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Original Article

Biological evaluation and spectral characterization of 4-hydroxy coumarin analogues

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الملخص

هدف البحث: إن زيادة مقاومة المضادات الحيوية أصبحت في الآونة الأخيرة مشكلة صحية عالمية. صممت هذه الدراسة لتطوير جزيئات فعالة جديدة بأقل تكلفة وأدنى حد من التسمم. درس البحث طريقة تصنيع جديدة بخطوة واحدة لمادة ٣- اريليزو النشطة حيويا واستبدالها بمشتقات الكومارين ومن ثم تقييم عملها الحيوي.

طرق البحث: تم استخدام فئران ويستار من الجنسين أوزانها ١٨٠-٢٠٠ جم، ومن العمر المناسب لتقييم شق الجرح، واستئصال الجرح، والتسمم الحاد. كما تم التفسير الهيكلي للمركبات المصنعة بالفحص الكالوريمتري التفاضلي، والرنين المغناطيسي النووي، والتحليل فوق البنفسجي المرئي، وتحليل العناصر. وتم فحص المركبات المصنعة للتحقق في المختبر من نشاطها كمضادات للميكروبات، ومضادات للأكسدة والتئام الجروح.

النتائج: تم تصنيع سلسلة من المركبات من اقتران خمسة مركبات مختلفة من أملاح أريل ديازونيم بإذابتها في تركيز ١٠٪ من هيدروكسيد الصوديوم. وأظهرت غالبية المركبات خصائص ذات أهمية كمضادات للميكروبات، ومساعدة في التئام الجروح، وكمضادات للأكسدة.

الاستنتاجات: أثبتت الدراسة أن نظائر الأريل ومتغير الأريل للمركب ٤- هيدروكسيد الكومارين معا يمتلكان خصائص ذات أهمية كمضادات للميكروبات، ومساعد في التئام الجروح وكمضادات للأكسدة.

الكلمات المفتاحية: مضادات الميكروبات؛ مضادات الأكسدة؛ التئام الجروح؛ هيدروكسيد كومارين

Abstract

Objective: The development of antibiotic resistance has recently been recognized as a global health problem. This study was designed to develop new potent molecules that are economic and minimally toxic. The research examined a novel one-step synthetic procedure of bioactive 3-arylazo-substituted coumarin derivatives (**4i**–**4v**) and further evaluated their biological actions.

Methods: Male and female Wistar rats of appropriate age weighing 180–200 g were used to assess the wound incision, wound excision, acute toxicity and 1,1-diphenyl-2-picrylhydrazyl (DPPH) models. The synthesized compounds were structurally interpreted with Differential Screening Calorimetry (DSC), FT/IR, 1H Nuclear Magnetic Resonance (NMR), LC-MS, UV-Visible and elemental analysis. The synthesized compounds were screened to investigate their *in vitro* antimicrobial, antioxidant and wound healing activities.

Results: A series of 4-hydroxy-3-(arylazo) coumarin (**4i**–**4v**) analogues were synthesized by coupling five different aryl diazonium salts with 4-HC in the presence of a 10% NaOH solution. The majority of the compounds showed significant antimicrobial, wound healing and antioxidant properties. The most potent compounds identified in the analysis were **4i**v and **4e**.

Conclusion: This study justifies that both aryl and hetero arylazo analogues of 4-hydroxy coumarin possess significant antimicrobial, wound healing and antioxidant properties.

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Keywords: Antimicrobial; Antioxidant; Wound healing; 4-Hydroxy coumarin

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Introduction

C-3 aryl-substituted 4-HC is essential for broad biological actions, such as anti-viral,[1] anti-bacterial,[2] anticancer,[3] anticoagulant[1] and antioxidant[4] activities. Heteroarylazo-substituted compounds have been widely used due to their excellent thermal,[5] optical[6] and biological properties includes antibacterial,[7] antiviral[8] and antioxidant[9] activities. The insertion of an aryl/heteroarylazo moiety at the C-3 position of coumarin has been reported to elicit good antimicrobial activity.[10] The 4-hydroxy 3-heteroaryl coumarin moiety is found in many natural and synthetic products and also exerts significant biological actions.[11] In our earlier work, we have reported the synthesis and characterization of several novel bioactive hetero aryl-conjugated 4-HC analogues and investigated their antimicrobial activity against four different bacterial pathogens.[12] Subsequently, we evaluated the antimicrobial, wound healing and antioxidant activities of earlier reported molecules as well as those of recently developed new aryl-conjugated 4-HC analogues against a wide range of bacterial and fungal strains and confirmed their structural characterization.

Materials and Methods

The chemicals used in this study were of synthetic grade and were obtained from Merck specialties Ltd. (Mumbai, India). The synthesized products were structurally confirmed with FT/IR (JASCO FT/IR 4100 Spectrophotometer using KBr disc), ^{1}H NMR (Bruker H^{1}NMR 400 MHZ) using TMS as an internal standard, LC-MS (Shimadzu-Mass spectrometer). differential scanning calorimetry (METTLER TOLEDO STAR^e system at a heating rate of 10 °C min^{-1}, temperature range 30–350 °C using aluminium cans calibrated with indium) and UV spectrophotometry (JASCO V-630 Spectrophotometer). An elemental analysis was carried out with a Perkin Elmer-2400 CHNO/S analyser system. The melting points were determined with the open capillary method (Elico) and are uncorrected.

4-hydroxy-3-(aryl substituted -2-yldiazenyl)-2H-chromen-2-one (**4i–4v**) was synthesized as described previously[12] (Figure 1).

*4-((4-hydroxy-2-oxo-2H-chromen-3-yl) diazenyl) benzenesulfonic acid, (**4i**)*

IR (K Br) cm^{-1}: 3446 (O–H str), 2996 (Ar–H), 1695 (C=O str. lactone carbonyl), 1612 (C=C str. of coumarin), 1510 (–N=N-), 1300, 1134 (SO_2 str.), 1197 (C-Ostr.), 831 (1, 4 disubst. Ar); ^{1}H NMR (DMSO-d_6) δ: 7.81 (d, coumarin H-5,

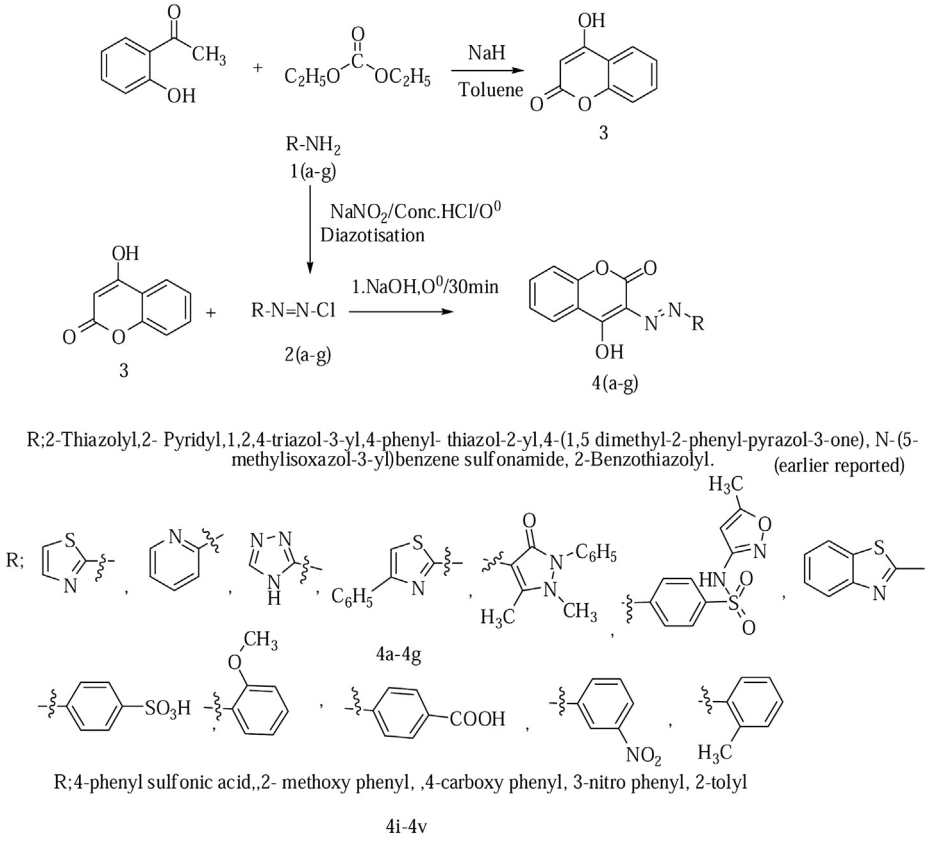

Figure 1: Synthesis of azo derived 4-Hydroxy Coumarin analogues.

Table 1: Physical characteristic data of newly synthesized 4-HC aryl analogues (4i–4v).

Comps.	Ar	M. formula	m/z	Rf	m. p.(°C)	Colour	Yield (%)
4i	phenyl 4-sulfonic acid	$C_{15}H_{10}N_2O_6S$	346.03	0.8	200–210	Bright Yellow	89
4ii	2-methoxyphenyl	$C_{16}H_{12}N_2O_4$	296.08	0.8	190–200	Coffee red	95
4iii	4- carboxy phenyl	$C_{16}H_{10}N_2O_5$	310.06	0.8	280–290	Bright Yellow	92
4iv	3-nitrophenyl	$C_{15}H_9N_3O_5$	311.05	0.9	200–210	Buff Yellow	95
4v	2-methyl phenyl	$C_{16}H_{12}N_2O_3$	280.08	0.8	160–170	Dark Brown	87

$J = 8.1$ Hz), 7.75–7.85 (m, 4H, Ar–H), 7.42 (d, coumarin H-8, $J = 8.1$ Hz), 7.37–7.65 (m, coumarin H-6 & 7); LC-MS (% area); 92.21; m/z; 344.96 (M–2); Analysis calcd % for $C_{15}H_{10}N_2O_6S$: C, 52.02; H, 2.91; N, 8.09; S, 9.26 Found %: C, 52.03; H, 2.89; N, 8.12; S,9.28.

*4-hydroxy-3-(2-methoxyphenyl) diazenyl)-2H-chromen-2-one, (**4ii**)*

IR (K Br) cm^{-1}: 3477 (O–H str), 3021 (Ar–H), 2925 (CH_2str. of OCH_3), 1745 (C=O str. lactone carbonyl), 1608 (C=C str. of coumarin), 1505 (–N=N-), 1483, 1110 (=C–O–CH_3 str.), 1264 (C-Ostr.), 750 (1, 2 disubst. Ar); ^{1}H NMR (DMSO-d_6) δ: 16.81 (s,1H,4-enolic OH), 8.09 (d, coumarin H-5, $J = 8.1$ Hz), 7.57 (d, coumarin H-8, $J = 8.1$ Hz), 7.46–7.65 (m, coumarin H-6 & 7), 6.94–7.34 (m, 4H, Ar–H), 3.83 (s, 3H, Ar-OCH_3); LC-MS (% area); 60.04; m/z; 296.99 (M + 1); Analysis calcd % for $C_{16}H_{12}N_2O_4$: C, 64.86; H, 4.08; N, 9.46 Found %: C, 64.87; H, 4.11; N, 9.45.

*4-((4-hydroxy-2-oxo-2H-chromen-3-yl) diazenyl) benzoic acid, (**4iii**)*

IR (K Br) cm^{-1}: 3446 (O–H str), 2987 (Ar–H), 1742 (C=O str. of carboxylic acid), 1697 (C=O str. lactone carbonyl), 1609 (C=C str. Of coumarin), 1509 (–N=N–), 1280 (OH bend. / C-Ostr.), 833 (1, 4 disubst. Ar); ^{1}H NMR (DMSO-d_6) δ: 16.85 (s, 1H, 4-enolic OH), 7.84(d, coumarinH-5, $J = 8.1$ Hz), 7.54–8.21 (m, 4H, Ar–H), 7.47 (d, coumarin H-8, $J = 8.1$ Hz), 7.42–7.67 (m, coumarinH-6 & 7); LC-MS (% area); 58.74; m/z; 310.95 (M^+) Analysis calcd% for $C_{16}H_{10}N_2O_5$: C, 61.94; H, 3.25; N, 9.03. Found%: C, 61.93; H, 3.23; N, 9.05.

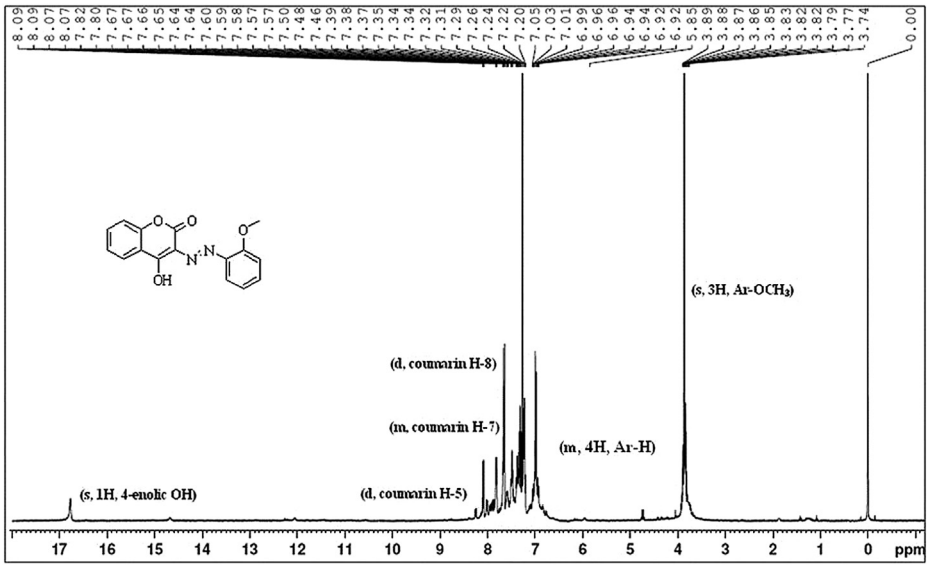

Figure 2: ^{1}H NMR of compound **4ii**.

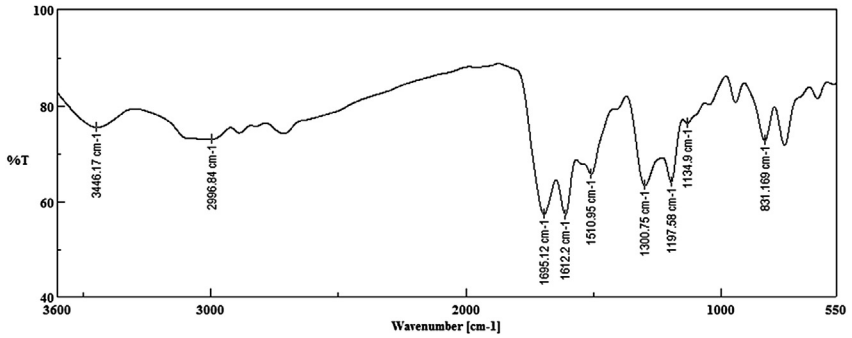

Figure 3: FT/IR of compound **4i**.

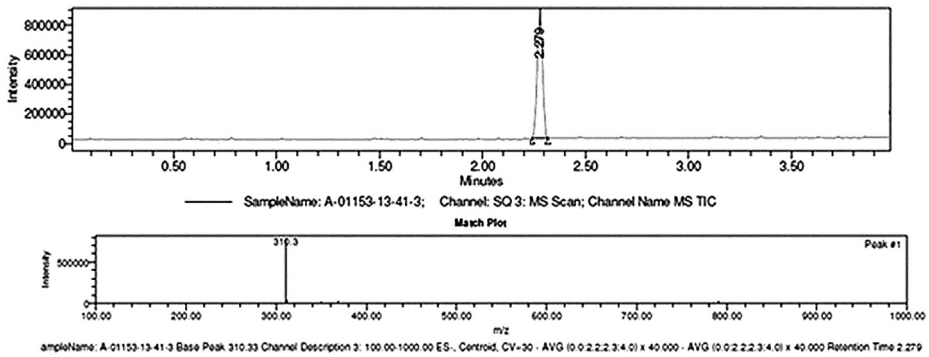

Figure 4: LC-MS of compound **4iv**.

Table 2: Electronic absorption spectral data (λ_{max}) nm of newly synthesized 4-HC aryl analogues (4i–4v).

Comps.	λ_{max} (Acetonitrile)	λ_{max} (Methanol)	λ_{max} (DMF)	λ_{max} (Dioxane)	λ_{max} (DMSO)	λ_{max} (THF)
4i	426	420	306, 427	390, 429	430	–
4ii	304, 445	450	435	305, 447	438	303, 448
4iii	302, 417	420	273, 405	257, 420	304, 421	285, 294, 421
4iv	254, 393	417	283, 387	255, 404	393	397
4v	429	430	284, 431	270, 303, 430	304, 432	303, 430

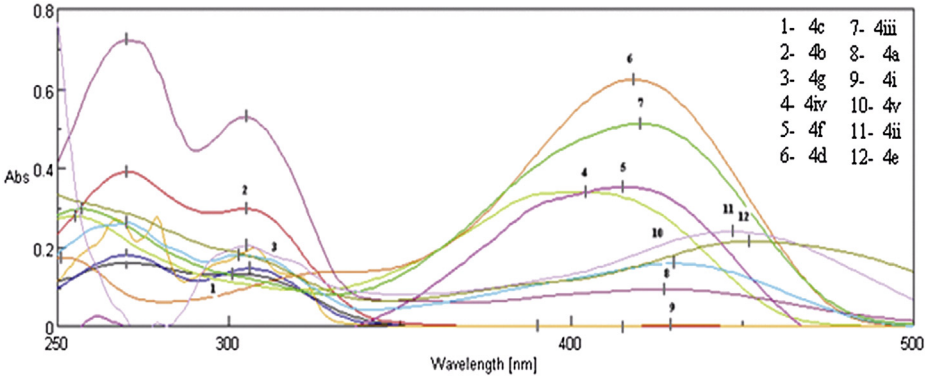

Figure 5: Solvatochromism of compounds (**4i–4v**, **4a–4g**) using 1, 4- dioxane.

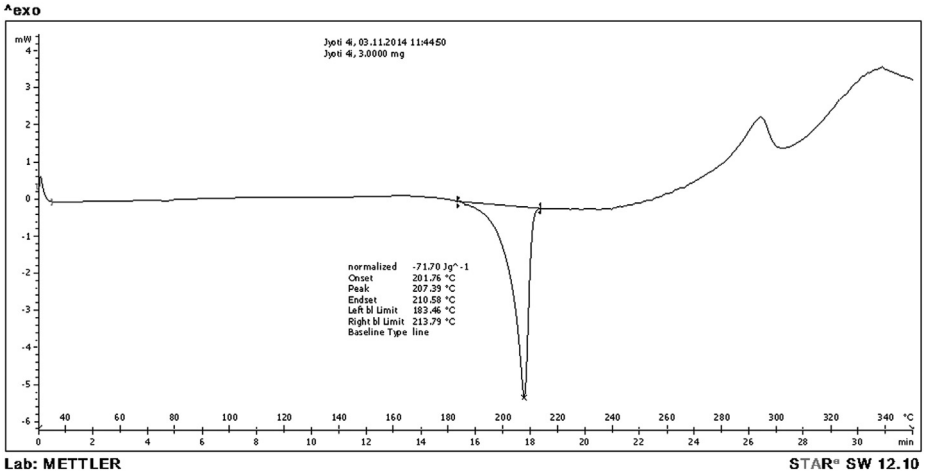

Figure 6: Thermogram of 4i by DSC.

*4-hydroxy-3-((3-nitrophenyl) diazenyl)-2H-chromen-2-one, (**4iv**)*

IR (K Br) cm^{-1}: 3555 (O−H str), 3088 (Ar−H), 1687 (C=O str. lactone carbonyl), 1618 (C=C str. of coumarin), 1508 (−N=N−/NO_2 str.), 1271 C-Ostr.), 743 (1, 3 disubst. Ar); ^{1}H NMR (DMSO-d_6) δ: 7.83 (d, coumarin H-5, J = 8.1 Hz), 7.71−8.37 (m, 4H, Ar−H), 7.38−7.71 (m, coumarin H-6 & 7), 7.34 (d, coumarin H-8, J = 8.1 Hz); LC-MS (% area); 100.00; m/z; 310.33 (M−1); Analysis calcd % for $C_{15}H_9N_3O_5$: C, 57.88; H, 2.91; N, 13.50. Found %: C, 57.85; H, 2.92; N, 13.48.

*4-hydroxy-3-(o-tolyldiazenyl)-2H-chromen-2-one, (**4v**)*

IR (K Br) cm^{-1}: 3023 (Ar−H), 2925 (CH_2str. of CH_3), 1709 (C=O str. lactone carbonyl), 1607 (C=C str. of coumarin), 1511 (−N=N−), 1286 (C-Ostr.), 770 (1, 2 disubst. Ar); ^{1}H NMR (DMSO-d_6) δ: 16.73 (s, 1H, 4-enolic

Table 3.1: Zone of inhibition (mm) of newly synthesized 4-HC aryl and heteroarylazo analogues (4i−4v, 4a−4g) against bacterial strains (Mean ± S.D.).

Compd	*E. coli* [a]	*S. ser. typhi* [b]	*S. typhimurium* [c]	*S. paratyphi* [d]	*S. flexneri* [e]	*P. aeruginosa* [f]	*V. cholera* [g]
	Mean ± S.D.	Mean ± S.D	Mean ± S.D	Mean ± S.D	Mean ± S.D.	Mean ± S.D.	Mean ± S.D
4i	11.33 ± 0.82	8.00 ± 0.63	11.33 ± 1.75	8.67 ± 0.52	11.33 ± 1.51	18.17 ± 0.98	16.33 ± 1.86
4ii	13.33 ± 0.52	15.00 ± 1.67	10.33 ± 0.52	18.33 ± 1.03	–	11.33 ± 0.82	11.67 ± 0.82
4iii	15.33 ± 1.37	9.17 ± 0.75	10.50 ± 2.07	10.33 ± 2.58	13.33 ± 1.63	8.33 ± 0.52	9.33 ± 1.75
4iv	17.50 ± 1.23	14.33 ± 1.37	10.50 ± 2.51	13.33 ± 3.93	19.33 ± 0.52	19.50 ± 1.23	16.50 ± 2.07
4v	15.50 ± 0.55	12.00 ± 0.00	15.33 ± 2.16	10.00 ± 1.27	12.50 ± 0.55	17.33 ± 0.52	11.33 ± 1.03
4a	16.00 ± 0.89	11.50 ± 2.07	18.17 ± 0.41	11.50 ± 0.55	11.50 ± 2.81	15.00 ± 1.41	13.00 ± 0.63
4b	18.00 ± 2.10	10.50 ± 2.26	10.33 ± 2.25	10.83 ± 1.47	14.50 ± 0.55	24.00 ± 0.63	8.83 ± 0.41
4c	13.00 ± 1.67	8.50 ± 0.84	12.17 ± 0.98	9.50 ± 1.23	13.33 ± 0.52	18.00 ± 0.89	–
4d	19.00 ± 1.27	11.50 ± 0.84	14.17 ± 0.75	13.33 ± 0.52	12.50 ± 2.81	17.83 ± 1.84	12.33 ± 1.03
4e	18.00 ± 2.10	15.50 ± 0.55	13.50 ± 0.55	16.50 ± 1.38	24.50 ± 0.55	18.00 ± 1.27	19.83 ± 0.75
4f	–	–	11.17 ± 0.98	–	11.50 ± 2.07	17.33 ± 0.52	8.83 ± 0.41
4g	–	10.83 ± 1.33	12.50 ± 1.64	11.50 ± 0.55	12.50 ± 0.55	8.00 ± 0.89	12.50 ± 3.39
RA	22.50 ± 1.98	15.33 ± 1.86	12.83 ± 0.98	17.83 ± 0.75	19.50 ± 2.07	20.67 ± 1.51	19.83 ± 1.33
Total	13.81 ± 6.66	10.94 ± 4.19	12.53 ± 2.65	11.67 ± 4.78	13.56 ± 5.69	16.42 ± 4.62	12.33 ± 5.27
ANOVA 'p' value	0	0	0	0	0	0	0

Compounds	*K. pneumoniae* [h]	*M. luteus* [i]	*B. circulans* [j]	*S. mitis* [k]	*P. carotovarum* [l]	*B. subtilis* [m]	*S. aureus* [n]
	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D.	Mean ± S.D	Mean ± S.D	Mean ± S.D
4i	–	–	11.00 ± 1.27	12.17 ± 1.33	8.33 ± 0.52	12.33 ± 0.52	–
4ii	14.33 ± 0.82	9.33 ± 1.37	15.33 ± 1.86	14.83 ± 1.17	14.33 ± 1.03	13.00 ± 0.89	10.67 ± 0.82
4iii	–	11.33 ± 1.37	14.33 ± 1.37	17.33 ± 1.37	12.33 ± 1.37	10.33 ± 1.63	10.17 ± 2.04
4iv	18.50 ± 0.55	18.33 ± 1.37	35.50 ± 1.23	25.17 ± 0.75	17.50 ± 0.55	15.33 ± 1.37	–
4v	–	10.17 ± 0.41	17.50 ± 2.51	–	12.17 ± 1.33	11.33 ± 0.82	10.67 ± 1.75
4a	11.83 ± 2.23	11.33 ± 1.86	12.50 ± 1.23	11.50 ± 1.64	11.17 ± 2.48	20.00 ± 0.89	22.00 ± 0.63
4b	13.50 ± 1.23	–	11.50 ± 1.23	13.50 ± 0.55	13.17 ± 0.98	15.00 ± 0.63	21.83 ± 2.14
4c	–	–	–	–	11.50 ± 1.98	10.00 ± 1.27	–
4d	12.50 ± 0.55	11.50 ± 1.23	–	11.50 ± 1.98	13.50 ± 2.95	20.00 ± 0.89	23.00 ± 1.67
4e	26.50 ± 0.55	18.50 ± 0.55	20.17 ± 0.98	25.17 ± 1.60	14.50 ± 1.23	23.00 ± 0.89	25.00 ± 0.89
4f	–	11.50 ± 0.55	–	11.50 ± 0.55	14.50 ± 1.64	9.67 ± 1.03	24.00 ± 0.63
4g	12.50 ± 1.38	11.50 ± 0.55	12.50 ± 0.55	10.50 ± 0.55	12.50 ± 0.55	11.00 ± 0.89	10.00 ± 1.41
RA	21.33 ± 1.51	15.67 ± 1.51	23.00 ± 4.00	17.67 ± 1.75	20.33 ± 2.50	20.33 ± 2.07	22.00 ± 1.67
Total	10.08 ± 8.96	9.94 ± 6.22	13.33 ± 9.78	13.14 ± 7.42	13.53 ± 3.25	14.72 ± 4.60	13.79 ± 9.47
ANOVA 'p' value	0	0	0	0	0	0	0

– No zone of inhibition.
[a] *Escherichia coli.*
[b] *Salmonella enterica ser. typhi.*
[c] *Salmonella enterica typhimurium.*
[d] *Salmonella enterica paratyphi.*
[e] *Shigella flexneri.*
[f] *Pseudomonas aeruginosa.*
[g] *Vibrio cholera.*
[h] *Klebsiella pneumonia.*
[i] *Micrococcus luteus.*
[j] *Bacillus circulans.*
[k] *Streptococcus mitis.*
[l] *Pectobacterium carotovorum.*
[m] *Bacillus subtilis.*
[n] *Staphylococcus aureus.*

Table 3.2: Zone of inhibition (mm) of newly synthesized 4-HC aryl and heteroarylazo analogues (4i−4v, 4a−4g) against bacterial strains (Mean difference ± S.E.M).

Compound		*E. coli* [a]		*S. ser. typhi* [b]		*S. typhimerium* [c]		*S. paratyphi* [d]		*S. flexneri* [e]		*P. aeruginosa* [f]		*V. cholera* [g]	
(I)	(J)	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.M	p' value
4i	RA	−11.17 ± 0.76	1.000	−7.33 ± 0.75	1.000	−1.50 ± 0.88	1.000	−9.17 ± 0.91	1.000	−8.17 ± 0.89	1.000	−2.50 ± 0.62	1.000	−3.50 ± 0.85	1.000
4ii	RA	−9.17 ± 0.76	1.000	−0.33 ± 0.75	0.976	−2.50 ± 0.88	1.000	0.50 ± 0.91	0.769	–	1	−9.33 ± 0.62	1.000	−8.17 ± 0.85	1.000
4iii	RA	−7.17 ± 0.76	1.000	−6.17 ± 0.75	1.000	−2.33 ± 0.88	1.000	−7.50 ± 0.91	1.000	−6.17 ± 0.89	1.000	−12.33 ± 0.62	1.000	−10.50 ± 0.85	1.000
4iv	RA	−5.00 ± 0.76	1.000	−1.00 ± 0.75	0.999	−2.33 ± 0.88	1.000	−4.50 ± 0.91	1.000	−0.17 ± 0.89	0.952	−1.17 ± 0.62	1.000	−3.33 ± 0.85	1.000
4v	RA	−7.00 ± 0.76	1.000	−3.33 ± 0.75	1.000	2.50 ± 0.88*	0.026	−7.83 ± 0.91	1.000	−7.00 ± 0.89	1.000	−3.33 ± 0.62	1.000	−8.50 ± 0.85	1.000
4a	RA	−6.50 ± 0.76	1.000	−3.83 ± 0.75	1.000	5.33 ± 0.88*	0	−6.33 ± 0.91	1.000	−8.00 ± 0.89	1.000	−5.67 ± 0.62	1.000	−6.83 ± 0.85	1.000
4b	RA	−4.50 ± 0.76	1.000	−4.83 ± 0.75	1.000	−2.50 ± 0.88	1.000	−7.00 ± 0.91	1.000	−5.00 ± 0.89	1.000	3.33 ± 0.62*	0	−11.00 ± 0.85	1.000
4c	RA	−9.50 ± 0.76	1.000	−6.83 ± 0.75	1.000	−0.67 ± 0.88	0.991	−8.33 ± 0.91	1.000	−6.17 ± 0.89	1.000	−2.67 ± 0.62	1.000	–	1
4d	RA	−3.50 ± 0.76	1.000	−3.83 ± 0.75	1.000	1.33 ± 0.88	0.331	−4.50 ± 0.91	1.000	−7.00 ± 0.89	1.000	−2.83 ± 0.62	1.000	−7.50 ± 0.85	1.000
4e	RA	−4.50 ± 0.76	1.000	0.17 ± 0.75	0.875	0.67 ± 0.88	0.685	−1.33 ± 0.91	0.999	5.00 ± 0.89*	0	−2.67 ± 0.62	1.000	0.00 ± 0.85	0.923
4f	RA	–	1	–	1	−1.67 ± 0.88	1.000	–	1	−8.00 ± 0.89	1.000	−3.33 ± 0.62	1.000	−11.00 ± 0.85	1.000
4g	RA	–	1	−4.50 ± 0.75	1.000	−0.33 ± 0.88	0.971	−6.33 ± 0.91	1.000	−7.00 ± 0.89	1.000	−12.67 ± 0.62	1.000	−7.33 ± 0.85	1.000

Compound		*K. pneumoniae* [h]		*M. luteus* [i]		*B. circulans* [j]		*S. mitis* [k]		*P. carotovarum* [l]		*B. subtilis* [m]		*S. aureus* [n]	
(I)	(J)	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.M	p' value	Mean (I−J) ± S.E.	P' value	Mean (I−J) ± S.E.M	P' value
4i	RA	–	1	–	1	−12.00 ± 0.95	1.000	−5.50 ± 0.69	1.000	−12.00 ± 0.96	1.000	−8.00 ± 0.66	1.000	–	1
4ii	RA	−7.00 ± 0.56	1.000	−6.33 ± 0.60	1.000	−7.67 ± 0.95	1.000	−2.83 ± 0.69	1.000	−6.00 ± 0.96	1.000	−7.33 ± 0.66	1.000	−11.33 ± 0.75	1.000
4iii	RA	–	1	−4.33 ± 0.60	1.000	−8.67 ± 0.95	1.000	−0.33 ± 0.69	0.979	−8.00 ± 0.96	1.000	−10.00 ± 0.66	1.000	−11.83 ± 0.75	1.000
4iv	RA	−2.83 ± 0.56	1.000	2.67 ± 0.60*	0	12.50 ± 0.95*	0	7.50 ± 0.69*	0	−2.83 ± 0.96	1.000	−5.00 ± 0.66	1.000	–	1
4v	RA	–	1	−5.50 ± 0.60	1.000	−5.50 ± 0.95	1.000	–	1	−8.17 ± 0.96	1.000	−9.00 ± 0.66	1.000	−11.33 ± 0.75	1.000
4a	RA	−9.50 ± 0.56	1.000	−4.33 ± 0.60	1.000	−10.50 ± 0.95	1.000	−6.17 ± 0.69	1.000	−9.17 ± 0.96	1.000	−0.33 ± 0.66	0.980	0.00 ± 0.75	0.923
4b	RA	−7.83 ± 0.56	1.000	–	1	−11.50 ± 0.95	1.000	−4.17 ± 0.69	1.000	−7.17 ± 0.96	1.000	−5.33 ± 0.66	1.000	−0.17 ± 0.75	0.956
4c	RA	–	1	–	1	–	1	–	1	−8.83 ± 0.96	1.000	−10.33 ± 0.66	1.000	–	1
4d	RA	−8.83 ± 0.56	1.000	−4.17 ± 0.60	1.000	–	1	−6.17 ± 0.69	1.000	−6.83 ± 0.96	1.000	−0.33 ± 0.66	0.980	1.00 ± 0.75	0.408
4e	RA	5.17 ± 0.56*	0	2.83 ± 0.60*	0	−2.83 ± 0.95	1.000	7.50 ± 0.69*	0	−5.83 ± 0.96	1.000	2.67 ± 0.66*	0.001	3.00 ± 0.75*	0.001
4f	RA	–	1	−4.17 ± 0.60	1.000	–	1	−6.17 ± 0.69	1.000	−5.83 ± 0.96	1.000	−10.67 ± 0.66	1.000	2.00 ± 0.75*	0.038
4g	RA	−8.83 ± 0.56	1.000	−4.17 ± 0.60	1.000	−10.50 ± 0.95	1.000	−7.17 ± 0.69	1.000	−7.83 ± 0.96	1.000	−9.33 ± 0.66	1.000	−12.00 ± 0.75	1

I= Test compound, J = RA (Reference antibiotic), S.E.M. = standard Error Mean (n = 6), *p value<0.05, − No zone of inhibition.

[a] *Escherichia coli.*
[b] *Salmonella enterica ser. typhi.*
[c] *Salmonella enterica typhimurium.*
[d] *Salmonella enterica paratyphi.*
[e] *Shigella flexneri.*
[f] *Pseudomonas aeruginosa.*
[g] *Vibrio cholera.*
[h] *Klebsiella pneumonia.*
[i] *Micrococcus luteus.*
[j] *Bacillus circulans.*
[k] *Streptococcus mitis.*
[l] *Pectobacterium carotovorum.*
[m] *Bacillus subtilis.*
[n] *Staphylococcus aureus.*

OH), 7.81(d, coumarin H-5, $J = 8.1$ Hz), 7.47 (d, coumarin H-8, $J = 8.1$ Hz), 7.37–7.73 (m, coumarin H-6 &7), 6.89–7.26 (m, 4H, Ar–H), 2.43 (s, 3H, Ar-CH_3); LC-MS (% area); 87.63; m/z; 281.13 (M + 1); Analysis calcd % for $C_{16}H_{12}N_2O_3$: C, 68.56; H, 4.32; N, 9.99 Found %: C, 68.57; H, 4.31; N, 9.97.

Antimicrobial activity

The antimicrobial activities of the newly synthesized 4-HC analogues were investigated using different microbial strains, *i.e., Escherichia coli* (MTCC 614), *Salmonella enterica ser. typhi* (MTCC 773), *S. enterica typhimurium* (MTCC 98), *S. enterica paratyphi* (MTCC 3220), *Shigella flexneri* (MTCC 1457), *Pseudomonas aeruginosa* (MTCC 1035), *Vibrio cholera* (MTCC 3906), *Klebsiella pneumonia* (MTCC 109), *Micrococcus luteus* (MTCC 1809), *Bacillus circulans* (MTCC 490), *Streptococcus mitis* (MTCC 2695), *Pectobacterium carotovorum* (MTCC 1428), *Aspergillus niger* (MTCC 9933), *Candida albicans* (MTCC 3017), *Candida glabrata, Cryptococcus neoformans* and *Trichophyton rubrum*, which were procured from the Institute of Microbial Technology and Gene bank, Chandigarh, India. *Staphylococcus aureus* and *Bacillus subtilis* strain hswx88[13] were isolated at the Department of Pharmaceutical Biotechnology, Utkal University. Freshly sub-cultured microorganisms were used. Ampicillin and Fluconazole were used as reference antibiotics.

The antimicrobial activity of the novel 4-HC analogues (**4i–4v** and earlier reported **4a–4g**) was assessed with the well and plate method using molten nutrient agar (antibacterial) and Sabouraud dextrose agar (antifungal).[14] After solidification, the media were inoculated, and 6-mm diameter wells were punched into the media. The wells were filled with a stock solution of test and reference compounds (1 μgmL^{-1}) and incubated for 24 h and 72 h for the bacterial and fungal strains, respectively, at 37 °C. The diameter of the zone of inhibition was measured using the Hi-Antibiotic Zone Scale.

Determination of minimum inhibitory concentration (MIC)[15]

A 1 μgmL^{-1} stock solution of synthesized compounds and reference antibiotic was prepared in 10% DMF. Furthermore, five different concentrations of (500–31.25 μgml^{-1}) were prepared by serial dilution. The different concentrations of respective compounds were loaded into the wells and incubated at 37 °C for 18–24 h. The MIC was determined after incubation.

Pharmacological activity

Animals

Male and female Wistar rats of appropriate age weighing 180–200 g were used in this study. The experiments were carried under the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals and approved by the Institutional Animal Ethical Committee with registration number 1171/C/08/CPCSEA and Ref. No. 60/SPS/IAEC/SOAU.

Acute toxicity study

An acute oral toxicity study was performed on female Wistar rats to establish the safe dose of the synthesized compounds. OECD guideline No. 420 (2000) for the Acute Oral Toxicity-Fixed Dose Procedure was followed (sighting study and main study) for a period of 14 days to study the acute toxic symptoms and the behavioural changes of the animals.

Excision wound model

The test compound showing significant antimicrobial activity against a maximum number of bacterial strains was selected for evaluation of wound healing activity. A wound

Table 4.1: Zone of inhibition (mm) of newly synthesized 4-HC aryl and heteroarylazo analogues (4i–4v, 4a–4g) against fungal strains (Mean ± S.D.).

Compounds	*A. niger*[a]	*T. rubrum*[b]	*C. albicans*[c]	*C. glabrata*[d]	*C. neoformans*[e]
4i	25.50 ± 0.55	12.17 ± 0.75	20.50 ± 1.38	15.67 ± 1.03	27.00 ± 0.89
4ii	25.00 ± 0.63	15.17 ± 0.75	17.33 ± 0.52	13.50 ± 0.55	30.17 ± 0.75
4iii	22.00 ± 1.27	18.17 ± 0.41	16.17 ± 0.98	13.17 ± 0.75	30.17 ± 1.84
4iv	30.00 ± 1.79	15.00 ± 0.63	22.00 ± 0.89	13.00 ± 1.79	32.00 ± 0.89
4v	25.17 ± 0.75	12.17 ± 1.17	18.00 ± 0.89	13.17 ± 0.98	30.17 ± 0.75
4a	27.00 ± 0.89	10.00 ± 1.27	23.00 ± 0.89	18.00 ± 1.27	30.00 ± 1.41
4b	22.17 ± 1.17	10.83 ± 0.98	24.00 ± 1.79	13.00 ± 2.10	15.33 ± 0.52
4c	16.17 ± 0.41	16.17 ± 0.75	11.17 ± 0.75	13.33 ± 1.03	26.50 ± 1.23
4d	21.17 ± 1.17	10.83 ± 0.75	17.83 ± 0.75	13.17 ± 0.75	23.83 ± 0.41
4e	25.00 ± 1.41	13.17 ± 0.98	21.00 ± 1.27	12.00 ± 0.89	35.00 ± 1.27
4f	29.33 ± 1.63	14.50 ± 2.51	24.17 ± 0.75	12.83 ± 2.32	31.50 ± 0.84
4g	27.33 ± 0.82	12.00 ± 0.63	16.00 ± 1.79	12.00 ± 0.89	27.33 ± 1.03
RA	19.67 ± 1.51	17.00 ± 0.63	25.83 ± 1.17	19.00 ± 1.41	29.50 ± 1.05
Total	24.27 ± 3.94	13.63 ± 2.67	19.77 ± 4.13	13.99 ± 2.45	28.35 ± 4.75

RA (Reference Antibiotic).
[a] *Aspergillus niger*.
[b] *Trichophyton rubrum*.
[c] *Candida albicans*.
[d] *Candida glabrata*.
[e] *Cryptococcus neoformans*.

excision model was employed using male Wistar rats to assess the wound healing activity.[16] Group-1 served as the control, group-2 was treated with 2% w/w nitrofurazone cream (standard) and remaining groups (group 3–10) were topically treated with 5% and 10% w/w ointments containing test compounds once per day for 16 days. The area of the wounds was recorded on the 4th, 8th, 12th and 16th day, and the closing percentage and % inhibition were calculated.

Incision wound model

Light incisions were made by cutting the skin of the Wistar rats under mild anaesthesia.[17] The parted skin was stitched together with black silk at both ends of the incisions. The test samples were applied as above. The tensile strength was measured with a tensiometer, 16 days after wounding.

Antioxidant activity assay by DPPH model

The free radical scavenging activity of the novel 4-HC analogues (**4i–4v** and **4a–4g**) was measured using the DPPH method with some modification.[14] The antioxidant activity of synthesized compounds was compared with that of standard butylated hydroxytoluene. The optical density was measured at 517 nm, and the inhibition concentration was calculated. Solution of DPPH in methanol were used as a control.

$$\% \text{ of inhibition} = [(A_{cont} - A_{test})/A_{test}] \times 100$$

A_{cont} = absorbance of control
A_{test} = Absorbance of the test compound.

Statistical analysis

The observed zone of inhibition data were subjected to a one way analysis of variance. The mean zone of inhibition for each compound and each strain was compared with the reference antibiotic with a Dunnett post hoc test. Significance was tested at the 5% level of a type one error. The null hypothesis was a zone of inhibition for the test compound that was larger than that of the reference antibiotic.

Sample size determination

A minimum sample size of five was calculated based on a probability of type 1 error (d) = 0.05, Power $(1 - \beta) = 0.8$, Number of groups 13 within group SD = 2. However, a sample size of six was used in the study for each compound and each strain.

Results

Chemistry

Spectral analysis

A series of 4-hydroxy-3-(arylazo) coumarin analogues (**4i–4v**) were synthesized by coupling five different aryl diazonium salts to 4-HC in the presence of a 10% NaOH solution (Figure 1). Diazotization was carried out in presences of nitrosyl chloride, and any excess nitrous acid was removed via the addition of urea. The crude products were recrystallized from ethanol. The infrared spectra of the prepared starting material (3) showed a strong absorption band at 3417 cm^{-1} corresponding to –OH group and band at 1698 cm^{-1} corresponding to the lactone carbonyl moiety of 4-HC. The involved coupling reactions that initially generated strong aryl diazonium electrophiles from different aryl amines then ultimately coupled at the C-3 position of 4-hydroxy coumarin to produce the desired 3-aryl azohydroxy coumarin. The physical data of the prepared compounds are reported in Table 1.

The infrared spectra of the synthesized compounds (**4i–4v**, **4a–4g**) showed strong absorption bands at 1745–1687 cm^{-1},

Table 4.2: Zone of inhibition (mm) of newly synthesized 4-HC aryl and heteroarylazo analogues (4i–4v, 4a–4g) against various fungal strains (Mean difference ± S.E.M.).

Compd.	*A. niger*[a]		*T. rubrum*[b]		*C. albicans*[c]		*C. glabrata*[d]		*C. neoformans*[e]	
(I)	Mean (I–J) ± S.E.	p' value	Mean (I–J) ± S.E.M	p' value	Mean (I–J) ± S.E.M	p' value	Mean (I–J) ± S.E.M	p' value	Mean (I–J) ± S.E.M	p' value
4i	5.83 ± 0.67*	0.000	−4.83 ± 0.62	1.000	−5.33 ± 0.65	1.000	−3.33 ± 0.76	1.000	−2.50 ± 0.61	1.000
4ii	5.33 ± 0.67*	0.000	−1.83 ± 0.62	1.000	−8.50 ± 0.65	1.000	−5.50 ± 0.76	1.000	0.67 ± 0.61	0.527
4iii	2.33 ± 0.67*	0.004	1.17 ± 0.62	0.188	−9.67 ± 0.65	1.000	−5.83 ± 0.76	1.000	0.67 ± 0.61	0.527
4iv	10.33 ± 0.67*	0.000	−2.00 ± 0.62	1.000	−3.83 ± 0.65	1.000	−6.00 ± 0.76	1.000	2.50 ± 0.61*	0.001
4v	5.50 ± 0.67*	0.000	−4.83 ± 0.62	1.000	−7.83 ± 0.65	1.000	−5.83 ± 0.76	1.000	0.67 ± 0.61	0.527
4a	7.33 ± 0.67*	0.000	−7.00 ± 0.62	1.000	−2.83 ± 0.65	1.000	−1.00 ± 0.76	0.999	0.50 ± 0.61	0.657
4b	2.50 ± 0.67*	0.002	−6.17 ± 0.62	1.000	−1.83 ± 0.65	1.000	−6.00 ± 0.76	1.000	−14.17 ± 0.61	1.000
4c	−3.50 ± 0.67	1.000	−0.83 ± 0.62	0.999	−14.67 ± 0.65	1.000	−5.67 ± 0.76	1.000	−3.00 ± 0.61	1.000
4d	1.50 ± 0.67	0.098	−6.17 ± 0.62	1.000	−8.00 ± 0.65	1.000	−5.83 ± 0.76	1.000	−5.67 ± 0.61	1.000
4e	5.33 ± 0.67*	0.000	−3.83 ± 0.62	1.000	−4.83 ± 0.65	1.000	−7.00 ± 0.76	1.000	5.50 ± 0.61*	0.000
4f	9.67 ± 0.67*	0.000	−2.50 ± 0.62	1.000	−1.67 ± 0.65	1.000	−6.17 ± 0.76	1.000	2.00 ± 0.61*	0.008
4g	7.67 ± 0.67*	0.000	−5.00 ± 0.62	1.000	−9.83 ± 0.65	1.000	−7.00 ± 0.76	1.000	−2.17 ± 0.61	1.000

I= Test compound, J = RA (RA) Reference Antibiotic, S.E.M. = Standard Error Mean (n = 6), *p value<0.05.
RA (Reference Antibiotic).
[a] *Aspergillus niger*.
[b] *Trichophyton rubrum*.
[c] *Candida albicans*.
[d] *Candida glabrata*.
[e] *Cryptococcus neoformans*.

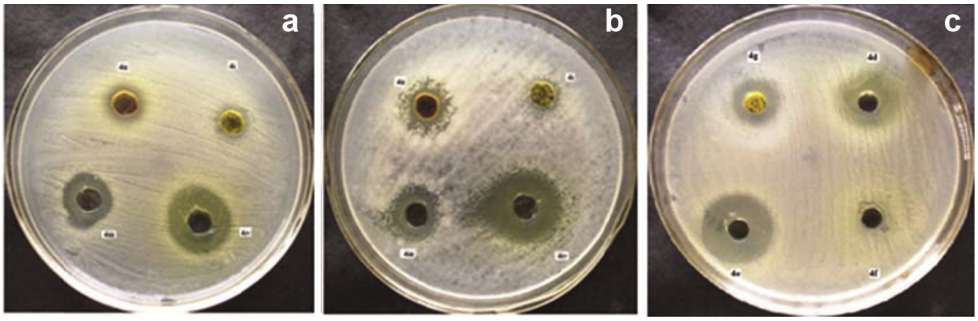

Figure 7: Bacteria zone of inhibition showed by 4-HC arylazo analogues: plate-a (**4i**. **4ii**, **4iii**, **4iv**) against E. coli and plate-b (**4i**. **4ii**, **4iii**, **4iv**) against S. mitis and 4-HC heteroarylazo analogues plate-c (**4d**, **4e**, **4f**, **4g**) against *V. cholera*.

3555−3446 cm^{-1}, 1618−1607 cm^{-1} and 1511−1505 cm^{-1} due to the presence of lactone carbonyl (C═Ostr), O-Hstr, C═Cstr and −N═N-str functional groups, respectively. This broad value (3555−3446 cm^{-1}) reveals that an enolic hydroxyl group was involved due to the intermolecular and intramolecular hydrogen bonding.[14] Compound **4i** showed three strong absorption bands at 1695 cm^{-1}, 1300 and 1137 cm^{-1}, which were assigned to the stretching of the coumarin lactone carbonyl and the asym/sym stretching of the sulfonyl group, respectively. The medium frequency bands at 1510 and 1280 cm^{-1} were assigned to (−N═N−) str and C-Ostr, respectively. Compound **4iv** showed four absorption bands at 1742, 1697, 1609 and 1509 cm^{-1}, which were assigned to the stretching of the carboxylic acid C═O, the carbonyl group of lactone, the C═C str. of coumarin and −N═N−, respectively. A signal attributable to an aryl amine group was not observed in the ^{1}H NMR spectral data, and amine absorption bands were also absent in the in FT/IR spectral data for all compounds. All ^{1}H NMR spectra, except for those of **4i** and **4iv**, showed board singlet at δ 16.73−16.85 ppm, which suggests the presence of enolic OH in the compounds. For compound **4ii**, the proton of the $-OCH_3$ group was observed in the H^1 NMR at δ3.83 ppm as singlet, and the aromatic protons of coumarin were also observed at δ 6.94−7.34 ppm as a multiplet (Figure 2). In addition, the three medium absorption bands at 2925, 1264 and 1110 cm^{-1} were assigned to the CH_2 str., $=C-OCH_3$ and C−O str. of the methoxy group, respectively. Compound **4i** showed two sharp strong vibration bands at 1300 and 1134 cm^{-1} due to SO_2 asymmetrical and symmetrical stretching, respectively (Figure 3). In addition, two series of protons were evident as a multiplet at δ 7.75−7.85 and 7.37−7.81 ppm, which were assigned to the aromatic phenyl protons and coumarin protons, respectively.

The predicted molecular weights of synthesized compounds were confirmed by LC-MS and provide strong evidence for the molecular formulas of these compounds. Compounds **4i**, **4ii**, **4iii**, **4iv** and **4v** had *m*/*z* values of 344.96, 296.99, 310.95, 310.33 and 281.13, respectively, which provides strong evidence for the molecular formulas of these compounds. The LC-MS of compound **4iv** is given in Figure 4.

Solvatochromic effect on 4-HC arylazo analogues

The solvent effects of the products were studied by UV-Visible spectrophotometry. The absorption spectra of these compounds (**4i**−**4v**) were observed in different solvents at a concentration of 10^{-5} to 10^{-6} M, and the results are reported in Table 2. Compound **4ii** showed highest bathochromic shift in all solvents with respect to the λ_{max}. The λ_{max} of compound **4ii** was 450 nm in methanol. The solvatochromic effect of 4-HC aryl and hetero aryl analogues in dioxane are reported in Figure 5. These bathochromic shifts can be attributed to the interaction of the amino protons in the compounds with polar aprotic solvents, such as THF, because the polarity of the dyes generally increases in an excited state. The introduction of an antipyrinyl substituent to the coumarin nucleus at the C-3 position yielded the largest bathochromic shift (λ_{max} 447 nm) compared with the other hetero-aryl compounds in

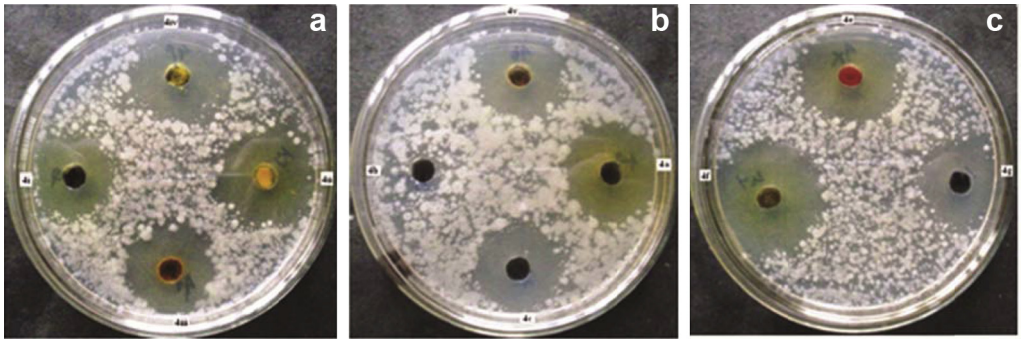

Figure 8: Fungal zone of inhibition showed by 4-HC aryl and hetero aryl analogues plate-a (**4i**. **4ii**, **4iii**, **4iv**), plate-b (4v, 4b, 4c, 4a), plate-c (**4e**, **4f** and **4g**) against *Cryptococcus neoformans*.

all solvents. The presence of a 2-methoxy phenyl (electron donating substituent) moiety in **4ii** attached in the same ring system resulted in a bathochromic shift in all solvents compared with the 3-nitro phenyl (electron withdrawing substituent) of **4iv**. The λ_{max} did not appreciably change for compounds **4i** and **4f**.

Thermal analysis

The sharp endothermic peak yielded by compound **4i** at 207.39 °C corresponds to its melting point (Figure 6).

Antimicrobial evaluation

The mean ± SD of the zone of inhibition for the newly synthesized 4-HC aryl and hetero aryl analogues was compared with that of the standard (RA) for each microbial strain with a one way-analysis of variance. The mean zone of inhibition significantly differed by compound, with a p value of 0.00. The pair-wise comparisons of each newly synthesized compound with the standard are expressed as the mean difference ± SEM.

The zones of inhibition were tested against *E. coli, S. enterica ser. typhi, S. enterica typhimurium, S. enterica paratyphi, S. flexneri, P. aeruginosa, V. cholera, K. pneumoniae, M. luteus, B. circulans, S. mitis, P. carotovarum, B. subtilis* and *S. aureus*. The highest mean zones of inhibition (mm) for these strains observed for compounds **4d**, **4e**, **4a**, **4ii**, **4e**, **4b**, **4e**, **4e**, **4e**, **4iv**, **4iv**, **4iv**, **4e** and **4e** were 19.00 ± 1.27, 15.50 ± 0.55, 18.17 ± 0.41, 18.33 ± 1.03, 24.50 ± 0.55, 24.00 ± 0.63, 19.83 ± 0.75, 26.50 ± 0.55, 18.50 ± 0.55, 35.50 ± 1.23, 25.17 ± 0.75, 17.50 ± 0.55, 23.00 ± 0.89 and 25.00 ± 0.89, respectively, as presented in Table 3.1.

The pair-wise comparisons of each newly synthesized compound with the standard revealed that **4iv** and **4e** inhibited *S. mitis* and *M. luteus* significantly more ($p < 0.05$) than RA; whereas compound **4iv** was significantly more active than RA against *B. circulans*. Compound **4e** showed a significantly larger zone of inhibition than RA against the bacterial strains *S. mitis* and *M. luteus, S. flexneri, K. pneumonia, B. subtilis* and *S. aureus*, as presented in Table 3.2.

The largest mean zones of inhibition (mm) of compounds **4iv** (30.00 ± 1.79), **4iii** (18.17 ± 0.41), **4f** (24.17 ± 0.75), **4a** (18.00 ± 1.27) and **4e** (35.00 ± 1.27) were observed against *A. niger, T. rubrum, C. albicans, C. glabrata* and *C. neoformans*, respectively, as presented in Table 4.1.

The pair-wise comparison of each newly synthesized 4-HC analogues with RA revealed that all compounds except **4c** and **4d** were significantly more active ($p < 0.05$) against *A. niger* than RA. Moreover, compounds **4iv**, **4e** and **4f** were significantly more active against the fungal strain *C. neoformans* than RA, as reported in Table 4.2.

The zones of inhibition of the 4-HC arylazo analogues against different microorganisms are reported in Figures 7 and 8, and the significant antimicrobial activity of **4e** is graphically presented in Figure 9.

Evaluation of minimum inhibitory concentration (MIC)

The inhibitory property of the 4-HC analogues was determined in terms of the MIC (μg ml^{-1}) (Table 5). The 4-HC analogues **4ii**, **4iv, 4b** and **4e** exhibited potential antibacterial activity at 31.25 μg ml^{-1} against most bacterial strains. The 4-HC hetero aryl analogue **4e** inhibited the growth of twelve pathogens at a concentration of 31.25 μg ml^{-1}. The 2-methoxy-substituted 4-HC analogue **4ii** was the next most potent compound; it inhibited the growth of seven bacterial pathogens at a concentration of 31.25 μg ml^{-1}. The reference antibiotic (Ampicillin) inhibited all bacterial strains at 31.25 μg ml^{-1}. Thus, **4e** and **4iv** may be considered highly effective, and **4v**, **4a**, **4b** and **4f** may be considered moderately active compounds, which corroborates the above findings.

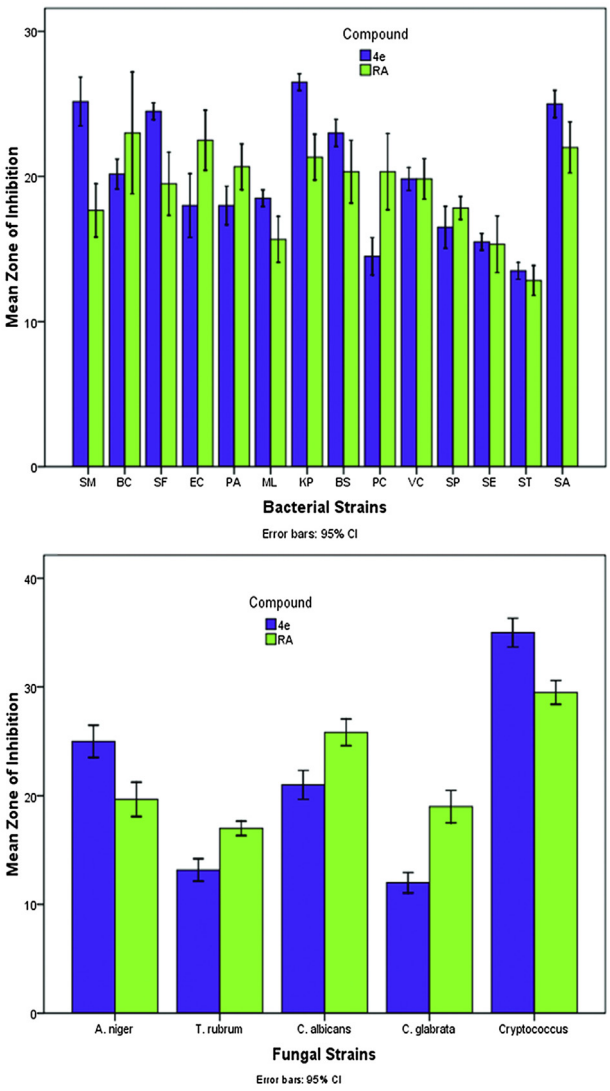

Figure 9: Statistical interpretation of significant antimicrobial effect of compound **4e** with the help of error bars.

Pharmacological evaluations

Acute toxic dose determination

The test compounds were safe at concentrations of up to 2000 mg/kg body weight. No toxic symptoms, gross behavioural changes and mortality were observed.

Table 5: MIC (μg ml^{-1}) values of newly synthesized 4-HC aryl and heteroarylazo analogues (4i–4v, 4a–4g) against various bacterial strains.

Compd.	*E. coli*[a]	*S. ser. typhi*[b]	*S. typhimerium*[c]	*S. paratyphi*[d]	*S. flexneri*[e]	*P. aeruginosa*[f]	*V. cholera*[g]
4i	500	250	250	250	500	31.25	31.25
4ii	62.5	31.25	250	31.25	–	250	125
4iii	31.25	125	125	125	125	250	500
4iv	31.25	62.5	125	62.5	31.25	31.25	125
4v	31.25	125	31.25	125	500	31.25	500
4a	31.25	500	31.25	500	500	62.5	>500
4b	31.25	125	125	125	125	31.25	>500
4c	62.5	500	62.5	500	125	31.25	–
4d	62.5	125	31.25	31.25	500	62.5	500
4e	31.25	31.25	125	31.25	31.25	31.25	31.25
4f	–	–	500	–	125	62.5	500
4g	–	500	500	500	500	500	125
RA	31.25	31.25	31.25	31.25	31.25	31.25	31.25

Compd.	*K. pneumoniae*[h]	*M. luteus*[i]	*B. circulans*[j]	*S. mitis*[k]	*P. carotovarum*[l]	*B. subtilis*[m]	*S. aureus*[n]
4i	–	–	125	125	500	125	–
4ii	31.25	250	31.25	31.25	31.25	31.25	250
4iii	–	125	500	31.25	125	500	500
4iv	125	31.25	31.25	31.25	125	125	–
4v	–	500	31.25	–	125	500	500
4a	500	500	500	500	125	31.25	31.25
4b	31.25	–	125	31.25	125	62.05	31.25
4c	–	–	–	–	500	62.05	–
4d	125	125	–	125	500	31.25	31.25
4e	31.25	31.25	31.25	31.25	125	31.25	31.25
4f	–	500	–	500	125	>500	31.25
4g	125	125	125	500	125	>500	>500
RA	31.25	31.25	31.25	31.25	31.25	31.25	31.25

– No zone of inhibition.
[a] *Escherichia coli.*
[b] *Salmonella enterica ser. typhi.*
[c] *Salmonella enterica typhimurium.*
[d] *Salmonella enterica paratyphi.*
[e] *Shigella flexneri.*
[f] *Pseudomonas aeruginosa.*
[g] *Vibrio cholera.*
[h] *Klebsiella pneumonia.*
[i] *Micrococcus luteus.*
[j] *Bacillus circulans.*
[k] *Streptococcus mitis.*
[l] *Pectobacterium carotovorum.*
[m] *Bacillus subtilis.*
[n] *Staphylococcus aureus.*

Table 6: Wound healing activity of some newly synthesized 4-HC aryl and heteroarylazo analogues by excision and incision wound model.

Compd.	Wound contraction (%)						Incision wound model
	0 Day	4th Day	8th Day	12th Day	16th Day	%inhibition	Tensile strength(g)
Control	318.1 ± 6.4	237.3 ± 6.5	182.8 ± 5.3	134.3 ± 4.4	82 ± 4.8	74.22	310 ± 7.2
Nitro furazone (Standard)	326 ± 6.2	201.8 ± 7.7**	123.6 ± 4.5***	80.1 ± 4.7***	40 ± 5.6***	87.73	530 ± 6.8***
4ii (5%)	320.1 ± 5.5	234 ± 7.4	181.6 ± 5.1	130.6 ± 6.2	70.6 ± 5.8	77.94	317 ± 4
4ii (10%)	311.5 ± 5.6	230.6 ± 8.5	177.8 ± 6.5	127.3 ± 5.4	67 ± 4.8	78.49	326 ± 7.6
4iv (5%)	311.3 ± 8.6	213.5 ± 6.7*	160.6 ± 8.8*	107 ± 5.1**	51.3 ± 3.7**	83.52	365 ± 8.5***
4iv (10%)	319 ± 3.1	208.6 ± 8.6*	146.6 ± 5.3**	90.6 ± 5.***	45 ± 4.8**	85.89	415.5 ± 6.5**
4a (5%)	316.1 ± 6.6	225.1 ± 5	175.3 ± 5.1	126.1 ± 4.7	65.3 ± 6	79.34	323 ± 6.9
4a (10%)	311.5 ± 6.5	224.3 ± 7	168.8 ± 3.7	124.6 ± 5.3	60.1 ± 6*	80.70	333 ± 7.8*
4e (5%)	319.8 ± 3.9	221 ± 6.5	164.3 ± 5.7a	116.6 ± 5.1*	53.5 ± 6.8*	83.27	349 ± 7.9**
4e (10%)	313.3 ± 4.7	214 ± 6.9^{a*}	162.3 ± 7.2*	100.5 ± 4.9***	60.1 ± 6*	80.81	376 ± 6.5***

Values are expressed in MEAN ± S.E.M of six animals. One Way ANOVA followed by Dunnett's t-test. (F-value denotes statistical significance at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$), respectively, in comparison to group-I.

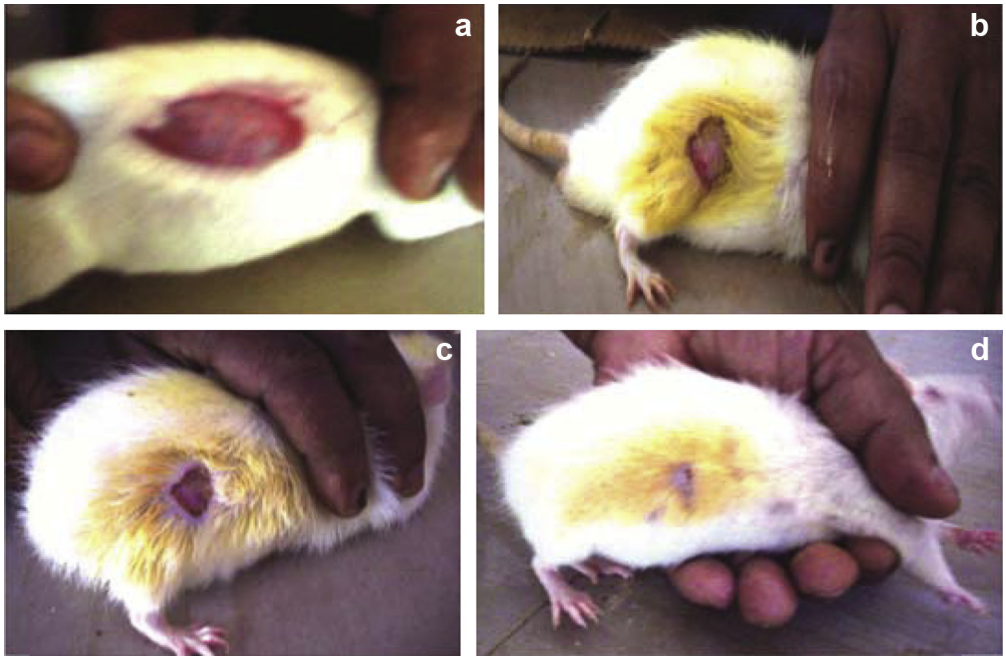

Figure 10: Wound treated with 4-HC aryl analogue (**4iv**) showed progressive healing on 0 day (a), 4th day (b), 12th day (c) and 16th day (d).

Screening of wound healing activity

In the excision wound model, the wounds progressively contracted until the end of observation on the 16th day. Optimum healing was defined as a minimum remaining wound area or % inhibition for the subjected compounds compared with the standard. The compounds included in the experiment showed significant wound healing activity, with more than 80% of wound inhibition by day 16, as reported in Table 6. The remaining wound areas of the control and standard were 82 ± 4.8 and 40 ± 5.6 mm^2, respectively with 74.22% and 87.73% inhibition, respectively (Figure 10).

The incision study revealed that compounds **4iv**, **4a** and **4e** showed significant breaking strength (force responsible for opening of the closed wound) more than the control (Table 6).

In vitro antioxidant screening

DPPH radicals accept a hydrogen atom or electron from organic molecules and can form stable diamagnetic molecules. The scavenging effects of newly synthesized compounds **4ii**, **4iii**, **4iv**, **4a**, **4b**, **4c**, **4e** and **4g** as well as that of the butylated hydroxy toluene standard were examined. The 50% of inhibition (IC_{50}) concentrations of the newly synthesized compounds were 50 ± 0.88, 22 ± 0.75, 20 ± 0.5, 30 ± 0.78, 9 ± 0.97, 7 ± 0.84, 9 ± 0.87 and 10 ± 0.94 μg/ml, respectively, whereas that of BHT was 32 ± 0.4 μg/ml (Table 7). The antioxidant activities of the synthesized analogues are reported in Figure 11. The study reveals that the presence of electron withdrawing groups (−COOH, NO_2) increases the antioxidant potential, which may be

Table 7: Antioxidant activity of newly synthesized 4-HC aryl and heteroarylazo analogues.

Conc. μgml^{-1}	10	50	100	200	IC_{50}
Compound	% Inhibition				
4ii	43 ± 0.88	50 ± 0.75	56 ± 0.78	63 ± 0.67	50 ± 0.88
4iii	46 ± 0.78	57 ± 0.67	67 ± 0.86	79 ± 0.75	22 ± 0.75
4iv	48 ± 0.67	58 ± 0.67	69 ± 0.78	80 ± 0.75	20 ± 0.5
4a	41 ± 0.75	56 ± 0.67	61 ± 0.88	78 ± 0.78	30 ± 0.78
BHT	42 ± 0.86	57 ± 0.67	72 ± 0.78	97 ± 0.69	32 ± 0.4
Conc. μgml^{-1}	2	4	6	8	10	IC_{50}
Compound	% Inhibition					
4b	5 ± 0.78	19 ± 0.88	31 ± 0.75	45 ± 0.67	58 ± 0.67	9 ± 0.97
4c	11 ± 0.75	19 ± 0.67	37 ± 0.88	59 ± 0.86	72 ± 0.78	7 ± 0.84
4e	8 ± 0.78	19 ± 0.86	30 ± 0.86	41 ± 0.75	59 ± 0.67	9 ± 0.87
4g	4 ± 0.88	13 ± 0.75	25 ± 0.67	35 ± 0.78	52 ± 0.67	10 ± 0.94

Free radical scavenging activity showed the results of % inhibition and (IC_{50}). IC_{50} expressed mean ± SD (n = 3).

due to the increase in the positive charge on the enolic hydroxyl group of 4-HC associated with the negative inductive effect of these groups. The positive charge intensification may lead to free radical inhibition. Moreover, the substitution electron-accepting groups are good free radical quenchers. The substitution of an electron-releasing group ($-OCH_3$) was found to decrease the free radical scavenging activity, which may be due to a positive inductive effect.

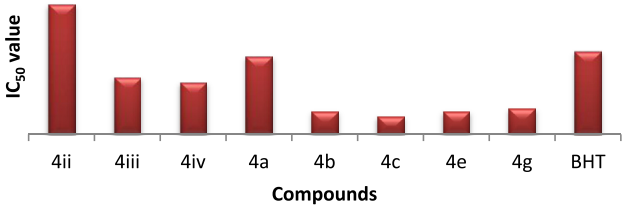

Figure 11: Graphical interpretation of radical scavenging activity of 4-HC analogues (compounds against IC50).

Discussion

All newly synthesized compounds exhibited good antimicrobial activity against most strains. However, the 4-HC arylazo analogue **4iv** exhibited significant antibacterial and antifungal activity, which may be due to nitro substitution.[18] The presence of N-alkyl substituted pyrazolone **4e** derived from 4-HC may be responsible for the excellent antimicrobial and antioxidant activity.

Based on the antimicrobial screening, five (**4ii, 4iii, 4iv, 4a and 4e**) out of twelve (**4i–4v**, **4a–4g** earlier reported) newly synthesized **4-HC** analogues showed maximum activity against pathogenic bacterial strains. The wound healing activity of the most potent four compounds (**4ii, 4iv, 4a** and **4e**) was evaluated with an excision and incision model. The reactive oxygen species secreted at wound site are responsible for delayed healing.[19] Moreover, invading microorganisms and free radicals[20] also prevent healing. Antioxidants terminate the formation of free radical intermediates and enable quick recovery of the wound and thus help prevent cell mortality.[21] Our synthesized compounds showed excellent antimicrobial activity and significant antioxidant potential in the wound healing study.

Conclusions

This research details an interesting, easily executed and simple technique for a one step synthesis of coumarin analogues to provide bioactive molecules that inhibit against various pathogens. The synthesized compounds were successfully characterized, and their *in vitro* antimicrobial and antioxidant activities were explored such that they can be used as ligands for new azo-metal complexes.

Authors' contributions

The authors of this research work have substantially contributed to fulfill the needs as follows: 1) data acquisition, data interpretation, drafting the article; 2) conceptualization of the study designing, critically examining the interpreted data & the drafted manuscript and finally its approval.

Conflict of interest

The authors have no conflict of interest to declare.

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