accordance with the proposed structures (see Experimental Section).

The IR spectrum of **4d** showed a broad band at 3057 cm^{-1} due to the presence of the NH$_2$ group, a strong band at 3316 cm^{-1} was attributed for OH. Since chromone carbonyl groups usually appear as sharp absorption bands in the region 1620–1650 cm^{-1} [27], the band at 1680 cm^{-1} was assigned to coumarin rather than the chromone carbonyl group.

In addition, the detection of a strong C=N stretching band at 1643 cm^{-1} was indicative of the formation of the pyridine-3-carbonitriles ring. The ^{1}H NMR spectra of **4d** displays a signal at δ 3.75 ppm ascribable to the OCH$_3$ protons of the pyridine-3-carbonitriles ring. A characteristic singlet proton signal at δ 4.81 ppm was assigned to NH$_2$ protons. In addition, the aromatic protons (both coumarinic and aromatic) are observed between δ 6.40 and δ 8.20 ppm .

In the ^{13}C NMR spectrum of **4d** indicated carbon atoms at δ 56.1(OCH$_3$), 97.6 (C$_3$); 109.5 (C$_4'$); 122.6 (C$_{2'}$); 151.9 (C$_{3'}$); 153.7 (C$_{5'}$), 162.1 (C$_2$), 168.2 (C$_4$), 114.1-129.5 (C$_{arom}$+ C$_{coumarinic}$), 160.4 (C$_{1'}$).

The structure of **4d** was finally elucidated through the analysis of the ^{1}H, ^{13}C HMBC spectrum which correlates the protons at δ 4.81 ppm with C$_{5'}$ (δ 153.07 ppm) and C$_4$ (δ 109.5 ppm). The aromatic protons correlate with C$_{3'}$ (δ 151. 9 ppm).

Table 1

Correlations between HMBC and NOESY for compound **4d**

Proton H-n	HMBC H-n-C-j	NOESY H-n-H-j
OCH3	9', 10', 11'	H$_{11'}$,H12'
H-5'	5',4'	
H-arom	3',4'	
OH	4,3,2	

Similarly the spectral values for all the compounds and C, H, N analyses are given in the experimental part

A mechanistic rationalization for this reaction is straightforward and is provided in **Scheme 3**

ii: *Malonitrile ; ammonium acetate, reflux, 5h*
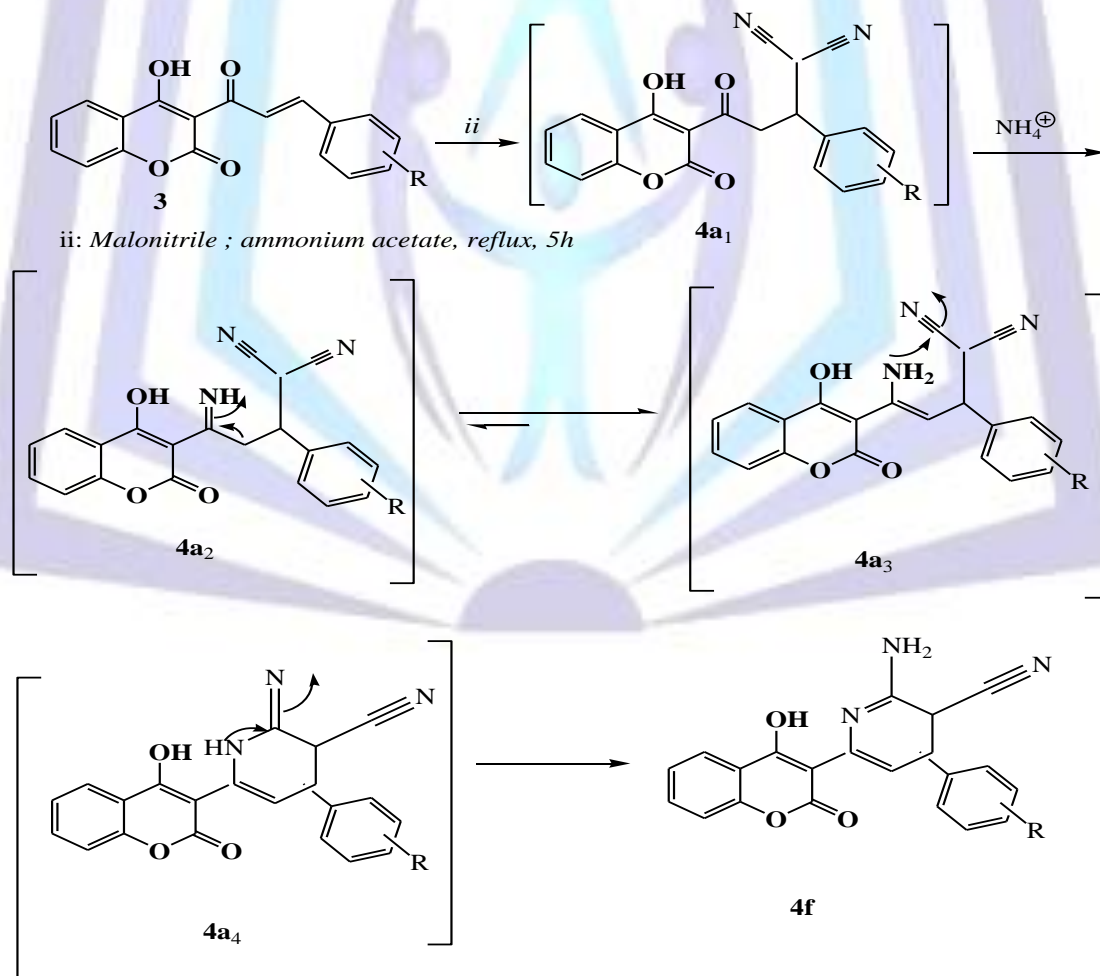
Scheme 3: Proposed mechanism for compound 4

The first reaction step consists of a nucleophilic attack on the unsaturated carbon, followed by intermolecular cyclization follow by a tautomeric enamine amine balance.

4. Pharmacology

Antibacterial activity

The synthesized 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4 a-f** were screened for *in vitro* antibacterial activity against four Gram Negative bacterial strains (*E. coli, K. Pnemoniae, S. typhi* and *S. Flexneri*) and the results are given in Table 2. All compounds except **4a** showed good zone of inhibition against pathogens *S. typhi* and *S. flexneri*. On the otherhand, all compounds displayed moderate activity against *E. coli*, but remained poor against *K. pnemoniae.* The compounds **4b,4f** with electron withdrawing groups on the phenyl ring showed good antibacterial activity. Whereas the compounds **4c** and **4f** with electron releasing groups on the phenyl ring showed moderate antibacterial activity on most of tested bacterial strains and 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivative **4d** without substitution on the phenyl ring showed least antibacterial activity. Also, all compounds except **4a** showed high potency against *S. typhi*. Thus, deactivating groups on phenyl ring enhances the antimicrobial behaviour of 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivatives rather than the activating groups.

Table 2 Antibacterial activity of the 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivatives 4a-f

Compound	Zone of inhibition in mm*			
	Escherichia coli	*Klebsiella pneumoniae*	*Salmonella typhi*	*Shigella flexneri*
4a	12	11	12	11
4b	12	-	21	23
4c	12	-	26	15
4d	15	12	23	22
4e	14	12	21	20
4f	13	11	20	19

* Inhibition zones including cup borer (8.5 mm) diameter; Positive control zone is 35 to 40 mm; '-' = Not active

4. Antioxydant activity

We considered that it was worthwhile to study the potential aspects of these new 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** derivatives for antioxidant activity according to our initial planning. The synthesized compounds **4a-f** were tested for *in vitro* antioxidant activity by DPPH radical, hydroxyl radical and superoxide radical scavenging assays. The IC_{50} values of the standards and test samples are summarized in Table 3. In all three antioxidant assays, Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivatives **4c,4f** bearing electron withdrawing groups on the phenyl ring showed antioxidant activities at 9.8 - 12.8 µM concentration. But, 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivative **4b** and **4e** with electron releasing fluore group and nitro phenyl ring showed good antioxidant activity (7.0 - 8.8 µM). Surprisingly, 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4a** without substitution on the phenyl ring is inactive in all antioxidant assays. Also, it should be noted that it is not possible to give any rational explanation for antioxidant activities even in the absence of essential enolic group.

Table 3 IC_{50} values of 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** in anti-oxidant assays

Compounds	IC_{50} values in µM		
	DPPH radical scavenging assay	Hydroxy radical Scavenging assay	Superoxide radical scavenging assay
4a	NA	NA	NA
4b	12.4	12.8	9.8
4c	11.1	11.0	12.7
4d	11.2	11.2	11.8
4e	11.1	11.0	11.5
4f	11.0	10.8	11.4
Ascorbic acid	3.8	3.9	-
Quercetin	-	-	4.9

Anti-inflammatory activity

As per our objective, we next examined the anti-inflammatory activities of 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivatives **4a-f** by lipoxygenase inhibition and phospholipase A_2 (PLA$_2$) inhibition assays. The IC$_{50}$ values of the standards and test samples in both assays are given Table 4. In both the assays, 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivatives **4b** and **4c** bearing strong electron withdrawing nitro groups showed potent anti-inflammatory activity in lipoxygenase inhibition assay (5.0 – 5.1 µM) and PLA$_2$ inhibition assay (26.5 - 34.9 µM). While, the other derivatives substituted with weak deactivating (**4b-e**). Notably, compound **4a** with no substitution on phenyl ring is not active. It should be noted that, **4b** and **4c** nearly have anti-inflammatory activities, as that of standards Indomethacin and Aristolochic acid. Thus, 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** derivatives with strong deactivating groups are more potent than 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** derivatives with weak deactivating/activating groups .

Table 4. IC$_{50}$ values of 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** for anti-inflammatory activity

Compounds	IC$_{50}$ values in µM	
	Lipoxygenase inhibition assay	PLA$_2$ inhibition assay
4a	NA	NA
4b	5.0	26.5
4c	5.1	34.9
4d	11.4	61.1
4e	11.3	61.4
4f	11.1	61.8
Indomethacin	4.8	-
Aristolochic acid	-	25.0

CONCLUSIONS

In summary, we have synthesized a new 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** derivatives and examined their antimicrobial, antioxidant and anti-inflammatory activities. The synthesized compounds showed moderate *in vitro* antibacterial activity. 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** derivatives are good antibacterial agents than the 4-Alkyl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles **4** derivatives. On contrary, in all the anti-oxidant assays, 4-Aryl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivatives are poor antioxidants compared to 4-Alkyl-2-amino-6-(4-hydroxy coumarin-3-yl) pyridine-3-carbonitriles derivatives. Also, aryl derivatives bearing deactivating groups are less anti-oxidant properties than those bearing activating groups. But, it is not possible to give any explanation for the antioxidant activities of these compounds even in the absence of essential enolic group.

Acknowledgment

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