SYNTHESIS AND ANTIMICROBIAL EVALUATION OF SOME NEW ASYMMETRICALLY SUBSTITUTED 4-ARYL- 2,6-DI(COUMARINYL) PYRIDINES

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ABSTRACT

In the present work the synthesis of various 4-aryl-2-(coumarin-3-yl)-6-(4-methyl-3-phenyl coumarin-6-yl)pyridines **(6a-i)** and 4-aryl-2-(coumarin-3-yl)-6-(4-methyl-7-methoxy coumarin-8-yl)pyridines (**7a-i)** have been carried out by reacting 1-[2(*H*)-1-benzopyran-3-yl]-3-aryl-prop-2-en-1-ones (coumarinoyl chalcones) (**3a-f)** with appropriate coumarinoyl methyl pyridinium bromide salt **4** and **5** respectively. The target compounds were characterized by the IR, ^{1}H-NMR, ^{13}C-APT and mass spectral analysis. Preliminary examination of target compounds as antimicrobial agents has been carried out using Broth dilution method.

Keywords: Dicoumarinyl pyridines, Krohnke's reaction, antimicrobial activity, Broth dilution method

INTRODUCTION

Over past few decades, resistance to antimicrobial agents among bacterial and fungal pathogens represent major global health problem in terms of morbidity and mortality[1]. Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness, higher health care expenditures, and a greater risk of death. Thus, to conquer such multidrug resistant pathogens is challenging task in present era. Therefore, there is crucial need to discover and develop effective antimicrobial agents with novel mechanisms of action and enhanced activity.

At present, coumarin represents one of the classes of versatile biodynamic agents, which are plentiful in plant kingdom and present as basic frame in potent pharmaceutical compounds. The promising biological profile offered by coumarin and its derivatives attributed to enormous diversity in substitution patterns on core scaffold. Coumarins play pivotal role in drug discovery exhibiting various therapeutic actions such as anticoagulant[2], antitumor[3], anti-inflammatory[4], hypolipidaemic[5], vasorelaxant[6], CNS depressant[7], antioxidant[8], anti-TB[9] and antimalarial[10] activities.

The interesting biological profiles of the coumarins make them adaptable targets in organic synthesis. Therefore, a detailed literature survey concerning the coumarins derivatives was carried out, through which we came across some 3-(2-pyridyl) and 3-(3-pridyl)coumarins which are recognized for their useful bioactivities viz. antifungal[11], bactericidal[12], fish toxicity[13], moth proofing activity[13] and CNS depressant activity[14]. Encouraged from the significant biological essence of pyridine substituted coumarins, a series of new 3-(2-pyridyl)[15-20], 6-(2-pyridyl)[21] and 8-(2-pyridyl)coumarins[22] and their antimicrobial assessment were reported from our laboratory. Additionally, we also reported some new dicoumarinyl substituted pyridines[23] in which synthesized coumarins adjoined pyridine by 3rd position. Thus two cumarin moieties were having symmetrical linkage with pyridine moiety and the attachment was by (3,3') positions of coumarins as shown in figure 1.

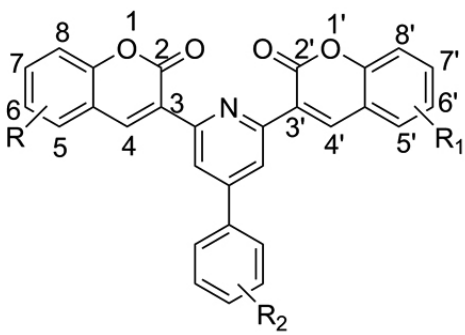
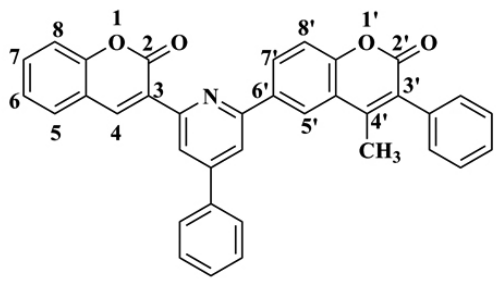
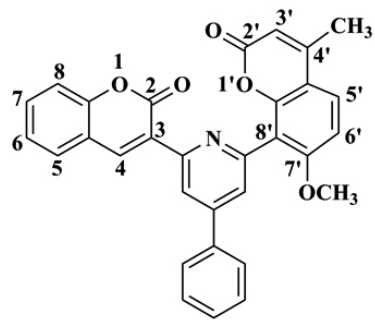


Figure 1: Attachment of two coumarin moieties with pyridine by (3,3') linkage.

In glance of our previous study here in, we report the synthesis of dicoumarinyl substituted pyridine in which the coumarin moieties are attached with pyridine moiety by different positions i.e by (3,6') [A] and (3,8') [B] as shown in figure 2 respectively. Additionally, the synthesized compounds were also evaluated for their antimicrobial potential.



(3,6') [A]



(3,8') [B]

Figure 2: Attachment of two coumarin moieties with pyridine by different positions i.e by (3,6') [A] and (3,8') [B].

RESULTS AND DISCUSSION

Chemistry

In the present work, various 4-aryl-2-(coumarin-3-yl)-6- (4-methyl-3-phenyl coumarin-6-yl)pyridines **6a-i** and 4-aryl-2-(coumarin-3-yl)-6-(7-methoxy-4-methylcoumarin-8-yl) pyridines **7a-i** have been synthesized by the reacting 1-[2(*H*)-1-benzopyran-3-yl]-3-aryl-prop-2-en-1-ones **3a-f** with 4-methyl-3-phenyl-6-coumarinoyl methyl pyridinium bromide salt **4** and 7-methoxy-4-methyl-8-coumarinoyl methyl pyridinium bromide salt **5** respectively in the presence of ammonium acetate and acetic acid under Kröhnke's reaction condition[24] as displayed in **scheme 1**.

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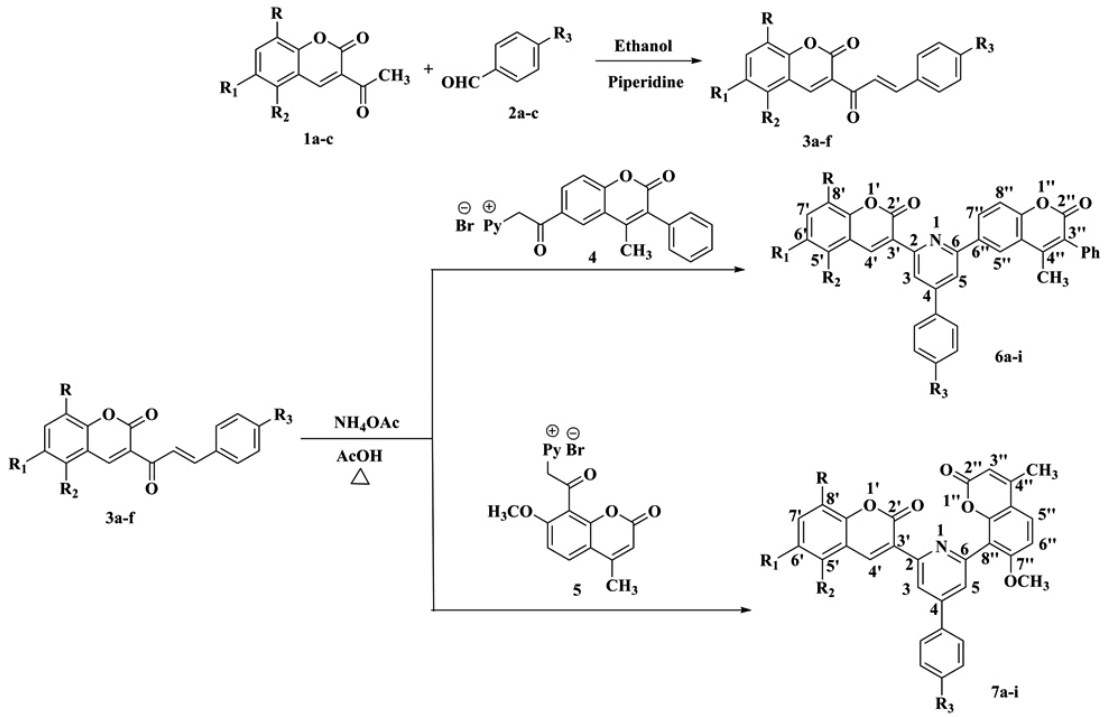
Compounds	R	R_1	R_2	R_3		Compounds	R	R_1	R_2	R_3
6a, 7a	H	H	H	H		**6f, 7f**	OCH_3	H	H	OCH_3
6b, 7b	H	H	H	CH_3		**6g, 7g**	H	Benzo		H
6c, 7c	H	H	H	OCH_3		**6h, 7h**	H	Benzo		CH_3
6d, 7d	OCH_3	H	H	H		**6i, 7i**	H	Benzo		OCH_3
6e, 7e	OCH_3	H	H	CH_3						



Scheme 1: Synthetic pathway for the preparation of starting precursor 3a-f, target compounds 6a-1 and 7a-i.

The structures of all the synthesized compounds were confirmed on the basis of ^{1}H-NMR; ^{13}C-APT; IR; elemental analysis and representative mass spectral data.

Amongst the compounds **6a-i**, the IR spectrum of compound **6a** exhibited the band at 3054 and 2924 cm^{-1} are due to aliphatic C-H and aromatic C-H stretching vibrations. The characteristic strong and sharp band observed at 1716 cm^{-1} for carbonyl stretching of δ-lactone ring of coumarin nucleus. The bands observed at 1611 and 1457 cm^{-1} are due to aromatic C=C and C=N stretching vibrations. A sharp and intense band observed at 761 cm^{-1} is due to C-H out of plane bending vibrations for mono substituted benzene ring. The ^{1}H-NMR spectrum of compound **6a** showed a singlet at 2.47 δ integrating for three protons, which is due to methyl group protons. The signal for C''$_7$-H appeared as doublet of doublet in the downfield region centered at 8.35 δ (*J*= 8.8 and 1.6 Hz). The C''$_5$-H appeared as meta coupled doublet at 8.42 δ (*J*= 1.6 Hz). A poorly resolved meta coupled doublet centered at 8.68 δ corresponds to proton attached to C_3 of the pyridine ring. The C_3-H of pyridine ring appeared in the downfield region due to peri effect of carbonyl group of δ-lactone. The signal for C'$_4$ of the coumarin ring appeared in the most downfield region at 8.95 δ is due to peri effect of nitrogen N_1. The remaining sixteen aromatic protons appeared as a multiplet between 7.28-7.93δ. The ^{13}C-APT spectrum of compound **6a** showed signals at 16.80 δ is due to methyl carbon. The signal appeared at 160.33 and 160.76 δ are due to carbonyl carbon of the δ-lactone ring of two coumarin moieties. The signals for thirty non equivalent carbons present in the compound appeared between116.46 δ to 156.00δ. The mass spectrum of compound **6a** showed molecular ion peak at m/z 533.0 along with other fragments corresponding to molecular formula $C_{36}H_{23}NO_4$.

Among the compounds **7a-i**, the IR spectrum of compound **7a** showed the band at 3057 and 2924 cm^{-1} are due to aliphatic C-H and aromatic C-H stretching vibrations respectively. A strong band at 1724 cm^{-1} due to carbonyl stretching of δ-lactone ring present in coumarin nucleus. The bands observed at 1611 and 1457 cm^{-1} are due to aromatic C=C and C=N stretching vibrations respectively. A sharp and intense band observed at 748 cm^{-1} is due to C-H out of plane bending vibrations for mono substituted benzene ring. The ^{1}H-NMR spectrum of compound **7a** showed a two singlet appeared at 2.69 and 3.90 δ integrating for three protons are of methyl group and methoxyl group respectively. A singlet appeared at 6.21δ correspond to proton attached to C_3''-H. A meta coupled doublet observed at 8.59 δ (*J*= 1.6 Hz) is due to C_3-H proton of pyridine ring. A singlet appeared at 8.76 δ and integrating for one proton is due to proton attached at C'$_4$. The C'$_4$ proton appears in the downfield region due to the peri effect of nitrogen. The remaining twelve aromatic protons appeared as multiplet between 7.79-7.06 δ. The ^{13}C-APT spectrum of compound **7a** showed signals at 19.61 and 56.47 δ are due to methyl carbon and methoxyl carbon respectively. The signal appeared at 160.11 and 160.53 δ can be assigned to carbonyl carbon of the δ-lactone ring of two coumarin moieties. The signals for the other aromatic carbons appeared between 108.33 δ to 159.18 δ. The mass spectrum of compound **7a** showed molecular ion peak at m/z 443.0 along with other fragments corresponding to molecular formula $C_{31}H_{21}NO_4$. The analytical data for the other derivatives were discussed in experimental section.

The analytic and spectroscopic data of remaining synthesized compounds are given in the Supplementary Material to this paper.

Evaluation of Antimicrobial activity

All the synthesized compounds were screened for their *in vitro* antimicrobial activity by Broth dilution method[25]. All the newly synthesized compounds **6a-i** and **7a-i** exerted significant inhibitory activity against all the employed strains. Upon evaluating antimicrobial activity data in **Table I**, it was observed that compounds **7b** and **7h** (MIC= 62.5 µg/ml) exhibited superior activity compare to Ampicillin (MIC= 250 µg/ml) against gram positive bacteria *S. aureus*, while compounds **6e** and **7g** (MIC= 6.25 µg/ml) were found to be more potent than Ampicillin (MIC= 250 µg/ml) against gram positive bacteria *B. Subtilis*. Against *S. aureus*, compounds **6d**, **6h**, **7a**, **7e**, **7g** and **7i** (MIC= 100 µg/ml) and against *B. Subtilis* compounds **6c**, **7a** and **7e** (MIC= 100 µg/ml) demonstrated

more inhibition than Ampicillin (MIC= 250 µg/ml) and comparable activity to Norfloxacin (MIC= 100 µg/ml). Compounds **6a**, **6c**, **6e**, **6g** and **7f** (MIC= 125 µg/ml) against *S. aureus*, while compounds **6a**, **6b**, **6f**, **6g**, **6h**, **7b**, **7d** and **7i** (MIC= 125 µg/ml) against *B. Subtilis* displayed better activity as compare to Ampicillin (MIC= 250 µg/ml). Moderate inhibition were shown by compounds **6b**, **6i**, **7c** and **7d** (MIC= 200 µg/ml) against *S. aureus* than Ampicillin (MIC= 250 µg/ml). Similarly, compound **6i** (MIC= 200 µg/ml) displayed moderate activity against *B.subtilis*. Compound **6f** (MIC= 250 µg/ml) against *S. aureus* and compounds **7c**, **7f** and **7h** (MIC= 250 µg/ml) against *B. Subtilis* showed comparable activity to Ampicillin (MIC= 250µg/ml).

In case of gram negative bacteria, compound **7b** (MIC= 50 µg/ml) was displayed outstanding inhibitory effect against *E. Coli* compare to Ampicillin (MIC= 100 µg/ml) and comparable activity to Chloramphenicol (MIC= 50 µg/ml). Against *E. Coli*, compounds **6d** and **6i** (MIC= 62.5 µg/ml) depicted remarkable activity compare to Ampicillin (MIC= 100 µg/ml). Compound **7h** (MIC= 100 µg/ml) against *E. Coli* and compounds **6c**, **6d** and **7i** (MIC= 100 µg/ml) against *S. Typhi* were found to equipotant to Ampicillin (MIC= 100 µg/ml).

Antifungal assessment data of target compounds revealed that Compounds **7e**, **7h** (MIC= 100 µg/ml) against *A. Niger* exhibited equal inhibition to Griseofulvin (MIC= 100 µg/ml) and Nystatin (MIC= 100 µg/ml). Compounds **6d**, **7b** (MIC= 100 µg/ml) against *C. Albicans* exerted excellent inhibition compare to Griseofulvin (MIC= 500 µg/ml) and equal potency to Nystatin (MIC= 100 µg/ml). Against *C. Albicans*, compounds **6b** and **6g** (MIC= 200 µg/ml); compounds

6h, **7d** and **7i** (MIC= 250 µg/ml) exhibited significant activity compare to Griseofulvin (MIC= 500 µg/ml).

Table I. Antibacterial Activity MIC (µg/mL) of the compounds 6a-i and 7a-i.

Compounds	Gram Positive Bacteria		Gram Negative Bacteria		Fungi	
	S.Aureus MTCC96	B.Subtilis MTCC441	E.Coli MTCC443	S.Typhi MTCCC98	A.Niger MTCC282	C.Albicans MTCC227
6a	125	125	250	125	500	>1000
6b	200	125	200	200	250	200
6c	125	100	125	100	500	500
6d	100	200	62.5	100	500	100
6e	125	62.5	250	250	>1000	500
6f	250	125	125	200	>1000	500
6g	125	125	250	125	250	200
6h	100	125	200	200	500	250
6i	200	200	62.5	200	1000	200
7a	100	100	250	250	500	>1000
7b	62.5	125	50	200	500	100
7c	200	250	250	200	>1000	500
7d	200	125	250	250	500	250
7e	100	100	200	200	100	>1000
7f	125	250	250	250	500	500
7g	100	62.5	125	200	1000	1000
7h	62.5	250	100	200	100	500
7i	100	125	200	100	250	250
Ampicillin	250	250	100	100	-	-
Chloramphenicol	50	50	50	50	-	-
Norfloxacin	10	100	10	10	-	-
Griseofulvin	-	-	-	-	100	500
Nystatin	-	-	-	-	100	100

Structure activity relationship established from the analysis of data reported in **Table.1**, lead to some general conclusions that mainly two structural features have influence on biological potential of synthesized compounds: 1) Substitution on phenyl ring. 2) Position of attachment of coumarin and pyridine moieties to each other. Observations indicate that the compounds **6b**, **6e**, **6h**, **7b**, **7e** and **7h** bearing weak electron releasing methyl group (R_3=CH_3) showed outstanding activity compare to parent targets. The increased efficiency attributed to lipophilicity of methyl group. Introduction methoxyl group (R_3=OCH_3) exerted reduced activity compare to parent analogs. It is interesting to note that compounds **7a-i** possess promising antimicrobial activity against gram positive bacteria *B. Subtilis* and *S. aureus*, while compounds **6a-i** demonstrate excellent activity against gram negative bacteria *E. Coli* and *S. Typhi*. We also noticed that among the compounds **6a-i** and **7a-i**, compounds bearing 4-methyl-7-methoxycoumarin-8-yl moiety at 6th position of pyridine ring, i.e. compounds **7a-i** were more efficient compare to compounds **6a-i**.

EXPERIMENTAL SECTION

All the melting points were determined on µ Thermocal 10 apparatus. All the IR spectra (KBr disc) were recorded on Shimadzu FT-IR 8400-S spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker Avance 400 spectrometer operating at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-APT. The chemical shift (δ) is reported in ppm using chloroform-d as a solvent and calibrated standard solvent signal. Mass spectra were recorded on Shimadzu QP 2010 spectrometer. Elemental analysis was carried out on Perkin-Elmer 2400 C-H-N-S-O Analyzer Series-II.

1-[2(*H*)-1-benzopyran-3-yl]-3-aryl-prop-2-en-1-ones (coumarinoyl Chalcone) **3a-f**[25], 4-methyl-3-phenyl-6-coumarinoyl methyl pyridinium bromide salt **4**[26] and 7-methoxy-4-methyl-8-coumarinoyl methyl pyridinium bromide salt **5**[27] were prepared according to literature procedure.

***General procedure for the synthesis of 4-aryl*-2-(coumarin-3-yl)-6-(4-methyl-3-phenyl coumarin-6-yl)pyridines** ***compounds 6a-i*:**

A solution of 4-methyl-3-phenyl-6-coumarinoyl methyl pyridinium salt **(4)** (0.003 mol) in glacial acetic acid (15 mL) was charged in a round bottom flask. Ammonium acetate (0.03 mol) and an appropriate 1-[2(*H*)-1-benzopyran-3-yl]-3-aryl-prop-2-en-1-ones (coumarinoyl Chalcone) **3a-f** (0.003 mol) in glacial acetic acid (15 mL) were add to the solution in the flask with stirring . The reaction mixture was further stirred for 30 minutes and then refluxed for 8 hours. It was allowed to cool at room temperature. The reaction mixture was poured into ice-cold water and extracted with chloroform (3x 30 ml). The organic layer was then washed with water and then dried over anhydrous sodium sulfate. The removal of chloroform under reduced pressure gave gummy material which was subjected to column chromatography using silica gel and chloroform- petroleum ether (60-80) (9:1) as an eluent to give compounds **6a-i**. The compounds were recrystallized from chloroform-hexane.

4-aryl-2-(coumarin-3-yl)-6-(4-methyl-3-phenyl coumarin-6-yl) pyridine (6a): White Solid, Yield 65%,; mp 282˚C; Anal. Calcd. for $C_{36}H_{23}NO_4$: C, 81.10; H, 4.38; N, 2.59%. Found: C, 81.04; H, 4.34; N, 2.63%; IR (KBr, ν_{max} cm^{-1}): 3054, 2924, 1716, 1611 and 1457; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.47 (3H, s, CH_3), 7.28-7.93 (16H, m, Ar-H), 8.35 (1H, dd, *J = 8.8 Hz and 1.6 Hz*, C''$_7$-H), 8.42 (1H, poorly resolved doublet, C''$_5$-H), 8.68 (1H, poorly resolved doublet, C_3-H), 8.95 (1H, s, C'$_4$-H); ^{13}C APT (100 MHz, $CDCl_3$): δ 16.8 (CH_3), 116.5(CH), 117.3(CH), 118.2(CH), 119.5(C), 120.8(C), 123.7(CH), 124.7(CH), 125.3(C), 127.2(CH), 127.3(CH), 127.8(C), 128.4(CH), 128.5(CH), 128.9(CH), 129.2(CH), 129.3(CH), 130.1(CH), 130.2(CH), 132.3(CH), 134.4(C), 135.7(C), 138.4(C), 142.8(CH), 147.7(C), 150.5(C), 151.7(C), 153.4(C), 154.0(C), 156.0(C), 160.3(CO) and 160.7 (CO).

***General procedure for the synthesis of 4-aryl-2-(coumarin-3-yl)-6-(7-methoxy-4-methyl coumarin-8-yl)pyridines 7a-i*:**

A solution of 4-methyl-7-methoxy-8-coumarinoyl methyl pyridinium salt **(5)** (0.003 mol) in glacial acetic acid (15 mL) was charged in a round bottom flask. Ammonium acetate (0.03 mol) and an appropriate 1-[2(*H*)-1-benzopyran-3-yl]-3-aryl-prop-2-en-1-ones (coumarinoyl Chalcone) **3a-f** (0.003 mol) in glacial acetic acid (15 mL) were add to the solution in the flask with stirring . The reaction mixture was further stirred for 30 minutes and then refluxed for 12 hours. It was allowed to cool at room temperature. The reaction mixture was poured into ice-cold water and extracted with chloroform (3x 30 ml). The organic layer was then washed with water and then dried over anhydrous sodium sulfate. The removal of chloroform under reduced pressure gave gummy material which was subjected to column chromatography using silica gel and chloroform-petroleum ether (80-20) as an eluent to give compounds **7a-i**. The compounds were recrystallized from chloroform.

4-aryl-2-(coumarin-3-yl)-6-(4-methyl-7-methoxycoumarin-8-yl) pyridine (7a):

White solid, Yield 60%; mp 280˚C; Anal. Calcd. for $C_{31}H_{21}NO_4$: C,78.90; H, 4.42; N, 3.01%. Found: C, 78.97; H, 4.49; N, 2.97%; IR (KBr, ν_{max} cm^{-1}): 3057, 2923, 1724, 1611 and 1457; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.69 (3H, s, CH_3), 3.90 (3H, s, OCH_3), 6.21 (1H, s, C_3''-H), 7.79-7.06 (12H, m, Ar-H), 8.59 (1H, d, *J*=2 Hz, C_3-H), 8.76 (1H, s, C'$_4$-H); ^{13}C-APT (100 MHz, $CDCl_3$): δ 19.61(CH_3), 56.47(OCH_3), 108.33(CH), 110.34(C), 111.63(CH), 112.23(CH), 114.31(C), 116.28(CH), 119.72(C), 121.36(CH), 124.09(CH), 124.50(CH), 126.22(CH), 127.46(CH), 129.04(CH), 129.19(CH), 131.98(CH), 138.30(C), 143.72(CH), 149.29(C), 150.81(C), 150.88(C), 151.48(C), 152.21(C), 153.99(C), 157.10(C), 159.18(C), 160.11(CO), 160.53(CO).

Biological Assay

All the synthesized compounds were screened for their antimicrobial activity against two Gram-positive bacteria *viz*. *Bacillus subtilis* (MTCC 441) and *Staphylococcus aureus* (MTCC 96), two Gram-negative bacteria *viz*. *Escherichia coli* (MTCC 443) and *Salmonella typhi* (MTCC 98) and two fungi *viz*. *Aspergillus niger* (MTCC 282) and *Candida albicans* (MTCC 227). All MTCC cultures were collected from Institute of Microbial Technology, Chandigarh and tested against above mentioned standard drugs. Mueller Hinton Broth was used as nutrient medium to grow and dilute the compound suspension for the test bacteria and Sabouraud Dextrose Broth was used for fungal nutrition. The size of the inoculum for the test strain was adjusted to 10^8 colony forming unit (CFU) per milliliter by comparing the turbidity. In the present study, ampicillin and norfloxacin were used as standard antibacterial drugs, whereas nystatin and griseofulvin were used as standard antifungal drugs. DMSO was used as a diluent to get the desired concentration of compounds to test upon standard bacterial strains. Each synthesized compound and standard drugs were diluted obtaining 2000 μgmL^{-1} concentration, as a stock solution. In primary screening 1000, 500 and 250 μgmL^{-1} concentrations of the synthesized drugs were taken. The active synthesized compounds found in this primary screening were further diluted to obtain 200, 125, 100, 62.5, 50, 25, 12.5 and 6.250 μgmL^{-1} concentrations for secondary screening to test in a second set of dilution against all microorganisms. The lowest concentration, which showed no visible growth (turbidity) after spot subculture was considered as MIC for each compound.

CONCLUSION

From present study, we summarized that employed synthetic strategy provide efficient route for the synthesis of asymmetrically substituted 4-aryl-2,6-di(coumarinyl) pyridines by Krohnke's protocol in good yield. Moreover the starting precursors were also easy to prepare from synthesis point of view. Antimicrobial study on target compounds concluded that the all the compounds exerted promising activity against gram positive bacteria and gram negative. The target compounds showed feeble activity against fungal pathogens. The compounds **6d**, **7b**, **7g** and **7h** were most proficient members of the series.

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