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Research Article

SYNTHESIS AND CHARACTERIZATION OF COUMARIN ANALOGS: EVALUATION OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES

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ABSTRACT

Coumarins and their analogs were found to be better antitumoral agents and still active against human retroviral invading system. This property could be ameliorated by coupling of few active groups to the parental molecule for enhancing its frequency of biofunctional diversity and multi spectrum characteristic, such an endeavor was done by 2-Oxo-2H-chromene-3-carboxylic acid phenylamide analogs. The structure were well characterized. Further the antimicrobial activity was examined by disc diffusion method and based on the minimum inhibitory concentration (MIC) values obtained the molecules were approximately 70% as active as the positive control especially halogenated compounds showed promising result. Even their free radical scavenging activity was considerably as good as 20% to that of ascorbic acid. Present research on coumarin analogs necessitate novel ideas in synthetic chemistry therein concerning the development of innovative synthetic strategies that could help in drug design.

Keywords: Coumarins, Knoevenagel reaction, Antimicrobial activity, Antioxidant activity, TBTU.

INTRODUCTION

Heterocyclic motifs have been much exploited in a drug discovery, development owing to their ample biological spectrum[1-3]. In particular, coumarins and their derivatives have acquired much attention from the pharmacological and pharmaceutical arena due to their broad range of therapeutic qualities[4-6]. Numerous coumarin analogs have been reported as potential antibacterial[7], antifungal[8], antiinflammatory[9], antioxidants[10], anticancer and anti-HIV[11] agents. Coumarins are widely used as additives in food, perfumes, cosmetics[12], pharmaceuticals and optical brighteners[13]. Modern researches demonstrated that different alteration in the structure of compounds having coumarin moiety would produce compounds with quantitatively as well as qualitatively changed biological activities[14]. The pharmacological, biochemical and therapeutic properties of coumarin derivatives strongly depend on the nature as well as the position of the structural substituents[15]. For example, introduction of the halogens, and in particular bromine, fluorine and fluoroalkyl substituents to coumarin moiety, has led to the enhancement of the lipophilicity[16]. Lipophilicity is an important physicochemical parameter in the development of antibacterial agent because it is known to be closely related to the permeation through a lipid coat of bacteria[17,18]. Studies have also shown that the number and position of substitutions in the coumarin ring influenced the antimicrobial potential[19].

With the development of new strains of bacteria resistant to many currently available antibiotic treatments, there is increasing interest in the discovery of new antibacterial agents. Antimicrobial resistance refers to microorganism that have developed the ability to inactivate, exclude or block the inhibition or lethal mechanism of the antimicrobial agents[20]. Identification of novel structure leads that may be of use in designing new, potent and broad spectrum antimicrobial agents remains a major challenge for researchers. Based on these findings recently, coumarin analog bearing p-fluoro phenyl oxadiazole moiety (Fig. 1A) was reported as significant antibacterial agent[21].

Antioxidants possess the ability to protect the cellular organelles from damage caused by free radicals induced oxidative stress. Free radicals used include hydroxyl radical, superoxide 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[22]. Generally, most of the free radicals generated from metabolism are scavenged by an endogenous defense system such as catalase, superoxide dismutase and peroxidase–glutathione system[23]. Prompted by these observations, researchers have been synthesized numerous hetrocyclic compounds as antioxidants. For instance, coumarin analog bearing p-methyl phenyl moiety (Fig. 1B) has been reported as potent antioxidant agent[24]. In view of the high degree of bio-activity shown by coumarin heterocyclic analogs and based on our consistent work on heterocycles having potent biological activities[25-30], prompted us to prepare new phenylamide coumarin analogs bearing different substituent and expected to possess potent antimicrobial and antioxidant activities.

MATERIAL AND METHODS

Chemicals were purchased from Sigma Aldrich Chemical Co. TLC was performed on aluminum-backed silica plates and

visualized by UV-light. Melting points were determined on a Thomas Hoover capillary melting point apparatus with a digital thermometer. IR spectra were recorded on FT-IR Shimadzu 8300 spectrophotometer, ^{1}H NMR ^{13}C NMR spectra were recorded on a Bruker 400 MHz NMR spectrophotometer in DMSO-d_6 and $CDCl_3$ then the chemical shifts were recorded in parts per million downfield from tetramethylsilane. Mass spectra were obtained with a VG70-70H spectrometer and important fragments are given with the relative intensities in brackets. Elemental analysis was done by Perkin Elmer 2400 and the results were within 0.4% of the calculated value.

Ethyl 2-oxo-2H-chromene-3-carboxylate **(3)** and of 2-Oxo-2H-chromene-3-carboxylic acid **(4)** were synthesized from the reported procedure[31].

SYNTHESIS OF 2-OXO-2H-CHROMENE-3-CARBOXYLIC ACID PHENYLAMIDE ANALOGS (6a-l)

To the compounds **4** (0.0037 mol) in dry dichloromethane (15 mL), lutidine (1.2 vol.) was added at 25-30° C, followed by the addition of **5a-l** (0.0037 mol) and the mixture was stirred at 25-30° C for 30 min. The reaction mixture was cooled to 0-5 °C and TBTU (0.0037 mol) was added over a period of 30 min while maintaining the temperature below 5° C. The reaction was stirred overnight and monitored by TLC using chloroform:methanol (9:1) as an eluent. The reaction mixture was diluted with 20 mL of dichloromethane and washed with 1.5 N hydrochloric acid (20 mL). The organic layer was washed with water (25 mL x 3), dried over anhydrous sodium sulfate, concentrated to a syrupy liquid and recrystallized twice from diethyl ether to afford compounds (**6a-l**) in good yield[32].

2-oxo-2h-chromene-3-carboxylic acid phenylamide (6a)

Yield 80%. mp 232-235°C; FT-IR (KBr, cm^{-1}): 3700-3500 (amide N-H), 1695 (amide C=O), 1665 (C=O); ^{1}H NMR (DMSO-d_6): δ 7.1-8.0 (m, 9H, Ar-H), 9.0 (s, 1H, coumarin ring-H), 10.8 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.0, 151.4, 150.8, 138.2, 128.7, 128.1, 127.8, 126.6, 125.2, 124.4, 124.1, 121.3, 120.4. LC-MS m/z 265 (M+1). Anal. Calcd. for $C_{16}H_{11}NO_3$: C, 72.45; H, 4.18; N, 5.28. Found: C, 72.36; H, 4.31; N, 5.17%.

2-oxo-2h-chromene-3-carboxylic acid (4-nitro-phenyl)-amide (6b)

Yield 85%. mp 240-241°C; FT-IR (KBr, cm^{-1}): 3710-3530 (amide N-H), 1695 (amide C=O), 1660 (C=O); ^{1}H NMR (DMSO-d_6): δ 7.3-8.1 (m, 8H, Ar-H), 9.1 (s, 1H, coumarin ring-H), 10.8 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.0, 151.4, 150.8, 144.3, 144.0,128.1, 127.8, 126.6, 125.2, 124.4, 123.8, 121.3. LC-MS m/z 310 (M+1). Anal. Calcd. for $C_{16}H_{10}N_2O_5$: C, 61.94; H, 3.25; N, 9.05. Found: C, 61.84; H, 3.35; N, 9.09%.

2-oxo-2h-chromene-3-carboxylic acid (4-methoxy-phenyl)-amide (6c)

Yield 79%. mp 215-217°C; FT-IR (KBr, cm^{-1}): 3715-3500 (amide N-H), 1690 (amide C=O), 1670 (C=O); ^{1}H NMR (DMSO-d_6): δ 3.7 (s, 3H, -OCH_3), 7.1-8.1 (m, 8H, Ar-H), 9.1 (s, 1H, coumarin ring-H), 10.8 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.0, 157.6, 151.4, 150.8, 130.5, 128.1, 127.8, 126.6, 125.2, 124.4, 121.4, 121.3, 114.3, 56.0. LC-MS m/z 295 (M+1). Anal. Calcd. for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. Found: C, 69.25; H, 4.54; N, 4.79. %.

2-oxo-2h-chromene-3-carboxylic acid (3-chloro-phenyl)-amide (6d)

Yield 87%. mp 225-227^0 C; FT-IR (KBr, cm^{-1}): 3730-3510 (amide N-H), 1685 (amide C=O), 1660 (C=O); ^{1}H NMR (DMSO-d_6): δ 7.0-8.1 (m, 8H, Ar-H), 9.5 (s, 1H, coumarin ring-H), 10.3 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.0, 151.4, 150.8, 139.6, 134.0, 130.1, 128.1, 1 27.8, 126.6, 125.2, 124.5, 124.4, 121.3, 120.8, 118.5. LC-MS m/z 299 (M+1). Anal. Calcd. for $C_{16}H_{10}ClNO_3$: C, 64.12; H, 3.36; 11.83; N, 4.67;. Found: C, 64.22; H, 3.42; 11.83; N, 4.57%.

2-oxo-2h-chromene-3-carboxylic acid o-tolylamide (6e)

Yield 90%. mp 208-210^0 C; FT-IR (KBr, cm^{-1}): 3730-3525 (amide N-H), 1695 (amide C=O), 1655 (C=O); ^{1}H NMR (DMSO-d_6): δ 2.30 (s, 3H, Ar-CH_3), 7.1-8.2 (m, 8H, Ar-H), 9.3 (s, 1H, coumarin ring-H), 10.5 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.0, 151.4, 150.8, 138.9, 129.6, 129.4, 128.1, 127.8, 126.6, 125.7, 125.2, 124.4, 124.0, 121.3, 120.3, 11.8. LC-MS m/z 279 (M+1). Anal. Calcd. for $C_{17}H_{13}NO_3$: C, 73.11; H, 4.69; N, 5.02. Found: C, 73.18; H, 4.76; N, 5.09%.

2-oxo-2h-chromene-3-carboxylic acid (4-fluoro-phenyl)-amide (6f)

Yield 82%. mp 252-254^0 C; FT-IR (KBr, cm^{-1}): 3700-3500 (amide N-H), 1695 (amide C=O), 1665 (C=O); ^{1}H NMR (DMSO-d_6): δ 7.3-8.4 (m, 7H, Ar-H), 9.2 (s, 1H, coumarin ring-H), 10.2 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.0, 157.7, 151.4, 150.8, 133.8, 128.1, 127.8, 126.6, 125.2, 124.4, 122.0, 121.3, 115.7. LC-MS m/z 317 (M+1). Anal. Calcd. for $C_{16}H_9ClFNO_3$: C, 60.49; H, 2.86; N, 4.41. Found: C, 60.59; H, 2.90; N, 4.51%.

2-oxo-2h-chromene-3-carboxylic acid (2-fluoro-phenyl)-amide (6g)

Yield 75%. mp 180-183^0 C; FT-IR (KBr, cm^{-1}): 3735-3525 (amide N-H), 1690 (amide C=O), 1675 (C=O); ^{1}H NMR (DMSO-d_6): δ 7.4-8.3 (m, 8H, Ar-H), 9.1 (s, 1H, coumarin ring-H), 10.6 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.0, 154.0, 151.4, 150.8, 128.1, 127.8, 126.6, 125.7, 125.2, 124.4, 124.3, 122.0, 121.3, 115.7. LC-MS m/z 283 (M+1). Anal. Calcd. for $C_{16}H_{10}NO_3$: C, 67.84; H, 3.56; N, 4.94. Found: C, 67.94; H, 3.59; N, 4.98%.

2-oxo-2h-chromene-3-carboxylic acid (2-bromo-phenyl)-amide (6h)

Yield 81%. mp 265-267^0 C; FT-IR (KBr, cm^{-1}): 3710-3520 (amide N-H), 1690 (amide C=O), 1655 (C=O); ^{1}H NMR (DMSO-d_6): δ 7.3-8.1 (m, 8H, Ar-H), 9.2 (s, 1H, coumarin ring-H), 10.4 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.0, 151.4, 150.8, 141.5, 132.0, 128.1, 127.8, 127.7, 126.6, 126.3, 125.2, 124.4, 122.6, 121.3, 115.0. LC-MS m/z 344 (M+1). Anal. Calcd. for $C_{16}H_{10}NO_3$: C, 55.84; H, 2.93; N, 4.07. Found: C, 55.89; H, 2.98; N, 4.10%.

2-oxo-2h-chromene-3-carboxylic acid (3-nitro-phenyl)-amide (6i)

Yield 85%. mp 260-262^0 C; FT-IR (KBr, cm^{-1}): 3710-3530 (amide N-H), 1695 (amide C=O), 1660 (C=O); ^{1}H NMR (DMSO-d_6): δ 7.3-8.1 (m, 8H, Ar-H), 9.1 (s, 1H, coumarin ring-H), 10.8 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.0, 151.4, 150.8, 148.6, 139.1, 129.6, 128.1, 127.8, 126.6, 126.5, 125.2, 124.4, 121.3, 119.2, 115.5. LC-MS m/z 310 (M+1). Anal. Calcd. for

$C_{16}H_{10}N_2O_5$: C, 61.94; H, 3.25; N, 9.05. Found: C, 61.84; H, 3.35; N, 9.09%.

2-oxo-2h-chromene-3-carboxylic acid (3-methoxy-phenyl)-amide (6j)

Yield 79%. mp 270-272^0 C; FT-IR (KBr, cm^{-1}): 3715-3500 (amide N-H), 1690 (amide C=O), 1670 (C=O); ^{1}H NMR (DMSO-d_6): δ 3.7 (s, 3H, -OCH_3), 7.1-8.1 (m, 8H, Ar-H), 9.1 (s, 1H, coumarin ring-H), 10.8 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.2, 162.0, 151.4, 150.8, 139.2, 129.7, 128.1, 128.5, 127.8, 126.6, 125.2, 128.5, 124.4, 121.3, 112.7, 109.7, 106.0, 56.0. LC-MS m/z 295 (M+1). Anal. Calcd. for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. Found: C, 69.25; H, 4.54; N, 4.79. %.

2-oxo-2h-chromene-3-carboxylic acid (3-methoxy-phenyl)-amide (6k)

Yield 85%. mp 230-233^0 C; FT-IR (KBr, cm^{-1}): 3725-3510 (amide N-H), 1695 (amide C=O), 1680 (C=O); ^{1}H NMR (DMSO-d_6): δ 3.8 (s, 3H, -OCH_3), 7.2-8.4 (m, 8H, Ar-H), 9.4 (s, 1H, coumarin ring-H), 10.8 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 163.8, 162.2, 162.0, 151.4, 150.8, 139.2, 129.7, 128.1, 127.8, 126.6, 125.2, 124.4, 121.3, 112.7, 109.7, 106.0, 56.0. LC-MS m/z 295 (M+1). Anal. Calcd. for $C_{17}H_{13}NO_4$: C, 69.15; H, 4.44; N, 4.74. Found: C, 69.25; H, 4.54; N, 4.79. %.

2-oxo-2h-chromene-3-carboxylic acid (4-acetyl-phenyl)-amide (6l)

Yield 72%. mp 240-242^0 C; FT-IR (KBr, cm^{-1}): 3725-3510 (amide N-H), 1680 (amide C=O), 1655 (C=O); ^{1}H NMR (DMSO-d_6): δ 2.5 (s, 3H, $COCH_3$), 7.1-8.0 (m, 8H, Ar-H), 9.7 (s, 1H, coumarin ring-H), 10.2 (s, 1H, CONH). ^{13}C NMR ($CDCl_3$): 196.5, 163.8, 162.0, 151.4, 150.8, 142.6, 133.0, 128.8, 128.8, 128.1, 127.8, 126.6, 125.2, 124.4, 121.3, 120.3, 120.3, 22.8. LC-MS m/z 307 (M+1). Anal. Calcd. for $C_{18}H_{13}NO_4$: C, 70.35; H, 4.26; N, 4.56. Found: C, 70.40; H, 4.31; N, 4.59%.

ANTIMICROBIAL ACTIVITY

Compounds **6a-l** were tested for their Antimicrobial Activity by disc diffusion assay and minimum inhibitory concentration (MIC) methods. Ampicillin (Sigma) was used as positive control against bacteria. ketoconazole (Himedia, Mumbai) were used as positive control against fungi.

Tested microbes

The following gram-positive bacteria were used for the experiments: *Streptococcus pyogenes* (SP), *Staphylococcus aureus* (SA) and *Bacillus subtilis* (BS). The gram-negative bacteria included, *Salmonella typhimurium* (ST), *Klebsiella pneumonia*, (KP) and *Escherichia coli* (EC). In addition, fungi *Candida albicans* (CA), *Aspergillus niger* (AN) and *Aspergillus flavus* (AF) were also used for the experiments. All cultures were obtained from the Department of Microbiology, Manasagangotri, Mysore.

Preparation of inoculums

Bacterial inoculums were prepared by growing cells in Mueller Hinton Broth (MHA) (Himedia) for 24 h at 37° C. These cell suspensions were diluted with sterile MHB to provide initial cell counts of about 104 CFU/mL. The filamentous fungi were grown on sabouraud dextrose agar (SDA) slants at 28° C for 10 days and the spores were collected using sterile doubled distilled water and homogenized.

Disc diffusion assay

Antibacterial activity was carried out using a disc diffusion method. Petri plates were prepared with 20mL of sterile Mueller Hinton Agar (MHA) (Himedia, Mumbai). The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min. The tests were conducted at 1000 μg/disc. The loaded discs were placed on the surface of the medium and left for 30min at room temperature for compound diffusion. Negative control was prepared using respective solvent. Streptomycin (10 μg/disc) was used as positive control. The plates were incubated for 24 h at 37 °C for bacteria and 48h at 27 °C for fungi. A zone of inhibition was recorded in millimeters and the experiment was repeated twice.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration studies of synthesized compounds were performed according to the standard reference method for bacteria and filamentous fungi by serial dilution method (compound were dissolved in DMSO). An inoculum of 100 L from each well was inoculated. The antifungal agent's ketoconazole, fluconazole for fungi and streptomycin and ciprofloxacin for bacteria were included in the assays as positive controls. For fungi, the plates were incubated for 48–72h at 28 °C and for bacteria the plates were incubated for 24 h at 37 °C. The MIC for fungi was defined as the lowest extract concentration, showing no visible fungal growth after incubation time. 5ml of tested broth was placed on the sterile MHA plates for bacteria and incubated at respective temperatures. The MIC for bacteria was determined as the lowest concentration of the compound inhibiting the visual growth of the test cultures on the agar plate.

ANTIOXIDANT ACTIVITY

Compounds **6a-l** were tested for their antioxidant property by 1-diphenyl-2-picryl-hydrazil (DPPH), Nitric oxide (NO) and Hydrogen peroxide (H_2O_2) scavenging methods by using ascorbic acid as a standard.

DPPH radical scavenging activity

The hydrogen atom or electron donating ability of the compounds was measured from the bleaching of the purple colored methanol solution of DPPH. The spectrophotometric assay uses the stable radical DPPH as a reagent. 1 ml of various concentrations of the test compounds (25, 50, 75, 100 and 100 µg/ml) in methanol was added to 4 ml of 0.004% (w/v) methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against blank at 517 nm. The percent of inhibition (I %) of free radical production from DPPH was calculated by the following equation

% of scavenging = [(A control – A sample)/ A blank] x 100 (1)

where A control is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound. Tests were carried at in triplicate.

Nitric oxide (NO) scavenging activity

Nitric oxide scavenging activity was measured by slightly modified methods of Green et al. and Marcocci et al. Nitric oxide radicals (NO) were generated from sodium nitroprusside. 1 ml of

sodium nitroprusside (10 mM) and 1.5 ml of phosphate buffer saline (0.2 M, pH 7.4) were added to different concentrations (25, 50, 75 and 100 µg/ml) of the test compounds and incubated for 150 min at 25°C and 1 ml of the reaction mixture was treated with 1 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromatophore was measured at 546 nm. Nitric oxide scavenging activity was calculated using Eq. (1).

Hydrogen peroxide (H_2O_2) scavenging activity

The H_2O_2 scavenging activity of the test compound was determined according to the method of Ruch et al. A solution of H_2O_2 (40 µM) was prepared in phosphate buffer (pH 7.4). 25, 50, 75 and 100 µg/ml concentrations of the test compounds in 3.4 ml phosphate buffer were added to H_2O_2 solution (0.6 ml, 40 µM). The absorbance value of the reaction mixture was recorded at 230 nm. The percentage of scavenging activity of H_2O_2 was calculated using Eq. (1).

RESULT AND DISCUSSION

Chemistry

Synthesis of 3-phenylamide coumarin analogs (**6a**-**l**) was achieved via Knoevenagel condensation as outlined in Scheme 1. Briefly, commercially available o-hydroxy benzaldehyde (**1**) reacts with diethyl malonate (**2**) to yield ethyl coumarin ester (**3**) under refluxing conditions in the presence of piperidine and ethanol as a solvent. The treatment of compound **3** with sodium hydroxide mainly gave the coumarin carboxylic acid (**4**). Further, compound **4** coupled with substituted phenyl amine in the presence of coupling agents 2,6-dimethylpyridine and N,N,N',N'-tetramethyl-o-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU) provided the final products (**6a–l**) . The structures of newly synthesized compounds were confirmed on the basis of ^{1}H NMR, LC-MS and IR spectrometry analysis. All the synthesized intermediates and titled chemical entities gave satisfactory analyses for the proposed structures on the basis of their spectral data.

Biology

Substituted-3- phenylamide coumarins (**6a**-**l**) were primarily tested for in vitro antibacterial activity against Gram-positive bacterial strains (Streptococcus pyrogenes, Staphylococcus aureus, and Bacillus subtilis) and Gram-negative bacterial strains (Salmonella typhimurium , Klebsiella pneumonia, and Escherichia coli) employing the agar diffusion assay[33,34]. Further, ampicillin the antibiotic drug, was used as a positive control. Antibacterial evaluation for coumarin analogs (**6a**-**l**) and positive control was performed at a fixed concentration of 1000 g/mL. All twelve compounds in Table 1 exhibited antibacterial activity against both Gram-positive and Gram-negative bacterial strains with zones of inhibition ranging from 20 mm to 44 mm. Compounds **6d** with the chloro group at meta position, 6**f** and **6g** with the fluoro group at para and ortho portion respectively, in the phenyl ring were identified as potent antibacterial agents against all Gram-positive and Gram-negative bacterial strains. Compound **6h** with bromo group at ortho position in the phenyl ring also showed good antibacterial activity against all Grampositive and Gram-negative bacterial strains. Nevertheless compounds **6c, 6j** and **6k** with the methoxy group at para, meta and ortho poition respectively and compound **6e** with the methyl group at ortho position showed poor antibacterial activity. Besides, other compounds showed moderate activity. Based on the data from the antibacterial studies, the following observations can be made. Both chloro and fluoro substituted analogs exhibited similar antibacterial activity as the standard antibiotic drug, ampicillin against all Gram-positive and Gram-negative bacterial strains. More importantly, introducing bromo substituents on the phenyl ring afforded less, but good activity compared to chloro and fluoro substituted analogs and the reason might be due to the bulkiness of the bromo group which renders the molecule to penetrate through the cell wall of the bacteria.

Analogs **6a**-**l** were also examined for antifungal activity against different fungal strains, i.e. *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus* (Table 1). The antifungal drug, ketoconazole was used as a positive control. The fungal strains were grown and maintained on Sabouraud glucose agar plates. The plates were incubated at 26° C for 72 h, and resulting zone of inhibitions were measured[35]. Antifungal screening for analogs and positive control was performed at a fixed concentration of 1000 g/mL. Interestingly compounds **6d, 6f** and **6g** were also identified as the most potent antifungal agent against all three fungal strains. Compound **6a** with no substitute in the phenyl ring showed least activity against all the strains and the other compounds showed moderate activity.

The minimum inhibitory concentration (MIC) values for analogs (**6a–l)** and the positive control drugs ampicillin and ketoconazole were also determined against the six bacterial strains and the three fungal strains by the liquid dilution method[36,37]. Concentrations of analogs and positive control drugs at 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 g/mL were prepared in an appropriate solvent. Inoculums of the bacterial and fungal cultures were also prepared. Inoculum (0.2 mL) and sterile water (3.8 mL) were added to a series of tubes, each containing 1 mL of test compound solution at the 11 different concentrations. The tubes were incubated for 24 h and carefully observed for the presence of turbidity. The minimum concentration at which no growth was observed was taken as the MIC value. The MIC values for all the analogs examined ranged from 2.5 to 50 g/mL. Several analogs exhibited superior antimicrobial activity compared to the positive control drugs, ampicillin and ketoconazole. Three halogenated analogs, **6d, 6f** and **6g** were identified as potent antibacterial agents against both Gram-positive and Gram-negative bacterial strains, these analogs showed superior as well as the same magnitude of antibacterial activity as the standard antibiotic, ampicillin against different bacterial strains. Compound **6h** showed good potency against *Streptococcus pyogenes*, *Bacillus subtilis* and *Escherichia coli*. Thus, based on the MIC data, analogs **6d, 6f** and **6g** were identified as the most potent antimicrobial agents examined. The MIC data for all the analogs against the different bacterial and fungal strains are shown in Table 2.

Compounds **6a**-**l** were also tested for in vitro antioxidant property by 1,1-diphenyl picrylhydrazyl (DPPH)[38,39] nitric oxide (NO)[40,41] and hydrogen peroxide (H_2O_2)[42] methods which were summarized in Tables 3-5 respectively. Compounds **6c** and **6e** showed good radical scavenging activity in all three methods due to the presence of electron donating groups such as methoxy and methyl groups attached to the phenyl ring when compared with the standard drug ascorbic acid. Compounds **6h, 6j, 6k** showed moderate antioxidant activity, whereas the other compounds displayed mild activity. In general, it was observed that electron donating substituted compounds exhibited greater activity when compared with the respective electron withdrawing substituted compounds.

Table 1 - Zone of inhibition of data for compounds 6a–l against different bacteria and fungi at 1000 g/mL concentration

Compound	Zone of inhibition (in mm)								
	Bacteria[a]			Bacteria[b]			Fungi[c]		
	SP	SA	BS	ST	KP	EC	CA	AN	AF
6a	26	23	28	32	32	35	20	30	26
6b	30	27	30	29	35	35	30	28	23
6c	24	21	27	28	25	31	28	31	32
6d	33	34	37	44	41	42	40	44	40
6e	23	20	21	33	22	34	25	37	34
6f	34	32	38	41	42	40	40	43	39
6g	32	31	35	40	40	40	41	40	40
6h	30	29	33	43	36	38	37	40	38
6i	28	25	29	31	32	33	29	36	30
6j	23	21	30	26	30	27	30	29	27
6k	22	20	26	22	26	22	27	33	24
6l	23	23	28	29	23	35	35	38	33
AMP[d]	33	33	38	43	42	40	-	-	-
KET[e]	-	-	-	-	-	-	41	44	41

[a] Gram-positive bacteria: SP- *Streptococcus pyogenes*; SA-*Staphylococcus aureus*; BS: *Bacillus subtilis*. [b] Gram-negative bacteria: ST- *Salmonella typhimurium*; KP- *Klebsiella pneumonia*; EC- *Escherichia coli*. [c] CA- Candida albicans; AN- Aspergillus niger; AF- Aspergillus flavus. [d] AMP- Ampicillin. [e] KET- Ketoconazole.

Table 2 - Minimum inhibitory concentration values for compounds 6a–l and positive control drugs against different bacteria and fungi

Compounds	Minimum inhibitory concentration (g/mL)								
	Bacteria[a]			Bacteria[b]			Fungi[c]		
	SP	SA	BS	ST	KP	EC	CA	AN	AF
6a	45	30	35	30	25	25	25	40	35
6b	30	35	25	45	30	40	40	45	50
6c	35	25	40	45	40	35	40	20	40
6d	10	2.5	5	10	2.5	10	35	50	35
6e	35	45	45	50	30	45	40	50	35
6f	10	2.5	5	10	2.5	5	30	40	35
6g	5	10	10	10	2.5	10	25	40	30
6h	10	25	5	15	20	10	35	30	35
6i	25	20	25	40	15	20	40	45	30
6j	25	30	25	15	30	25	25	40	50
6k	30	20	35	30	25	40	25	30	35
6l	35	30	25	15	20	25	35	40	30
AMP[d]	25	10	20	10	10	15	-	-	-
KET[e]	-	-	-	-	-	-	25	15	15

[a] Gram-positive bacteria: SP- *Streptococcus pyogenes*; SA-*Staphylococcus aureus*; BS: *Bacillus subtilis*. [b] Gram-negative bacteria: ST- *Salmonella typhimurium*; KP- *Klebsiella pneumonia*; EC- *Escherichia coli*. [c] CA- Candida albicans; AN- Aspergillus niger; AF- Aspergillus flavus. [d] AMP- Ampicillin. [e] KET- Ketoconazole.

Table 3 – *In vitro* antioxidant activity of compounds 6a-l in DPPH method

Compounds	Concentration (µg/ml)				
	25	50	75	100	IC_{50}
6a	52.75 ± 1.65	58.45± 1.08	61.65± 0.95	65.75 ± 1.56	23.88 ± 1.46
6b	48.89 ± 0.67	53.75± 1.28	57.67 ±0.58	62.64 ± 0.88	26.88 ± 1.17
6c	70. 86 ± 0.12	75.34 ± 0.37	77.33 ± 0.38	81.22± 0.61	15.12 ± 0.53
6d	57.24 ± 1.10	62.35 ± 1.16	66.19± 0.80	70.24 ± 0.86	18.68± 0.69
6e	74.55± 0.20	77.28 ± 0.40	81.59± 0.50	83.66 ± 0.67	15.09 ± 0.22
6f	68. 49 ±1.23	72.85 ± 1.39	74.75±1.53	79.25 ±1.05	18.29±1.10
6g	64.68 ± 1.04	69.53 ±1.38	73.75± 0.82	77.65 ± 1.62	19.66 ± 1.07
6h	67.46 ± 1.06	69.26 ± 0.71	74.16± 1.24	79.33 ± 1.00	16.37 ± 1.00
6i	61.66 ±0.60	65.26 ±1.25	70.01±1.22	74.55 ±1.27	17.55 ±1.14
6j	68.57 ± 0.18	73.55 ± 0.34	75.58 ± 0.59	79.21 ± 0.60	16.08 ± 0.49
6k	71.37±0.29	74.39 ± 0.51	76.19±0.60	81.58±0.79	16.26 ± 0.44
6l	62.66 ± 1.35	66.48 ± 1.12	71.37± 1.48	75.38 ± 0.84	17.67 ± 1.06
Ascorbic acid	81.72±0.07	82.32±0.32	84.34±0.37	86.16±0.44	14.35±0.38
Blank	-	-	-	-	-

(-) Showed no scavenging activity. Values were the means of three replicates ± SD.

Table 4 - The *In vitro* antioxidant activity of compounds 6a-l in nitric oxide (NO) method

Compounds	Concentration (µg/ml)				
	25	50	75	100	IC_{50}
6a	68.58 ± 0.80	71.88 ± 1.00	75.58±1.32	80.44 ± 1.20	15.45 ± 0.88
6b	60.75 ±1.55	63.48 ± 1.21	66.75± 1.31	72.55 ± 0.72	20.25 ± 0.95
6c	73.45 ± 0.20	77.33 ± 0.30	80.32 ± 0.49	83.49±0.65	14.44±0.49
6d	63.75 ± 1.32	71.68 ± 1.24	76.76 ±1.05	78.39 ±0.76	18.77±1.12
6e	75.88 ± 0.21	80.75 ± 0.40	81.89 ± 0.51	83.65±0.71	13.89 ± 0.70
6f	70.46 ±0.76	72.41 ± 0.87	76.38 ± 1.00	80.55 ± 1.30	15.78 ± 0.49
6g	68.37 ± 0.91	70.48 ± 1.37	74.49 ± 0.95	79.19 ± 1.24	19.45± 1.07
6h	60.07±1.31	65.58±1.56	69.77±0.69	72.66±1.03	17.27±0.81
6i	61.88± 1.09	66.92 ± 1.49	71.83 ± 1.29	76.87 ± 0.61	16.86 ± 1.17
6j	71.47 ± 0.20	76.35 ± 0.36	80.56±0.57	82.65 ± 0.69	15.64 ±0.79
6k	69.21 ±0.08	74.65 ± 0.25	79.19 ±0.44	81.19 ± 0.61	16.11 ± 0.82
6l	62.75±1.57	63.58 ± 1.26	69.73 ± 1.35	72.59±0.75	21.68 ± 0.97
Ascorbic acid	83.49±0.14	84.37±0.31	87.18±0.45	89. 19±0.73	13.64±0.50
Blank	-	-	-	-	-

(-) Showed no scavenging activity. Values were the means of three replicates ± SD.

Table 5 - The *In vitro* antioxidant activity of compounds 6a-l in hydrogen peroxide (H_2O_2) method

Compounds	Concentration (µg/ml)				
	25	50	75	100	IC_{50}
6a	60.47 ± 1.06	63.67 ± 1.27	67.78 ± 1.56	70.48 ± 0.85	20.29 ± 0.53
6b	56.76 ± 1. 04	60.53±1.11	63.57±1.39	67.28 ± 1.46	20.07 ± 1.00
6c	66.36 ± 0.19	69.48 ± 0.39	72.58 ± 0.51	77.28 ± 0.73	16.65 ± 0.88
6d	51.48 ± 0.80	54.58 ± 1.31	57.69 ± 0.70	62.68 ± 1.00	21.37 ± 0.34
6e	63.17±1.01	66.29± 1.21	68.38± 0.51	71.18±0.61	16.07±0.46
6f	51.68 ± 1.16	54.59 ± 0.86	58.38 ± 0.96	63.58 ± 0.64	24.63 ± 1.05
6g	54.64±1.15	58.57 ± 0.85	62.72 ± 1.45	66.53 ± 0.84	22.72±0.66
6h	63.66 ± 0.29	65.78 ± 0.45	68.79 ± 0.65	72.79 ± 0.75	19.58±1.25
6i	60.19 ± 0.78	61.88± 1.50	66.66 ± 1.00	70.19± 1.30	19.09 ± 1.15
6j	60.06 ±1.25	62.85 ± 1.11	67.19±1.00	71.48 ± 1.48	18.09±0.70
6k	64.58 ± 1.05	67.19 ± 1.21	69.27 ±0.51	72.47 ± 0.61	16.28±0.51
6l	62.47 ± 0.18	65.29 ± 0.55	67.47 ± 0.58	70.47 ± 0.69	18.18 ± 0.18
Ascorbic acid	75.59±0.14	77.28±0.26	81.18±0.55	85.18±0.62	15.16±0.08
Blank	-	-	-	-	-

(-) Showed no scavenging activity. Values were the means of three replicates ± SD.

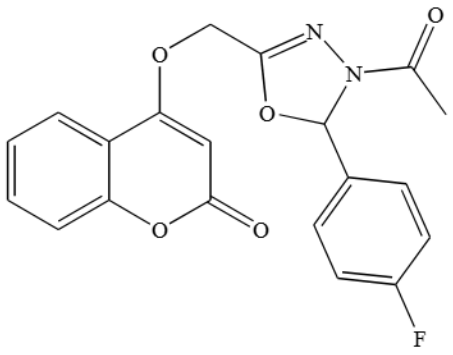


Fig A

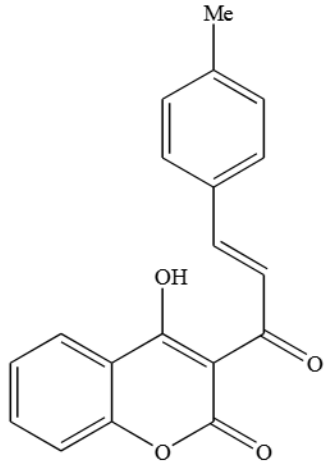
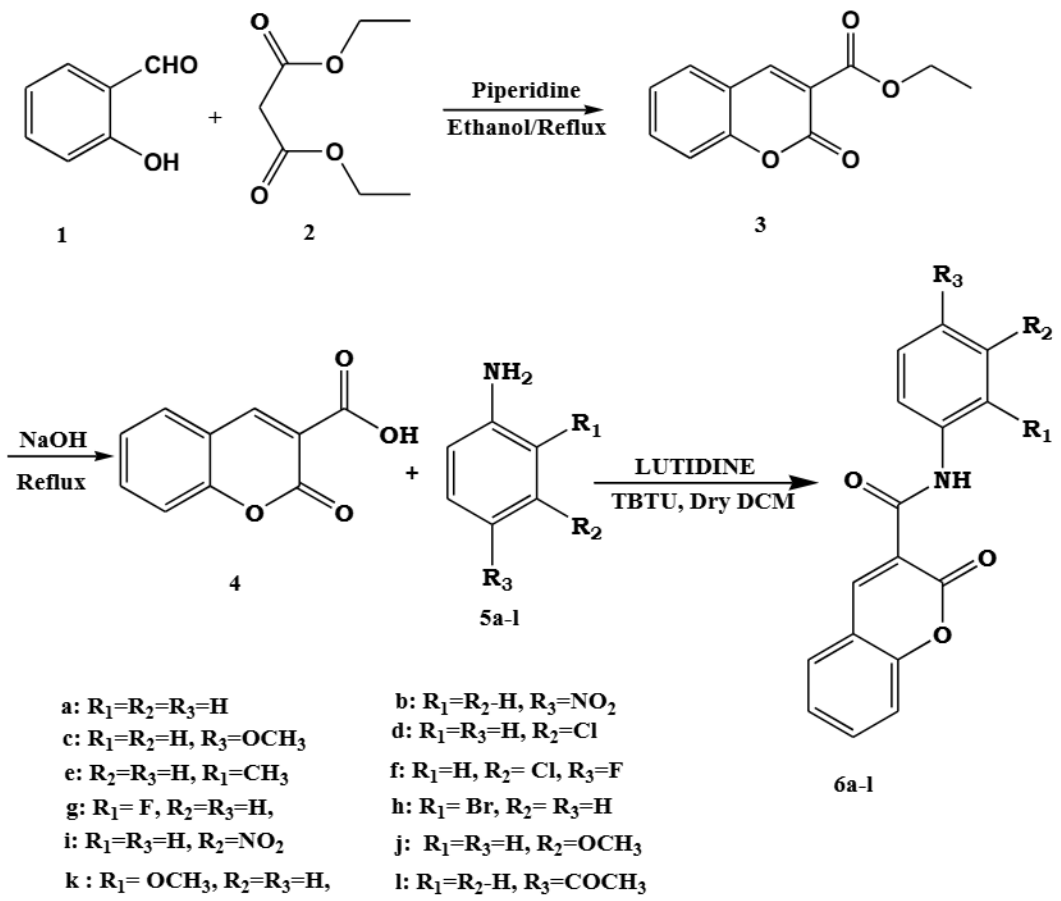


Fig B

Figure 1. Representative drug examples of coumarin derivatives



Scheme 1. Synthesis of 2-Oxo-2H-chromene-3-carboxylic acid phenylamide analogs

CONCLUSION

Finally, a small sub-library of phenylamide coumarins (**6a–l)** that integrates a variety of substituents at the ortho, meta and para position of the phenyl moiety have been synthesized and evaluated for their antimicrobial activity against a panel of bacterial and fungal strains. Incorporating lipophilic electron-withdrawing fluoro or chloro substituents in the phenyl ring afforded molecules with potent antimicrobial activity. Analogs **6d, 6f** and **6g** were considered lead compounds worthy of further structural optimization and development as potential antimicrobial agents for the treatment of bacterial and fungal infections. Further, the title compounds were also evaluated for their antioxidant activity. It was observed that the compounds having electron donating groups **6c** and **6e** exhibited greater antioxidant activity. The investigation of antioxidant screening data reveals that the remaining compounds showed moderate to mild inhibition activity. The presence of the electron donating substituent enhanced and electron withdrawing groups decreased the antioxidant activity.

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