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Research Article

SYNTHESIS, SPECTRAL STUDIES AND ANTIMICROBIAL ACTIVITY OF 8-METHYL-2-SUBSTITUTED-6H-CHROMENO [6,7-d] OXAZOLE-6-ONE DERIVATIVES

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ABSTRACT

Antibiotic resistance has been a global healthcare problem due to the indiscriminate use of antibiotics and the subsequent creation of bacteria that can survive traditional treatment. Present research work focused to synthesis of coumarin derivatives. Figure 2 shows synthesis of compounds 8-Methyl-2-substituted-6*H*-chromeno [6,7-d] oxazol-6-one. All the Molecules were evaluated against Gram positive bacteria *Staphylococus aureus* (MTCC 3160), Gram negative bacteria *E. Coli*. (MTCC 614) for antimicrobial activity. The derivative bearing 2-chloro (6f) substituent possessed maximum activity against Gram negative and minimum activity against Gram positive bacteria, whereas 4-chloro (6d) substituent exhibited good activity against both strains.

Keywords: Coumarin, Oxazole, Antimicrobial.

INTRODUCTION

Antibiotic resistance has been a global healthcare problem due to the indiscriminate use of antibiotics and the subsequent creation of bacteria that can survive traditional treatment[1]. In addition, antibiotic resistance has drastically outpaced new antibiotic discovery. For instance, methicillin-resistant *Staphylococcus aureus* is one of the main species of bacteria that cause nosocomial infections in hospitals worldwide [2, 3]. It was only two years after the introduction of the methicillin in 1959 that resistant strains of the gram-positive human pathogen *Staphylococcus aureus* emerged. Majority of these pathogens arise from the commensal bacteria in humans, which cause opportunistic infections when immune compromised or under certain medical conditions[4]. Additionally, many problems remain unresolved due to occasional serious side effects and the appearance of antibiotic-resistant mutant bacteria. Therefore, the search for new drugs effective for the treatment of bacterial infections is of high priority. Focusing on known validated intracellular targets remains a valid approach to identify new drug candidates with novel chemical structures[5].

It has long been believed that small alterations in the structure of certain molecules can greatly affect their receptor binding affinity and biological activity[6]. Several studies have demonstrated that the introduction of various heterocyclic rings on parent moiety is effective in the production of variety of compounds with potential biological activities[7].Oftentimes, known heterocyclic moieties possessing antibiotic properties are modified to prolong their use for a known target[8].These may include the variations in the substitution pattern around the main heterocyclic nuclei, ring opening and ring closer[9, 10].

Coumarins are important and privileged scaffolds required for designing new drugs to overcome the growing problem of drug resistance. Many natural, semisynthetic and synthetic drugs bearing coumarin moiety are available as well-known clinical agents, and exhibit diverse pharmacological properties including anticancer[11], antimutagenic, antioxidant[12], antimicrobial[13], anti-tuberculosis[14] and anti-inflammatory[15], with

high bioavailability. In addition, coumarins are endowed with unique structural active sites such as hydroxyl group (OH), oxygen as heteroatom and C-3, C-7 positions in the basic ring which are significant for their biological activities[16]. For example, novobiocin is an aminocoumarin antibiotic, an efficient anti-staphylococcal agent used to cure methicillinresistant *Staphylococcus aureus* (MRSA). Osthole was identified as a promising lead moiety for drug discovery and functions as a potential multitarget alternative medicine. Additionally, warfarin containing 4-hydoxycoumarin moiety acts as an anticoagulant drug. Other coumarin derivative drugs are Ammoresinol, Ostruthin, Anthogenol, Imperatorin, Grandivittin, Agasyllin, Agelinol benzoate and Osthole. Ostruthin exhibited against a wide spectrum of Gram positive bacteria such as *Bacillus megaterium, Micrococcus lysodeikticus*, and *Staphylococcus aureus* (**Figure 1**)[17].

Coumarin nucleus incorporating oxazole moiety had been reported to possess synergism with total eight conventional antibacterial agents, *i.e.* chloramphenicol, gentamycin, fosfomycin, levofloxacin, minocycline, tazobactam, teicoplanin, vancomycin, against methicillin-resistant *Staphylococcus aureus* (MRSA) strains[18].

MATERIAL AND METHODS

All the chemicals used were of LR grade and purchased from Spectrochem, Oswal, Merck India, Lobachemie. The solvents employed were also of LR grade and obtained from Lobachemie, Spectochem and S.D. fine chem Ltd. All the media used for anti-microbial activity were procured from HIMEDIA. All the solvents were distilled and dried where necessary before use.

The identification and characterization of the compounds were carried out by following to ascertain that all prepared compounds were of different chemical nature than the parent compounds:

1. Physical methods: Melting point, solubility, thin layer chromatography.
2. Spectroscopic Methods: IR, ^{1}HNMR.

The reaction were monitored with the help of TLC using either precoated aluminium sheets with silica gel, 0.2 mm layer thickness (E. Merck) or prepared using silica gel. The various solvent systems used for developing the chromatogram in variable ratio.

a. Chloroform: Methanol
b. Ethylacetate: Hexane

The spots were being located either under iodine vapor or by UV light. Melting points were taken by the capillary method using Melting point apparatus (Perfit India). All the infra red (IR) spectra were recorded on Brucker FTIR.^{1}HNMR spectra were recorded on JNM-ECS400 (400 *MHz*) spectrometer using DMSO as solvent. TMS was used as standard and chemical shift (δ) data are reported in parts per million *(ppm)* where s, bs, d, t and m designated as singlet, broad singlet, doublet, triplet and multiplet respectively. Mass spectra were run on Micromas Q-T of micro spectrometer using TOF based detector at SAIF, Punjab University, Chandigarh.

Synthesis of 7-hydroxy-4-methyl coumarin

In a beaker, a solution of resorcinol (5g, 0.03 mol) and ethylacetoacetate (4g, 0.03 mol) was added to concentrated sulphuric acid (3g, 0.03 mol) at 5°C with constant stirring for 30 minutes. Solution was poured on the crushed ice with vigorous stirring; 7-hydroxy-4-methyl coumarin was precipitated. Suspension was filtered; crude product was dissolved in cold aqueous sodium hydroxide (10%) solution and re-precipitated it by addition of dilute hydrochloric acid. Crude product was decolorized and re-crystallized from charcoal and ethanol respectively to get 7-hydroxy-4-methyl coumarin.

7-hydroxy-4-methyl coumarin (3) Yield: 42.4%; Melting Point: 190-192°C; R_f: 0.6 (Chloroform: Methanol 7:3); IR (cm^{-1}): 3489 (O-H), 1663 (C=O), 1600 (C=C); ^{1}HNMR(DMSO-d_6): δ 10.54 (s, 1H), 6.12-7.59(m, 4H, Ar-H),2.35 (s, 3H).

Synthesis of 7-Hydroxy-4-methyl-6-nitro-2*H*-Chromen-2 one

To a solution of 3 (8.8g, 0.04mol) in sulfuric acid (27.6g, 0.15mol) kept at a temperature below 5°C, a mixture of nitric acid (5.64g, 0.06mol) and sulfuric acid (9.2g, 0.05mol) , was added dropwise with stirring so as to keep the temperature below 5°C. After complete addition (about one hour), the reaction mixture was stirred for another hour at the same temperature and then poured onto ice- water. The yellow precipitate obtained was filtered off, washed with water several times and air-dried. The 6-nitro isomer was obtained using boiling ethanol to separate the freely soluble 8-nitro isomer.

7-Hydroxy-4-methyl-6-nitro-2*H*-Chromen-2 one (4): Yield: 25%; Melting Point: 260-262°C; R_f: 0.64 (Chloroform: Methanol 7:3); IR (cm^{-1}): 3083 (O-H), 1537 (Asymmetric NO_2), 1363 (Symmetric NO_2), 1633 (C=C) ; ^{1}HNMR (DMSO-d_6): δ 11.92 (s, 1H), δ 6.27-8.22 (m, 3H, Ar-H),δ 2.36-2.46 (s, 3H).

Synthesis of 6-Amino-7-hydroxy-4-methyl-2*H*-chromen-2-one

A suspension of 4 (3.86g,) and stannous chloride dehydrate (13g, 0.06mol) in ethyl alcohol (15.7g, 0.3mol) and conc. HCl (21.4g, 0.5mol) was boiled to give a clear solution. The separated solid obtained after cooling in the refrigerator for 2 days was filtered, suspended in water and neutralized with sodium bicarbonate. The resulted yellow precipitate was filtered, extracted several times with hot isopropyl alcohol and the extracts were collected, concentrated under reduced pressure and cooled. The separated products were filtered and crystallized from isopropyl alcohol.

6-Amino-7-hydroxy-4-methyl-2*H*-chromen-2-one (5): Yield: 39%; Melting Point: 268-270°C; R_f: 0.67 (Chloroform: Methanol 7:3); IR (cm^{-1}): 3229 (O-H), 3058 (NH_2), 1693 (C=O), 1619 (C=C).

Synthesis of 8-Methyl-2-substituted-6*H*-chromeno [6,7-d] oxazol-6-one

In a solution of 5 (0.38g, 0.002 mol) in glacial acetic acid (20.9g, 0.3mol), add appropriate aldehyde namely, benaldehyde, p-nitrobenzaldehyde, 4-bromo benzaldehyde, 4-chloro benzaldehyde, 2-nitro benzaldehyde, 2-chloro benzaldehyde (0.002 mol) and was refluxed for 15 hours, cooled, poured into ice/cold water. The precipitate formed was filtered off and recrystallized (**Figure 2**).

8-Methyl-2- phenyl-6*H*- chromeno [6, 7-d] oxazol-6-one (6a): Yield: 86%; Colour: Brown; Melting Point: 277-279°C; R_f: 0.60 (Hexane: Ethylacetate 7:3); IR (cm^{-1}): 1709 (C=O), 1612 (C=N), 1542 (C=C); ^{1}HNMR (DMSO-d_6): δ 6.16-9.33 (m, 7H, Ar-H), 2.49-2.50 (s, 3H).

2-(4-bromophenyl)-8-methyl-6*H*- chromeno [6, 7-d] oxazol-6-one (6b): Yield: 60%; Colour: Brown; Melting Point: 250-252°C; R_f: 0.65 (Hexane: Ethylacetate 7:3); IR (cm^{-1}): 1714 (C=O), 1612 (C=N), 1537 (C=C); ^{1}HNMR (DMSO-d_6): δ 6.12-9.95 (m, 7H, Ar-H), 2.36-2.45 (s, 3H).

8-methyl-2-(4-nitrophenyl)-6*H*-chromeno [6,7-d] oxazole-6-one (6c): Yield: 75%; Colour: Yellow; Melting Point: 251-253°C; R_f: 0.66 (Hexane: Ethylacetate 7:3); IR (cm^{-1}): 1705 (C=O), 1612 (C=N), 1583 (C=C), 1335 (NO_2); ^{1}HNMR (DMSO-d_6): δ 6.15-8.31 (m, 7H, Ar-H), 2.39 (s, 3H).

2-(4-chlorophenyl)-8-methyl-6*H*- chromeno [6, 7-d] oxazol-6-one (6d): Yield: 66%; Colour: Brown; Melting Point: 218-220°C; R_f: 0.62 (Hexane: Ethylacetate 7:3); IR (cm^{-1}): 1708 (C=O), 1652 (C=N), 1537 (C=C) 679 (C-Cl); ^{1}HNMR (DMSO-d_6): δ 6.11-9.28 (m, 7H, Ar-H), 2.44-2.45 (s, 3H).

8-methyl-2-(2-nitrophenyl)-6*H*-chromeno [6,7-d] oxazole-6-one (6e): Yield: 40%; Colour: Yellow; Melting Point: 254-256°C; R_f: 0.61 (Hexane: Ethylacetate 7:3); IR (cm^{-1}): 1686 (C=O), 1613 (C=N), 1513 (C=C), 1339 (NO_2); ^{1}HNMR (DMSO-d_6): δ 6.15-8.18 (m, 7H, Ar-H), 2.49 (s, 3H).

2-(2-chlorophenyl)-8-methyl-6*H*- chromeno [6, 7-d] oxazol-6-one (6f): Yield: 37%; Colour: Brown; Melting Point: 224-226°C; R_f: 0.64 (Hexane: Ethylacetate 7:3); IR (cm^{-1}): 1710 (C=O), 1653 (C=N), 1610 (C=C), 642 (C-Cl); ^{1}HNMR (DMSO-d_6): δ 6.12-10.30 (m, 7H, Ar-H), 2.49-2.50 (s, 3H).

Synthesis of 8-Methyl-2-substituted-6*H*-pyrano [6, 7-d] benzoxazol-6-ones

To a solution of compound 5 (0.38 g, 0.002 mol) in pyridine (9.8g, 0.12mol), an (0.002 mol) acid anhydride namely acetic anhydride, succinic anhydride was added. The reaction mixture was refluxed for 10 hours, the pyridine was distilled under reduced pressure, and the residue was washed with water and dissolved in sodium hydroxide solution (5%, 10mL). The reaction mixture was filtered off and neutralized by dilute hydrochloric acid. The precipitate formed was filtered off, washed with water, dried and recrystallized.

2,8-dimethyl-6*H*-chromeno[6,7-d] oxaazol-6-one (7a): Yield: 53%; Colour: Brown; Melting Point: 298-300°C; R_f: 0.72 (Hexane: Ethylacetate 7:3); IR (cm^{-1}): 1726 (C=O), 1623 (C=N), 1526 (C=C); ^{1}HNMR (DMSO-d_6): δ 6.28-8.23 (m, 3H, Ar-H), δ 2.45-2.46 (s, 3H), δ 2.44 (s,3H).

3-(8-methyl-6-oxo-6*H*-chromeno [6, 7-d] oxazol-2-yl) propanoic acid (7b): Yield: 38%; Colour: Brown; Melting Point: 258-256°C; R_f: 0.74 (Hexane: Ethylacetate 7:3); IR (cm^{-1}): 3299 (O-H), 1723 (C=O), 1607 (C=N), 1541 (C=C); ^{1}HNMR (DMSO-d_6): δ 12.09-12.24 (s,1H), δ 6.07-9.30 (m, 3H, Ar-H), δ 3.29-3.66 (d, 4H), δ 2.28-2.46 (s, 3H).

BIOLOGICAL SCREENING OF THE SYNTHESIZED COMPOUNDS

Antimicrobial activity of synthesized compounds

All the synthesized compounds were subjected to antimicrobial activity against Gram positive bacteria *Staphylococus aureus* (MTCC 3160), Gram negative bacteria *E. Coli*. (MTCC 614). The primary screening was carried out by Cup-Plate diffusion method using nutrient agar medium at concentration (10µg/ml) for standard and (100µg/ml) for test. Ciprofloxacin was used as a standard drug for antibacterial activity and DMSO was used as control. The zone of inhibition was observed in mm.

RESULTS AND DISCUSSION

A four step reaction was used to synthesize various substituted 6-Methyl-2-substituted-8*H*-pyrano [2, 3-e] benzoxazol-8-ones and 8-Methyl-2-substituted-6*H*-pyrano[6,7-d] benzoxazol-6-ones. In the initial step, 7-Hydroxy-4-methyl coumarin was synthesized by *via* the Pechmann condensation of resorcinol and ethylacetoacetate. The synthesized compound was treated with conc. nitric acid and sulphuric acid which result in formation of two isomers of nitro coumarin, These isomers were separated using suitable solvent system. The separated 6-Nitro-7-hydroxy-4-methylcoumarin was further treated with stannous chloride dihydrate in the presence of conc. hydrochloric acid and ethanol which result in formation of 6-Amino-7-hydroxy-4-methylcoumarin which was further treated with various aldehyde *viz.* benzaldehyde, 4-bromo benzaldehyde, 4-nitro benzaldehyde, 4-chlorobenzaldehyde, 2-nitrobenzaldehyde, 2-chlorobenzaldehyde in the presence of glacial acetic acid and various acid anhydrides *viz.* acetic anhydride, succinic anhydride in the presence of pyridine to produce final derivatives. All the reactions were monitored through TLC observation till the completion using suitable mobile phase each time. After completion of the reaction, the products were purified by using suitable solvents *e.g* ethanol. The outcome of the present work has been summarized in **Table 1**.

The purity of the compound was confirmed by TLC using precoated silica gel as a stationary phase, using appropriate solvent system as mobile phase and visualized under UV-light as well as analyzed. Structures of the title compounds were confirmed by FTR-ATR and ^{1}H NMR spectral studies.

The total 8 coumarin deivatives were synthesized and a yield was found to be in the range of 37-86%. The synthesized compounds were all yellow to brown in colour. The melting points were in between the range of 218-300° C and are uncorrected. The R_f* for all derivatives were found in the range of 0.6-0.8 by using Ethyl acetate: Hexane (7:3) as solvent system.

The structure of all synthesized derivatives was characterized on the basis of their physical and spectral data *i.e* IR, ^{1}HNMR. The IR spectra of compound 4 showed two characteristic peak at 1537 and 1363 cm^{-1} for asymmetric and symmetric -NO_2 group whereas compound 5 depicted a peak at 3058 for –NH stretch. The IR spectra of derivatives showed absorption band at 1686-1726 cm^{-1} which confirmed the presence of C=O group, 1583-1653 cm^{-1} confirmed the presence of C=N group, 1513-1610 cm^{-1} revealed the presence of C=C group. Solvent used for ^{1}HNMR spectra was DMSO, ^{1}HNMR spectra of compound 6d depicted multiplet in the region δ 6.11-8.22 because of presence of four aromatic protons that was different from compound 4 which illustrated broad singlet peak at δ 11.92 for hydroxyl group.

Antimicrobial activity

The synthesized derivatives were evaluated for antibacterial activity using two strains *viz.* Gram positive bacteria *staphylococcus aureus* (MTCC 3160) and Gram negative bacteria *Escherichia coli* (MTCC 614). *In vitro* testing was carried out using Agar plate assay protocols using Ciprofloxacin as a standard, each derivative was tested at dose level need to (1000mcg, 500mcg, 250mcg, 125mcg) **(Table 2).**

The antibacterial activity data indicates that synthesized derivatives *i.e.* 6a, 6b, 6c, and 6e exhibited marked inhibitory activity against test organisms. The compounds having electron withdrawing substituents at benzaldehyde *(e.g.* 4-bromo, 4-nitro, 4-chloro) exhibit a very good activity against Gram negative and Gram positive bacteria. The derivative bearing 2-chloro (6f) substituent possessed maximum activity against Gram negative and minimum activity against Gram positive bacteria, whereas 4-chloro (6d) substituent exhibited good activity against both strains. The compounds formed by reacting with electron donating substitutents had less activity against Gram positive and Gram negative bacteria as compared than aldehydes (**Figures 3-4**).

Table 1: Physical characteristic of synthesized compound

Sr. No.	Compounds	Molecular Formula	Molecular Weight (g)	Melting Point (°C)	Yield (%)	R_f*
1.	6a	$C_{17}H_{11}NO_3$	277.24	277-279	86	0.60
2.	6b	$C_{17}H_{10}BrNO_3$	356.17	250-252	60	0.65
3.	6c	$C_{17}H_{10}N_2O_5$	322.28	251-253	75	0.66
4.	6d	$C_{17}H_9Cl.NO_3$	346.16	218-220	66	0.62
5.	6e	$C_{17}H_{10}N_2O_5$	322.28	254-256	40	0.61
6.	6f	$C_{17}H_9Cl.NO_3$	346.16	224-226	37	0.64
7.	7a	$C_{12}H_9NO_3$	215.2	298-300	53	0.72
8.	7b	$C_{14}H_{11}NO_5$	273.24	258-260	38	0.74

Table 2: *In vitro* antimicrobial activity against standard drug

Sr.No.	Compound	Conc. (mcg)	Disc Size (mm)	Zone of inhibition in (mm)	
				E.coli (MTCC 614)	*Staphylococcus aureus* (MTCC 614)
1.	Standard*	1000(standard)	6	25.92	34.48
	6a	1000	6	16.23	10.32
		500	6	15.32	8.16
		250	6	13.71	7.11
		125	6	11.25	6.18
2.	Standard	1000(standard)	6	25.92	34.48
	6b	1000	6	17.44	12.91
		500	6	13.53	11.23
		250	6	12.76	8.66
		125	6	12.27	7.99
3.	Standard	1000(standard)	6	25.92	34.48
	6c	1000	6	14.82	12.72
		500	6	13.78	10.01
		250	6	12.81	Nil
		125	6	12.13	Nil
4.	Standard	1000(standard)	6	25.92	34.48
	6d	1000	6	14.04	8.85
		500	6	13.3	6.1
		250	6	11.36	5.21
		125	6	9.87	Nil
5.	Standard	1000(standard)	6	25.92	34.48
	6e	1000	6	13.17	11.71
		500	6	13	9.16
		250	6	12.68	Nil
		125	6	11.55	Nil
6.	Standard	1000(standard)	6	25.92	34.48
	6f	1000	6	24	5.16
		500	6	17.28	Nil
		250	6	12.19	Nil
		125	6	10.93	Nil
7.	Standard	1000(standard)	6	25.92	34.48
	7a	1000	6	12.11	9.99
		500	6	11.5	8.11
		250	6	10.26	4.03
		125	6	9	Nil
8.	Standard	1000(standard)	6	25.92	34.48
	7b	1000	6	11.48	10.18
		500	6	10.82	9.71
		250	6	10.08	8.29
		125	6	9.85	7.61

Standard*: Ciprofloxacin

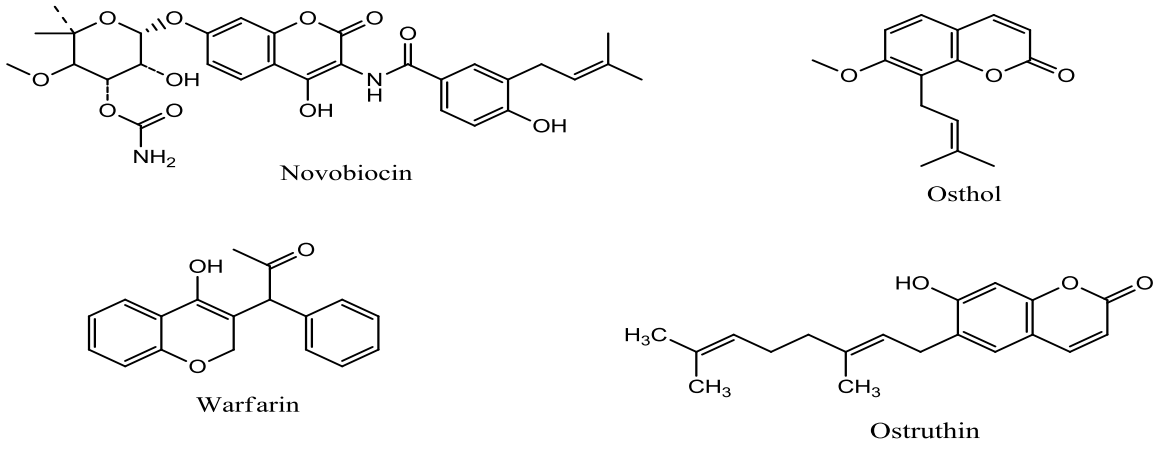
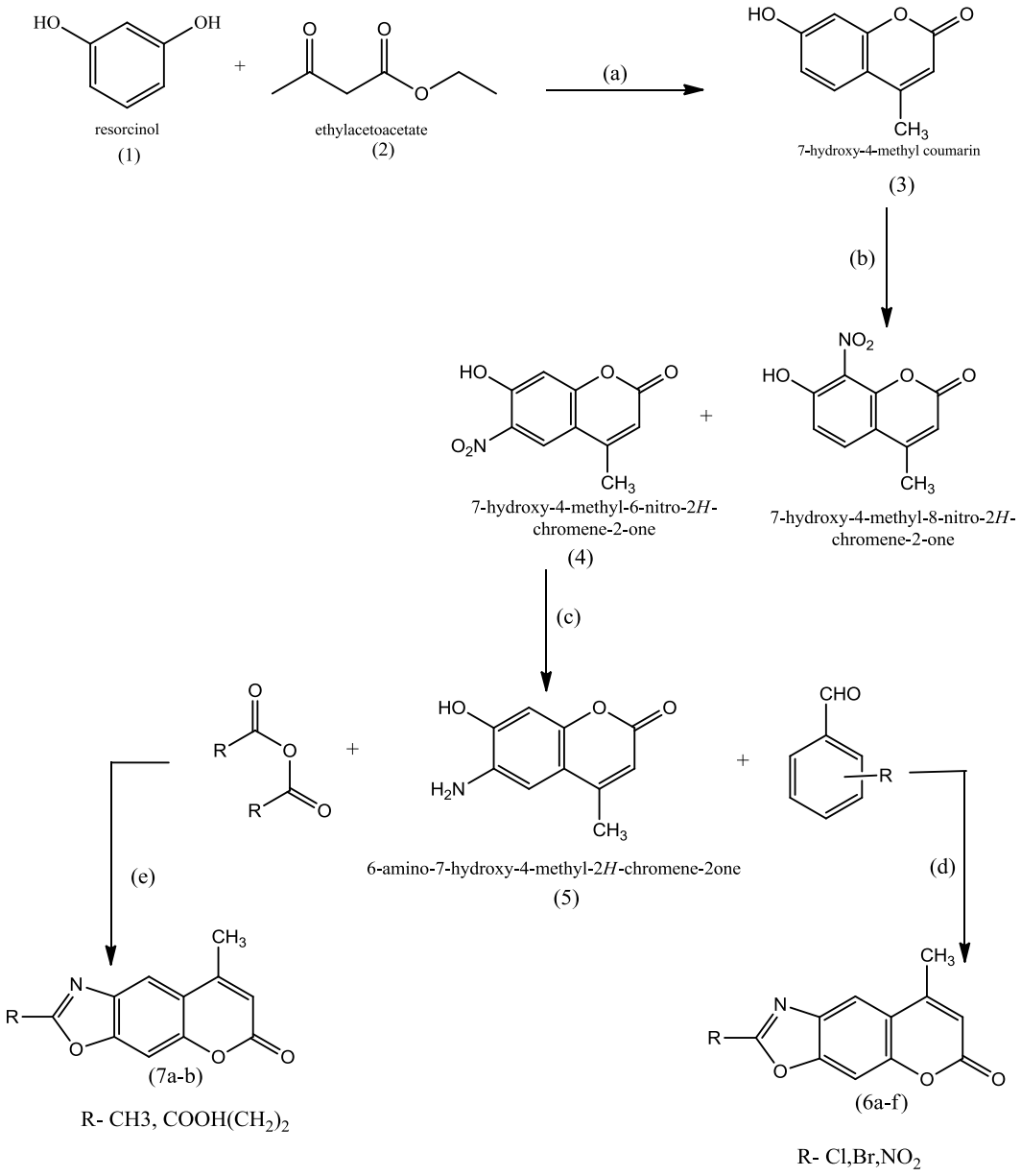


Fig. 1: Clinically known drugs bearing coumarin nucleus



Reagents and conditions: (a) Conc. H_2SO_4, stir for 1h at 5°C (b) Conc. HNO_3, H_2SO_4, stir for 1h at 10°C (c) $SnCl_2$, Conc. HCl, C_2H_5OH, kept for 2 days in refrigerator (d) Glacial acetic acid, reflux, 15h (e) Pyridine, reflux, 10h.

Fig. 2: Scheme for the synthesis of 8-methyl-2-substituted-6H-chromeno-[6,7-d] oxazol-6-one derivatives

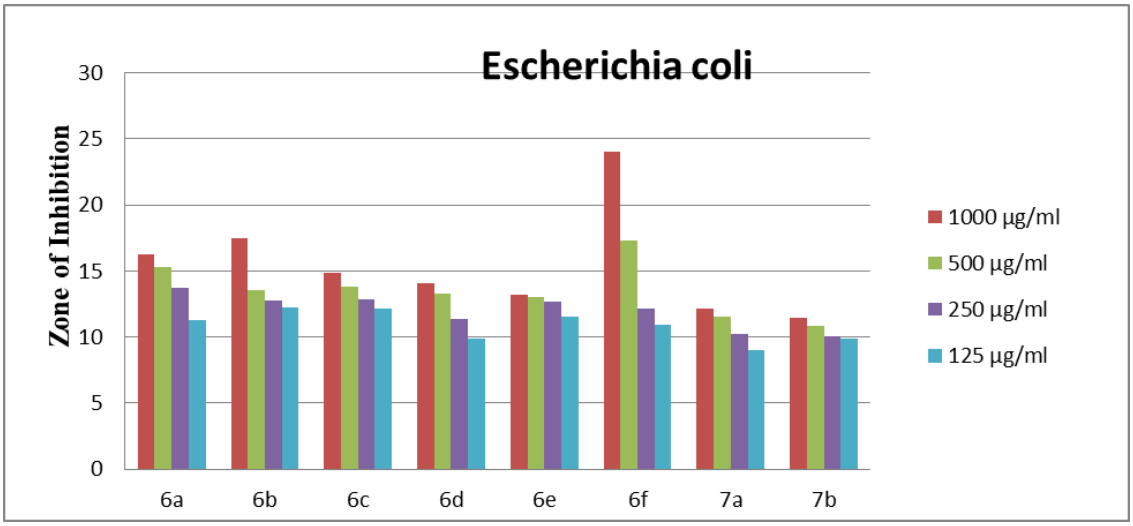


Fig. 3: Antibacterial activity of compounds (6a-f, 7a-b) against Gram positive bacterial strains

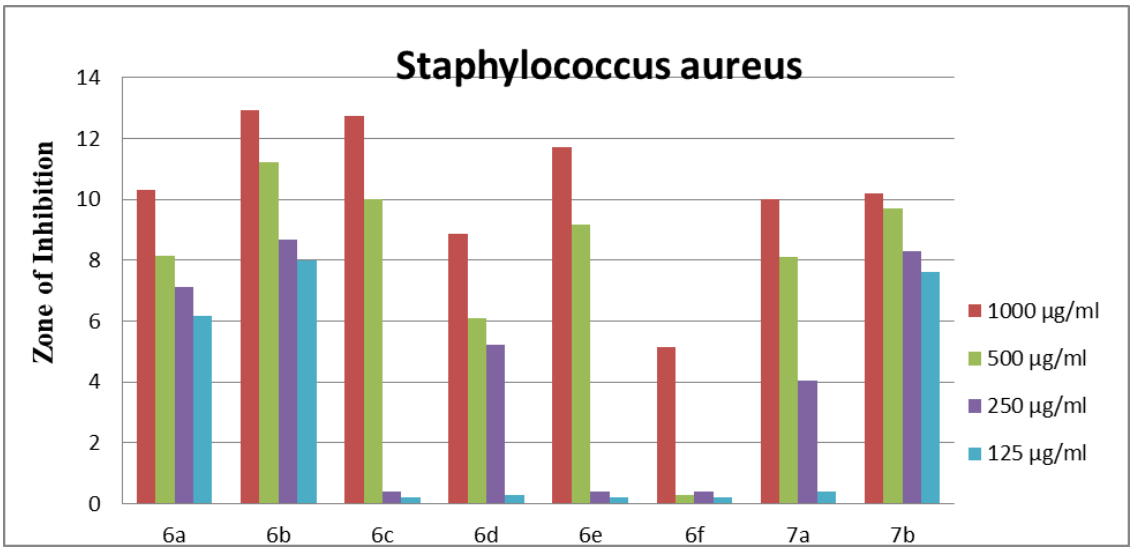


Fig. 4: Antibacterial activity of compounds (6a-f, 7a-b) against Gram negative bacterial strains

CONCLUSION

In the present study a total of 8 coumarin derivatives were synthesized. The derivative bearing 2-chloro (6f) substituent possessed maximum activity against Gram negative and minimum activity against Gram positive bacteria, whereas 4-chloro (6d) substituent exhibited good activity against both strains. The synthesized compounds can be further explored by study SAR and utilize various other substituted aldehydes as well as acid anhydrides to potentiate the antimicrobial activity.

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