Enzyme inhibition and antibacterial potential of 4-Hydroxycoumarin derivatives

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The 4-Hydroxycoumarin derivatives are known to show a broad spectrum of pharmacological applications. In this paper we are reporting the synthesis of a new series of 4-Hydroxycoumarin derivatives synthesized through Knovenegal condensation; they were characterized by using UV-Vis, FT-IR, NMR spectroscopies. The synthesized compounds were evaluated for antibacterial activity against Staphylococcus aureus and Salmonella typhimurium strains. The compounds (2), (3) and (8) showed favorable antibacterial activity with zone of inhibitions 26.5± 0.84, 26.0 ± 0.56 and 26.0 ± 0.26 against Staphylococcus aureus (Gram-positive) respectively. However, the compounds (5) and (9) were found more active with 19.5 ± 0.59 and 19.5 ± 0.32 zone of inhibitions against Salmonella typhimurium (Gram-negative). Whereas, in urease inhibition assay, none of the synthesized derivatives showed significant anti-urease activity; although, in carbonic anhydrase-II inhibition assay, the compound (2) and (6) showed enzyme inhibition activity with IC⁵₀ values 263±0.3 and 456±0.1, respectively.

Keywords: 4-Hydroxycoumarin. Anti-urease. Carbonic anhydrase. Aldehydes. Antibacterial.

INTRODUCTION

The derivatives of 4-Hydroxycoumarin (Figure 1) are of great interest due to their broad spectrum of pharmacological activities including antioxidant (Jung, Park, 2009), anti-allergy (Lee et al., 2004), antiviral (Yu et al., 2003), anti-diabetic (Kontogiorgis, Hadjipavlou-Litina, 2005), anti-HIV (Tosun et al., 2008), anticonvulsant (Sashidhara et al., 2010), anti-tubercular (Jin, Chen, Zhu, 2007), anti-hyperlipidemic (Hamdi et al., 2011), rodenticidal (Khan et al., 2004) and antifungal (Gao et al., 2010). Some of the 4-Hydroxycoumarin derivatives such as warfarin, coumatetralyl, bromadiolone, flocoumafen and brodifacoum are known due to their anticoagulant (Stanchev et al., 2009; Trivedi et al., 2007), anti-inflammatory (Cardoso et al., 2011), antipyretic, antibacterial (Jung et al., 2004; Manolov, Maichle-Moessmer, Danchev, 2006) antiviral as well as anticancer activities (Inoue et al., 1994; Kostova et al., 2001). The coumarin derivatives are also used in medicines for treatment of renal stones. These stones are known to form by the decomposition of urea to carbon dioxide and ammonia causing urological diseases (Gunnwegr, Hoefnagel, van Bekkum, 1995; De la Hoz, Moreno, Vazquez, 1999). The coumarin derivatives are also reported for the inhibition of carbonic anhydrase enzyme which are involved in osteoporosis and malfunctioning of the human kidneys (Stefanou et al., 2011). Therefore,
synthesizing new derivatives of 4-Hydroxycoumarin may lead towards the discovery of some new potent compounds which can prove to be beneficial while developing medicines of above mentioned diseases.

The organic compounds such as 4-Hydroxycoumarin having active methylene group can undergo Knovenegal condensation with compounds possessing carbonyl functionality (Kidwai et al., 2007). Knovenegal condensation is a known method for the formation of carbon to carbon double bond. Here, in this paper, we are reporting new derivatives of 4-Hydroxycoumarin which are synthesized through Knovenegal condensation.

**FIGURE 1** - Structure of 4-Hydroxycoumarin.

**MATERIAL AND METHODS**

The UV-Vis spectra of synthesized 4-Hydroxycoumarin derivatives (1-9) were recorded on PG instrument T80 + UV-Vis Spectrophotometer ranging from 200 nm to 800 nm. The FT-IR analysis of compounds was performed on PerkinElmer Spectrum 100 FT-IR Spectrometer. The 1H NMR δ (ppm) spectra were recorded in CDCl3/DMSO-d6 by using Bruker AM-400 & 500 MHz NMR spectrometer. All the chemicals and solvents used in this research were purchased from SigmaAldrich®/Fluka and were of analytical grade. Chemicals/solvents were used without any further purification, until and unless mentioned.

**Synthesis of 4-hydroxy-6-nitrocoumarin (1)**

To a pre-cooled sulfuric acid (10 mL), 4-Hydroxycoumarin (3.08 mmol; 1.0 equiv.) and sodium nitrate (4.60 mmol; 1.2 equiv.) were added. The resulting mixture was stirred at 0 °C for 1.0 h. The reaction progress was monitored on TLC (dichloromethane:methanol 4:1). On completion of the reaction, the reaction mixture was poured on ice-cooled water. The crude product precipitated out and was filtered; subsequently, the product was re-crystallized using a mixture of EtOAc/n-hexane (7:3) to obtain the final product as a white solid (Huang et al., 2007).

**General procedure for the synthesis of 4-hydroxy-6-nitrocoumarin derivatives (2-9)**

The derivatives of 4-Hydroxycoumarin (2-9) were synthesized by the reaction of (1) (0.48 mmol; 1.0 equiv.) with different substituted aromatic aldehydes (0.48 mmol; 1.0 equiv.) in 15 mL of ethanol. The reaction mixture was refluxed for 20-30 h as shown in (Scheme 1). Reaction progress was monitored by TLC (dichloromethane:methanol 4:1). Each product precipitated out as a solid which was subsequently filtered and washed with saturated solution of sodium bisulphite (3x) to get pure production moderate to good yield.

**SCHEME 1** - General synthetic route for 4-Hydroxycoumarin derivatives.
Antibacterial activity

The synthesized derivatives of 4-Hydroxycoumarin were subjected to an antimicrobial activity analysis by using agar well diffusion method (Patonay et al., 1984). During this study, two clinically isolated bacterial strains from the blood of diseased patients were used. One strain was Staphylococcus aureus (S. aureus; Gram-positive) which can cause food poisoning, skin infection, respiratory tract infections. Whereas, the second strain was Salmonella typhimurium (S. typhimurium; Gram-negative) which is known to cause typhoid fever, weakness, stomachache, headache or loss of appetite. The ciprofloxacin was used as a positive control (8.3 µg/µL in DMSO). The DMSO was used as a negative control. In this method, agar plates were prepared by pouring about 10 mL agar solution in each petri dish (94 mm x 16 mm). Next, the swishing of agar filled petri dish was carried out in order to smooth the surface. The inhibition zones were measured in millimeters (mm) by using a ruler. These plates were inoculated with bacterial strains under sterilized conditions and wells were filled with test samples (compound 1-9), which were prepared by dissolving 1 mg of each sample in 120 µL of DMSO. Specified wells were also filled with positive as well as negative controls. The 30 µL of each sample along with positive and negative controls were added to each well followed by incubation of petri dishes at 37 °C for 20-24 h. After completion of the growth period, the zones of inhibition were measured.

Enzyme inhibition

Urease inhibition assay

The reaction mixture containing 25 µL of enzyme (jack bean urease) solution, 55 µL of buffer solution (containing 100 mM urea) and 5 µL of test compound (0.5 mM) were incubated in a 96-well flat bottom plate at 30 °C for 15 min. Urease inhibition was measured by indophenol method, described by Weatherburn (Arfan et al., 2010). Briefly, 45 µL of each phenol reagent which contains [1% (w/v) phenol and 0.005% (w/v) sodium nitroprusside], and the 70 µL of alkali reagent containing [0.5% (w/v) sodium hydroxide and 0.1% NaOCl] were added to the each well. The increasing change in absorbance was measured at 630 nm after 50 min by using a microplate reader SpectraMax, M2 (Molecular Device, CA, USA). All the reactions were repeated three times in a final volume of 200 µL. The entire assay was accomplished at pH 6.8. The percentage inhibition was measured by using the above equation. In this method thiourea was used as the reference standard inhibitor of urease activity.

Carbonic anhydrase inhibition assay

The hydrolysis of 4-nitrophenyl acetate (4-NPA) produces 4-nitrophenol and CO2. The reaction was examined by quantifying the formation of a yellow colored compound, 4-nitrophenol at a wavelength of 400 nm. In this experiment, HEPES-tris buffer was used at a final concentration of 20 mM at pH 7.4. The total reaction volume of 200 µL was made by adding 140 µL of the HEPES-tris, 20 µL of test compounds (0.5 mM) dissolved in DMSO, 20 µL of purified bovine erythrocyte CA-II (0.1 mg/mL) prepared in de-ionized water, and 20 µL of 4-NPA (0.7 mM) dissolved in ethanol. As a control, 20 µL of DMSO was used instead of test compound. Initially test compounds were incubated in a 96-well flat bottom plate for a period of 15 min with the enzyme. The rate of product formation was measured by the addition of 20 µL substrate at 25 °C for a period of 30 min with regular interval of 1 min. SpectraMax M2 (Molecular Device, CA, USA) was used for the measurement of product (4-nitrophenol) formation in triplicate, at a wavelength of 400 nm (Supuran, Scozzafava, Casini, 2003).

RESULTS AND DISCUSSION

4-Hydroxy-6-nitrocoumarin (1)

The compound (1) was obtained by the nitration of 4-Hydroxycoumarin using sodium nitrate and sulfuric acid. Yield: 70%; Rf; 0.5 (dichloromethane:methanol 4:1); m.p.: 252-254 °C; UV-Vis (DMF) λmax: 315 nm; FT-IR (KBr) ν: 3500, 1753, 1608, 1515, 1343 cm⁻¹, 1H NMR (400 MHz, CDCl3) δ:8.67 (dd, J = 4, 8 Hz, 1H at C 1), 7.56 (d, J = 8 Hz, 1H at C 2), 9.07 (d, J = 4 Hz, 1H at C 5), 5.85 (s, 1H at C 9), 15.98 (s, 1H at C 10). The product was obtained in comparatively good yield with a considerable range of melting point and maximum absorbance at 315 nm. The presence of nitrogen containing functional group has been confirmed by the appearance of signals in FT-IR at 3140 cm⁻¹ and 1524 cm⁻¹. This confirms that a successful nitrogen containing coumarin has been synthesized. Furthermore, the possibility of nitrating occurring at alkene functionality has been ruled out by
the presence of stretching vibration of the alkene group at 1608 cm\(^{-1}\) which verifies that the alkene functionality is intact in the synthesized molecule. Similarly, the presence of vibration frequencies at 3500 cm\(^{-1}\) and 1753 cm\(^{-1}\) clearly confirm the presence of hydroxyl as well as carbonyl functionalities in the compound, respectively. The appearance of singlet at 15.98 in 1HNMR, taken in CDCl\(_3\), also confirms the presence of hydroxyl groups, whereas appearance of a downfield singlet at 5.85 shows the existence of alkene group. Moreover, the doublets appearing at 9.07, 8.67 and 7.56 ppm belong to protons appearing at C 5, 1 and 2, respectively.

(Z)-3-(4-Nitrobenzylidene)-6-nitro-3H-chromene-2,4-dione (2)

The compound (2) was synthesized by reacting (1) with 4-nitrobenzaldehyde. Yield: 65%; Rf: 0.7 (dichloromethane:methanol 4:1); m.p.: 274-276 °C; UV-Vis (DMF) \(\lambda_{\text{max}}\): 305 nm; FT-IR (KBr) \(\nu\): 3095, 1683, 1615, 1597, 1515, 1343, 1246 cm\(^{-1}\), 1H NMR (500 MHz, DMSO-d6) \(\delta\): 8.56 (s, 1H at C 1), 8.37 (d, J = 8 Hz, 1H at C 3), 8.07 (d, J = 8 Hz, 1H at C 4), 7.55 (d, J = 8 Hz, 2H at C 16 and 18), 7.43 (d, J = 8 Hz, 2H at C 15 and 19), 6.31 (s, 1H at C 20). The first derivative of 4-hydroxy-6-nitrocoumarin was obtained in good yield and considerable melting point range. The 10 nm shift in maximum UV/Vis absorbance of the compound from 4-hydroxy-6-nitrocoumarin as well as the appearance of the signal at 1615 cm\(^{-1}\) in FT-IR are the prime evidences of the successful synthesis of the compound under discussion. Moreover, the disappearance of FT-IR signal at 3500 cm\(^{-1}\) along with intense signal appearance at 1683 cm\(^{-1}\) prove that the hydroxyl group has been oxidized to form carbonyl group. In 1HNMR, the appearance of singlet at C 20 (6.31 ppm) confirms the presence of an exocyclic double bond which is next to 4-hydroxy-6-nitrocoumarin. Also the appearance of doublets with integration for 2 protons at 7.43 ppm and 7.55 ppm verifies the presence of 4-nitrobenzene moiety. The rest of the 1HNMR shifts are in line with the precursor and prove its integrity.

(Z)-3-(3-Nitrobenzylidene)-6-nitro-3H-chromene-2,4-dione (3)

The compound (3) was synthesized by reacting (1) with 3-nitrobenzaldehyde. Yield: 60%; Rf: 0.6 (dichloromethane:methanol 4:1); m.p.: 261-263 °C; UV-Vis (DMF) \(\lambda_{\text{max}}\): 304 nm; FT-IR (KBr) \(\nu\): 1349, 1533, 1620, 1543, 1249, 1682, 3097 cm\(^{-1}\), 1H NMR (500 MHz, DMSO-d6) \(\delta\): 8.57 (s, 1H at C 1), 8.37 (d, J = 8 Hz, 1H at C 3), 7.65 (d, J = 8 Hz, 1H at C 15), 7.50-7.56 (m, 2H at C 4 and 16), 8.03 (d, J = 8 Hz, 1H at C 17), 7.92 (s, 1H at C 19), 6.32 (s, 1H at C 20). This compound was synthesized in 60% yield with acceptable melting point range and similar absorbance in UV/Vis analysis to (2). This compound showed similar FT-IR analysis to (2) as well as comparable 1HNMR shifts as far as the integrity of 4-hydroxy-6-nitrocoumarin moiety and exocyclic double bond formation confirmation are concerned. However, in 1HNMR, at C 19 the appearance of singlet at 7.92 ppm along with two doublets and one multiplet at 7.65, 8.03 and 7.50-7.56 ppm for C 15, C 17 and C 16, respectively confirms that the C 18 is not attached to a proton as there is no proton signal related to C 18. Moreover, the downfield shifting of chemical shifts of protons at C 15-18 and C 19 can be attributed to the presence of a nitrogroup at C 18. This confirms the presence of 2-nitrobenzene moiety in the synthesized compound.

(Z)-3-(5-Fluoro-2-hydroxybenzylidene)-6-nitro-3H-chromene-2,4-dione (4)

The compound (4) was synthesized by the reaction of (1) with 5-fluoro-2-hydroxybenzaldehyde. Yield: 78%; Rf: 0.6 (dichloromethane:methanol 4:1); m.p.: 291-294 °C; UV-Vis (DMF) \(\lambda_{\text{max}}\): 307 nm; FT-IR (KBr) \(\nu\): 3069, 1716, 1526, 1425, 1340, 1194 cm\(^{-1}\), 1H NMR (400 MHz, CDCl3) \(\delta\): 8.50 (d, J = 4 Hz, 1H at C 1), 8.34 (dd, J = 4, 8 Hz, 1H at C 3), 7.56 (d, J = 8 Hz, 1H at C 4), 7.48 (d, J = 4 Hz, 1H at C 15), 7.35 (dd, J = 4, 8 Hz, 1H at C 17), 7.21 (d, J = 8 Hz, 1H at C 18), 5.64 (s, 1H at C 19), 8.06 (s, 1H at C 20). The synthesized compound was in good yield as compared to above described compounds. The shift in maximum absorbance by 7 nm compared to 4-hydroxy-6-nitrocoumarin along with the appearance of 1621 cm\(^{-1}\) signal in FT-IR spectrum, which determines the presence of an exocyclic double bond, are the results which point out towards successful synthesis of the compound under discussion. An intense signal at 1194 cm\(^{-1}\) refers to the presence of fluorine in the molecule. Furthermore, the remaining vibrations recorded in FT-IR spectrum are similar to compound (2) which shows that 4-hydroxy-6-nitrocoumarin moiety is intact with an exception of the conversion of the hydroxyl
group to a carbonyl group. In 1H NMR taken in CDCl3, the appearance of singlet at 8.06 ppm refers to the presence of olefinic proton, singlet at 5.64 ppm belongs to the hydroxyl group, whereas doublets at 7.48 ppm and 7.21 ppm belong to protons attached to C 15 and C 18, respectively. The doublet of doublet appearing at 7.35 ppm is of the proton attached to C 17. The spectroscopic analysis shows the successful synthesis of compound under discussion.

(Z)-3-(2-Hydroxy-5-methylbenzylidene)-6-nitro-3H-chromene-2,4-dione (5)

The compound (5) was synthesized by reacting (1) with 2-hydroxy-5-methylbenzaldehyde. Yield: 75%; Rf: 0.6 (dichloromethane:methanol 4:1); m.p.: 272-274 °C; UV-Vis (DMF) λmax: 306 nm; FT-IR (KBr) v: 3125, 1770, 1650, 1570, 1440, 1325 cm^-1. 1H NMR (400 MHz, CDCl3) δ: 8.48 (d, J = 8 Hz, 1H at C 1), 8.35 (d, J = 8 Hz, 1H at C 3), 7.48 (d, J = 8 Hz, 1H at C 4), 7.15 (s, 1H at C 15), 7.21 (dd, J = 4, 8 Hz, 1H at C 17), 6.95 (d, J = 8 Hz, 1H at C 18), 11.23 (s, 1H at C 19), 8.67 (s, 1H at C 20), 2.34 (s, 3H at C 22). The maximum absorption, FT-IR analysis and 1HNMR spectrum of compound (5) are similar to compound (4) with the exception that in FT-IR, the compound (5) did not show fluorine functionality related signal. Moreover, the chemical shifts in 1HNMR are also in line with the evidences showing that the compound under study has successfully been synthesized. For instance, singlet at 8.67 ppm shows presence of the exocyclic double bond. Moreover, the multiplicity and the coupling pattern along with coupling constant values in, 1HNMR, all the signals of 4-hydroxy-6-nitrocoumarin moiety were observed along with singlet at 8.95 ppm which shows the olefinic proton presence. Also, two doublets appearing at 6.90 ppm and 7.11 ppm showed the presence two chemically equivalent protons at both C 15 and C 19 as well as C 16 and C 18 positions. The presence of OH group was confirmed by the appearance of singlet at 5.31 ppm. These results prove the successful synthesis of compound under discussion.

(Z)-3-(4-Methoxybenzylidene)-6-nitro-3H-chromene-2,4-dione (7)

This compound (7) was synthesized by the reaction of (1) with 4-methoxybenzaldehyde. Yield: 67%; Rf: 0.6 (dichloromethane:methanol 4:1); m.p.: 255-257 °C; UV-Vis (DMF) λmax: 304 nm; FT-IR (KBr) v: 3115, 1780, 1640, 1580, 1465, 1325 cm^-1, 1H NMR (400 MHz, CDCl3) δ: 8.51 (d, J = 8 Hz, 2H at C 1 and 3), 6.90 (d, J = 8 Hz, 1H at C 4), 3.83 (s, 3H at C 17), 7.11 (d, J = 8 Hz, 2H at C 16 and 18), 7.59 (d, J = 8 Hz, 2H at C 15 and 19), 8.95 (s, 1H at C 20). The yield of compound (7) is also appreciable with acceptable melting point range and considerable difference in maximum absorption. The FT-IR spectrum of this compound is similar to compound (6) with stretching vibration of exocyclic double bond at 1660 cm^-1. In case of 1HNMR of this compound, the chemical shifts for 4-hydroxy-6-nitrocoumarin moiety are similar to previously described compounds, thereby confirming the integrity of 4-hydroxy-6-nitrocoumarin moiety with the conversion of the hydroxyl group to carbonyl group. The synthesis of this compound is evident by the signal appearing at 8.95 ppm as singlet which belongs to proton attached to olefinic carbon. Similarly, the multiplicity and coupling patterns along with coupling constants are following the same sequence as in compound (6). The signal at 3.83 ppm chemical shift is a singlet with integration of 3 protons which clearly explains the presence of the methoxy group in the molecule. Moreover, the chemical shifts of protons attached to C 15, 16, 18 and 19 shifted downfield with respect to methoxy group. All these results clearly emphasize on the successful synthesis of compound (7).
The compound (8) was synthesized by the reaction of (1) with 2-hydroxybenzaldehyde. Yield: 77%; Rf: 0.7 (dichloromethane:methanol 4:1); m.p.: 294-297 ºC; UV-Vis (DMF) λ.max: 306 nm, FT-IR (KBr) ν: 3072, 1716, 1619, 1526, 1486, 1343, 1221 cm⁻¹, 1H NMR (400 MHz, CDCl3) δ: 8.55 (d, J = 4 Hz, 1H at C 1), 8.42 (dd, J = 4, 8 Hz, 1H at C 3), 7.68 (d, J = 8 Hz, 1H at C 4), 7.50 (d, J = 8 Hz, 1H at C 15), 7.76 (t, J = 8 Hz, 1H at C 16), 7.20 (d, J = 8 Hz, 1H at C 18), 10.16 (s, 1H at C 19), 8.14 (s, 1H at C 20). The compound (8) has successfully been synthesized in 77% yield and its melting point was recorded close to 300 ºC. The maximum absorption shift in the UV/Vis analysis along with the presence of FT-IR vibration at 1619 cm⁻¹ for alkene functionality confirms the synthesis of compound (8). The FT-IR vibrations for carbonyl functionality along with nitro functionality were present in the molecule, thereby confirming that the 4-hydroxy-6-nitrocoumarin part of the molecule is present in this molecule as was present in the previously synthesized compounds. The 1HNMR also confirms the synthesis of compound (8) in similar fashion as for previous compounds. The presence of 2-hydroxybenzaldehyde is confirmed by appearance of chemical shift for hydroxyl group at 10.16 ppm as a singlet. Moreover, the signals and the multiplicity of protons attached to C 15-18 also confirms the successful synthesis of compound (8).

(Z)-3-(2-Hydroxy-4-methoxybenzylidene)-6-nitro-3H-chromene-2,4-dione (9)

The compound (9) was synthesized by reacting (1) with 2-hydroxy-4-methoxybenzaldehyde. Yield: 79%; Rf: 0.7 (dichloromethane:methanol 4:1); m.p.: 266-268 ºC; UV-Vis (DMF) λ.max: 305 nm, FT-IR (KBr) ν: 3124, 1719, 1623, 1589, 1487, 1343, 1207 cm⁻¹. 1H NMR (400 MHz, CDCl3) δ: 8.48 (d, J = 8 Hz, 1H at C 1), 8.35 (d, J = 8 Hz, 1H at C 3), 7.48 (d, J = 8 Hz, 1H at C 4), 6.85 (d, J = 4 Hz, 1H at C 15), 6.89 (t, J = 4 Hz, 1H at C 16), 3.89 (s, 1H at C 17), 5.29 (s, 1H at C 18), 12.25 (s, 1H at C 19), 8.04 (s, 1H at C 20).

Overall discussing the methods adopted in order to conduct this research, in the first step the nitration of 4-hydroxycoumarin was performed as reported (Huang et al., 2007). According to the literature, the reaction of 4-Hydroxycoumarin with sodium nitrate in the presence of sulfuric acid at 0 ºC introduces the nitro group to the 4-Hydroxycoumarin moiety at C 6 and yields the 6-nitro-4-hydroxycoumarin (1). In the second step, the compound (1) undergoes Knovenegal condition at C 3. The reaction conditions were optimized by the reaction of a compound (1) with different substituted aromatic aldehydes. The ethanol was taken as solvent in the synthesis of all compounds (2-9) (Table I). The completion time of this second step reaction is largely dependent upon the nature of substituents on aromatic aldehydes. The reaction completion was monitored on TLC by using Dichloromethane : Methanol in (4:1).

| Compound No. | Compounds                        | Yield (%) |
|--------------|----------------------------------|-----------|
| 1            | 4-hydroxy-6-nitrocoumarin        | 70        |
| 2            | R = 4-nitrobenzaldehyde          | 65        |
| 3            | R = 3-nitrobenzaldehyde          | 60        |
| 4            | R = 5-fluoro-2-hydroxybenzaldehyde | 78    |
| 5            | R = 2-hydroxy-5-methylbenzaldehyde     | 75    |
| 6            | R = 42-hydroxybenzaldehyde       | 71        |
| 7            | R = 4-methoxybenzaldehyde        | 67        |
| 8            | R = 2-hydroxybenzaldehyde        | 77        |
| 9            | R = 2-hydroxy-4-methoxybenzaldehyde | 79   |

The synthesized compounds (1-9) were characterized by using suitable analytical techniques. The λmax of compounds (1-9) was measured by making solutions of all compounds under study in dichloromethane. The absorbance measurements in the UV-Vis region were carried out using 10 mm quartz cuvettes. The λmax for compound (1) was 315 nm whereas for the compounds
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(2-9) it’s shifted considerable from 8 nm to 11 nm and blue shift was observed as compared to compound (1).

The FT-IR analysis of compound (1) showed two characteristic signals at 1340 cm\(^{-1}\) and 1524 cm\(^{-1}\) which confirm that the nitro group has been introduced in the 4-Hydroxycoumarin moiety. The FT-IR analysis of compound (1) gives a broad signal at 3500 cm\(^{-1}\) due to the stretching vibration of hydroxyl group present at C 4. The FT-IR analysis of compounds (2-9) shows the intense signal in the range of 1615 to 1650 cm\(^{-1}\) due to the stretching vibrations of aliphatic carbon-carbon double bonds which were formed by Knovenegal condensation. The disappearance of signal at 3500 cm\(^{-1}\) along with the appearance of signals either at 1682 cm\(^{-1}\) or 1683 cm\(^{-1}\) signifies that the hydroxyl group of compound (1) has effectively been converted to a carbonyl group.

The 1H NMR spectra of compounds (1-9) are in good agreement with the proposed structures. The 1H NMR spectrum of compound (1) gives singlet signal at \(\delta\) 5.82 ppm due to the presence of a proton at C 3; however, the disappearance of this signal confirms the Knovenegal condensation has taken place specifically at C 3. The proton at position 14 gives a singlet in the range of 8 ppm.

Antibacterial activity

In antibacterial assay, the ciprofloxacin was used as a positive control and it showed 38.5 \(\pm\) 0.74 mm and 33.5 \(\pm\) 0.77 mm zones of inhibition against \textit{S. aureus} and \textit{S. typhimurium}, respectively. The DMSO was used as negative control in all experiments. This negative control showed no zone of inhibition against both bacterial strains used in the study. The compounds (2), (3) and (8) were found highly active as compared to other synthesized compounds as they showed antibacterial activity with 26.5 \(\pm\) 0.84, 26.0 \(\pm\) 0.56 and 26.0 \(\pm\) 0.26 mm zones of inhibition against \textit{S. aureus}, respectively. On the other hand, the compound (5) and (9) showed moderate to good activity against \textit{S. typhimurium} as compared to other synthesized compounds with 19.5 \(\pm\) 0.59 and 19.5 \(\pm\) 0.32 mm zones of inhibition against \textit{S. typhimurium}, respectively. The antibacterial potential of all the synthesized compounds has been summarized in (Table II). Therefore, compounds (2), (3) and (8) can be considered as potent molecules for developing selective antibacterial drugs for \textit{S. aureus}.

TABLE II - Antibacterial bioassay screening of investigated compounds on two selected bacterial strains \textit{Staphylococcus aureus} and \textit{Salmonella typhimurium}

| Comp No. | \textit{Staphylococcus aureus} Zone of inhibition (mm) | \textit{Salmonella typhimurium} Zone of inhibition (mm) |
|----------|----------------------------------------------------|-----------------------------------------------------|
| 1        | 21.5 \(\pm\) 0.57                                  | 15.5 \(\pm\) 0.31                                    |
| 2        | 26.5 \(\pm\) 0.84                                  | 1.05 \(\pm\) 0.61                                   |
| 3        | 26.0 \(\pm\) 0.56                                  | 2.05 \(\pm\) 0.22                                   |
| 4        | 18.5 \(\pm\) 0.81                                  | 17.5 \(\pm\) 0.76                                   |
| 5        | 24.5 \(\pm\) 0.25                                  | 19.5 \(\pm\) 0.59                                   |
| 6        | 1.05 \(\pm\) 0.77                                  | 1.05 \(\pm\) 0.61                                   |
| 7        | 21.0 \(\pm\) 0.22                                  | 2.00 \(\pm\) 0.53                                   |
| 8        | 26.0 \(\pm\) 0.26                                  | 2.08 \(\pm\) 0.23                                   |
| 9        | 20.5 \(\pm\) 0.51                                  | 19.5 \(\pm\) 0.32                                   |
| DMSO     | 0.0 \(\pm\) 0.0                                   | 0.0 \(\pm\) 0.0                                     |
| CPX*     | 38.5 \(\pm\) 0.74                                  | 33.5 \(\pm\) 0.77                                   |

*CPX=Ciprofloxacin (Standard 8.3 µg of ciprofloxacin in per µL DMSO)

Zone of inhibitions are represented as mean \(\pm\) SEM.

Urease inhibition potential of synthesized compounds

The results of urease inhibition revealed that the compound (5) showed 38.6\% inhibition; whereas, compound (2) showed 0.6\% inhibition at 0.5 µM concentration. During urease inhibition studies thiourea was used as a standard and it showed 98.1\% inhibition at a concentration of 0.5 µM with IC\(_{50}\) value 21 \(\pm\) 0.11. However, the compounds (2-9) did not show significant % inhibition and, therefore, the IC\(_{50}\) values were not calculated. The anti-urease activity of all the compounds has been summarized in (Table III).

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synthesized compounds were evaluated for their antibacterial potential; the compounds (2), (3) and (8) showed favorable antibacterial activity, comparable to the standard drug (Ciprofloxacin), against *Staphylococcus aureus* whereas the compounds (5) and (9) were found active against *Salmonella typhimurium*. Moreover, all the synthesized compounds were also evaluated for their possible urease and carbonic anhydrase-II inhibition. However, none of the synthesized derivatives showed significant anti-urease activity. Furthermore, the compounds (2) and (6) showed carbonic anhydrase-II inhibition activity with IC$_{50}$ values of 263 ± 0.3 and 456 ± 0.1, respectively. Therefore, compound (2) is a good selective antibacterial agent as well as a good carbonic anhydrase-II inhibitor. Compound (3) and (8) are also good antibacterial agents and compound (6) is a considerably potent carbonic anhydrase-II inhibitor.

### TABLE III - Enzyme inhibition activity of 4-Hydroxycoumarin derivatives

| Compound No. | R$_1$ | R$_2$ | R$_3$ | R$_4$ | R$_5$ | Urease Inhibition % inhibition at 0.5 µM | IC$_{50}$ ± S.E.M | Carbonic anhydrase II Inhibition % inhibition at 0.5 µM | IC$_{50}$ ± S.E.M |
|--------------|-------|-------|-------|-------|-------|------------------------------------------|-------------------|-------------------------------------------------|-------------------|
| 1            | -     | -     | -     | -     | -     | 22.4                                     | -                 | -                                               | -                 |
| 2            | H     | H     | NO$_2$| H     | H     | 0.6                                       | 63                | 263 ± 0.3                                       | -                 |
| 3            | H     | NO$_2$| H     | H     | H     | 26.7                                      | -                 | -                                               | -                 |
| 4            | OH    | H     | H     | F     | H     | 17.2                                      | -                 | -                                               | -                 |
| 5            | OH    | H     | H     | CH$_3$| H     | 38.6                                      | -                 | -                                               | -                 |
| 6            | H     | H     | OH    | H     | H     | 27.9                                      | 54                | 456 ± 0.1                                       | -                 |
| 7            | H     | H     | OCH$_3$| H     | H     | 20.0                                      | -                 | -                                               | -                 |
| 8            | OH    | H     | H     | H     | H     | 16.7                                      | -                 | -                                               | -                 |
| 9            | OH    | H     | OCH$_3$| H     | H     | 16.1                                      | -                 | -                                               | -                 |
| Standard (thiourea for urease inhibition) | 98.1 | 21 ± 0.11 | | | | | | |
| Standard (acetazolamide for CA II inhibition) | 84 | 0.5 ± 0.1 | | | | | | |

### Potential carbonic anhydrase-II inhibition of synthesized compounds

The carbonic anhydrase-II inhibition assay was performed on all synthesized compounds. The compounds (2) and (6) showed 63% and 54% inhibition, respectively. The IC$_{50}$ values of these compounds were 263 µM and 456 µM, respectively. The standard taken for this study was acetazolamide, which showed 84% inhibition at a concentration of 0.5 µM with IC$_{50}$ value 0.5 ± 0.1. The carbonic anhydrase II activity are shown in (Table III). Therefore, the highest % inhibition was shown by compound (2) which can be considered as a potential drug candidate for inhibiting the activity of carbonic anhydrase-II.

### CONCLUSION

In this study, the successful synthesis and characterization of 4-hydroxy-6-nitrocoumarin was carried out along with its eight derivatives. All these synthesized compounds were evaluated for their antibacterial potential; the compounds (2), (3) and (8) showed favorable antibacterial activity, comparable to the standard drug (Ciprofloxacin), against *Staphylococcus aureus* whereas the compounds (5) and (9) were found active against *Salmonella typhimurium*. Moreover, all the synthesized compounds were also evaluated for their possible urease and carbonic anhydrase-II inhibition. However, none of the synthesized derivatives showed significant anti-urease activity. Furthermore, the compounds (2) and (6) showed carbonic anhydrase-II inhibition activity with IC$_{50}$ values of 263 ± 0.3 and 456 ± 0.1, respectively. Therefore, compound (2) is a good selective antibacterial agent as well as a good carbonic anhydrase-II inhibitor. Compound (3) and (8) are also good antibacterial agents and compound (6) is a considerably potent carbonic anhydrase-II inhibitor.
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