Research Article

Synthesis, Characterization and Biological Evaluations of some 2,6-bis(6-substituted-2H-chromen-2-one)-4-(2,5-disubstituted-1H-indol)pyridines and their Molecular docking studies

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Abstract

Objective: The aim of this study was to synthesize 2,6-bis (6-substituted-2H-chromen-2-one)-4-(2,5-disubstituted-1H-indol)pyridine derivatives as potent biological agents and their molecular docking studies. **Material and methods:** The structure of the newly synthesized compounds was confirmed by FT-IR, ^{1}HNMR and Mass spectroscopic methods and to evaluate the biological studies like antimicrobial and antioxidant activities. The mode of action of these active compounds was carried out by molecular docking studies. **Results and Conclusion:** Among all the synthesized compounds tested **3a** was found to be the most active with, *S. aureus*, **3b** with *S. aureus, E. coli*, **3e** with *E. coli, E. fecalis*, **3h** with *S. typhi* and **3d, 3h** with *A. nizer* at 100µg/ml and some of the compounds have shown promising antioxidant properties.

***Keywords*:** Indole, coumarin, pyridine, antimicrobial, antioxidant and molecular docking

Introduction

Antimicrobials are one of the most significant weapons in fighting bacterial infections. They have extremely benefited the health related quality of human life. Indole and its derivatives have been reported to possess a variety of physiological and pharmacological activities like antibacterial (Renukadevi et al., 1999), antifungal (Biradar et al., 2008; Biradar et al., 2009), antitumor (Doddappa et al., 2011; Biradar et al., 2004), antiviral (Biradar et al., 2016; Biradar et al., 2011), antioxidant (Biradar et al., 2016), and so forth. As part of interest in the synthesis of heterocyclic compounds that have been explored for developing pharmaceutically important molecules. Coumarins have played an important role in medicinal chemistry; coumarin derivatives containing pyridine heterocycle are known to exhibit anticoagulant, antibacterial and antifungal activities (Brahmbhatt et al., 2004; Brahmbhatt et al., 2010; Doddappa et al., 2011; Biradar et al., 2004). Pyridine scaffolds have been found in numerous naturally occurring compounds and are also frequently used in functional materials. Pyridine-diol derivatives are of particular interest as building blocks for the construction of dendritic nanostructures in supra-molecular chemistry, whereas N-protected pyridine-3,4-diols find applications as potent chelating agents in medicinal chemistry (Kenchappa et al., 2013a). Furthermore, halogenated heteroaromatic compounds are interesting synthetic intermediates for the development of novel pharmaceuticals (Kenchappa et al., 2013b). Coumarin nucleus has been the aim of many researchers as most of its derivatives were proved to be bioactive as antibacterial, antifungal, antioxidant, anticoagulant, anticancer, anthelmentic and antitumor (Doddappa et al., 2011; Biradar et al., 2004; Brahmbhatt et al., 2004). Much research has been focused on the inhibition of bacterial growth by naturally occurring coumarins (xanthoxin, herniarin, umbelliferone and scopoletin) and on the antifungal activity of umbelliferone, scopoletin and coumarin itself. Due to the remarkable importance of coumarins, indole and pyridine, in the present work we have developed a simple and convenient, one-pot, three-component synthesis of 2,6-bis (6-substituted-2H-chromen-2-one)-4-(2,5-disubstituted-1H-indol)pyridine derivatives **3(a–h)** by Chichibabin reaction and evaluated for antimicrobial and antioxidant activities.

In modern drug designing, molecular docking is routinely used for understanding drug-receptor interaction. This

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method has been frequently used to predict the binding affinity and orientation of small drug molecules at the active site of their protein targets and to rationalize the selectivity observed. A molecular docking study was performed with Tripos SYBYL X 2.2.1.Program.

Materials and methods

Chemistry

All chemicals used in this research were purchased from sigma Aldrich and SD Fine chemicals. Melting points were taken in open capillary tubes and are uncorrected. The purity of the compounds was checked by thin layer chromatography using Merck silica gel 60 F254 coated aluminum plates. IR spectra were recorded on Shimadzu-FTIR Infrared spectrometer in KBr pellets(100mg). ^{1}HNMR spectra were recorded on a Bruker (400MHz) $CDCL_3$ as an internal standard. Mass spectra were recorded on LCMS 2010A, SHIMADZU Mass Spectrophotometer.

Protocol for the synthesis of starting materials

Starting material 2,5-disubstituted-1H-indol-3-carboxaldehyde (1) were prepared by literature method (Biradar, 1982) and 3-acetyl-coumarin (2a) and 3-acetyl-6-bromocoumarin (2e) was prepared by the literature procedure (Knoevenagel, 1898).

General procedure for the synthesis of 2,6-bis (6-substituted-2H-chromen-2-one)-4-(2,5-disubstituted-1H-indol)pyridine derivatives 3(a–h)

One equivalent of 2,5-disubstituted-1H-indol-3-carboxaldehyde (1), two equivalents of 3-acetyl coumarin (2a) or 3-acetyl-6-bromocoumarin (2e) and 1.5 equivalent of ammonium acetate were dissolved in acetic acid followed by reported method (Kenchappa et al., 2013) and the reaction mixture was refluxed for about 8-10 hrs in an oil bath at the temperature of 110-115°C. After completion of the reaction, the reaction mixture was kept at room temperature overnight, and poured into ice cold water. The solid separated was filtered and washed with sodium bicarbonate solution and recrystallized using ethanol.

Spectral analysis of synthesized compounds

3, 3'-(4-(5-chloro-2-phenyl-1H-indol-3-yl)pyridine-2,6-diyl)bis(2H-chromen-2-one)(3a)

Light Orange Crystals, MP= 282-283 ºC, yield; 60%, MF=$C_{37}H_{21}ClN_2O_4$

IR (KBr, cm^{-1}); 3424(NH), 3002(Ar-H), 1654(C=O), 1437 (C=N), 1315(-O-) and 708(C-Cl). ^{1}H NMR (400 MHz, Bruker δ ppm) ($CDCL_3$); 7.28 - 9.24 (m, 21H, Ar-H) and 10.09 (s, 1H, NH). Mass; LCMS (m/z) =59(M^+)(100%), 593(M^++2)(33%).

3, 3'-(4-(5-methyl-2-phenyl-1H-indol-3-yl)pyridine-2,6-diyl)bis(2H-chromen-2-one)(3b)

Light Brown Crystals, MP= 248-249 ºC, yield; 65%, MF=$C_{38}H_{24}N_2O_4$

IR (KBr, cm^{-1}); 3401 (NH), 3002(Ar-H), 2916-2870(CH_3), 1654(C=O), 1437 (C=N) and 1316(-O-). ^{1}H NMR (400 MHz, Bruker δ ppm) ($CDCL_3$); 2.52 (s, 3H, CH_3), 7.17 - 9.24 (m, 21H, Ar-H) and 10.09 (s, 1H, NH). Mass; LCMS (m/z) =572 (M^+).

3, 3'-(4-(2-phenyl-1H-indol-3-yl)pyridine-2,6-diyl)bis(2H-chromen-2-one)(3c)

Light Yellow Crystals, MP= 214-215 ºC, yield; 70%, MF=$C_{37}H_{22}N_2O_4$

IR (KBr, cm^{-1}); 3429 (NH), 3002(Ar-H), 1654(C=O), 1437 (C=N) and 1315(-O-). ^{1}H NMR (400 MHz, Bruker δ ppm) ($CDCL_3$); 7.01 - 8.94 (m, 21H, Ar-H) and 10.25 (s, 1H, NH). Mass; LCMS (m/z) =558 (M^+).

3, 3'-(4-(1H-indol-3-yl)pyridine-2,6-diyl)bis(2H-chromen-2-one)(3d)

Light Yellow Crystals, MP= 120-121ºC, yield; 68%, MF=$C_{31}H_{18}N_2O_4$

IR (KBr, cm^{-1}); 3429 (NH), 3002(Ar-H), 1654(C=O), 1437 (C=N) and 1315(-O-). ^{1}H NMR (400 MHz, Bruker δ ppm) ($CDCL_3$); 7.22 - 9.06 (m, 17H, Ar-H) and 10.08 (s, 1H, NH). Mass; LCMS (m/z) =482 (M^+).

3, 3'-(4-(5-chloro-2-phenyl-1H-indol-3-yl)pyridine-2,6-diyl)bis(6-bromo-2H-chromen-2-one)(3e)

Yellow Needles, MP= 216-217 ºC, yield; 55%, MF=$C_{37}H_{19}Br_2ClN_2O_4$

IR (KBr, cm^{-1}); 3413 (NH), 3020(Ar-H), 1660(C=O), 1477 (C=N), 1320(-O-), 739(C-Br) and 704(C-Cl). ^{1}H NMR (400 MHz, Bruker δ ppm) ($CDCL_3$); 7.28 - 8.47 (m, 18H, Ar-H) and 10.09 (s, 1H, NH). Mass; LCMS (m/z) =749 (M^+), 751 (M^++2), 753 (M^++4).

3, 3'-(4-(5-methyl-2-phenyl-1H-indol-3-yl)pyridine-2,6-diyl)bis(6-bromo-2H-chromen-2-one)(3f)

Light Brown Crystals, MP= 212-213 ºC, yield; 75%, MF=$C_{38}H_{22}Br_2N_2O_4$

IR (KBr, cm^{-1}); 3450 (NH), 3100(Ar-H), 2910-2854(CH_3), 1644(C=O), 1470 (C=N), 1310(-O-) and 720(C-Br). ^{1}H NMR (400 MHz, Bruker δ ppm) ($CDCL_3$); 2.52 (s, 3H, CH_3), 7.28 - 8.43 (m, 18H, Ar-H) and 10.09 (s, 1H, NH). Mass; LCMS (m/z) =728 (M^+), 730 (M^++2) and 732 (M^++4).

3, 3'-(4-(2-phenyl-1H-indol-3-yl)pyridine-2,6-diyl)bis(6-bromo-2H-chromen-2-one)(3g)

Dark Gray Crystals, MP= 214-215 ºC, yield; 55%, MF=$C_{37}H_{20}Br_2N_2O_4$

IR (KBr, cm^{-1}); 3424 (NH), 3002(Ar-H), 1654(C=O), 1437 (C=N), 1315(-O-) and 712(C-Br). ^{1}H NMR (400 MHz, Bruker δ ppm) ($CDCL_3$); 6.09 - 8.24 (m, 19H, Ar-H) and 10.0 (s, 1H, NH). Mass; LCMS (m/z) =714 (M^+), 716 (M^++2) and 718 (M^++4).

3, 3'-(4-(1H-indol-3-yl)pyridine-2,6-diyl)bis(6-bromo-2H-chromen-2-one)(3h)

Dark Yellow Crystals, MP= 220-221 ºC, yield; 60%, MF=$C_{31}H_{16}Br_2N_2O_4$

IR (KBr, cm^{-1}); 3424 (NH), 3002(Ar-H), 1654(C=O), 1437 (C=N), 1315(-O-), 709(C-Br). ^{1}H NMR (400 MHz, Bruker δ ppm) ($CDCL_3$); 6.18 - 7.92 (m, 15H, Ar-H) and 10. 20 (s, 1H, NH). Mass; LCMS (m/z) =638 (M^+), 640 (M^++2) and 642 (M^++4).

Biological studies

Antimicrobial activity

The newly synthesized compounds **3(a-h)** were screened for *in-vitro* antibacterial activity against two gram-positive bacterium, *Enterococcus fecalis* (*E. fecalis*) and *Staphylococcus aureus* (*S. aureus*) and three Gram-negative bacteria *Escherichia coli* (*E. coli*), *Shegella* and *Salmonella typhi* (*S. typhi*) as reported earlier by our group (Godipurge et al., 2016).

For Antibacterial, preparation of nutrient broth, subculture, base layer medium, agar medium and peptone water was done as per the standard procedure. Each test compound (5mg) was dissolved in 5ml of dimethyl sulfoxide (1000µg/ml).Volumes of 0.05ml and 0.1ml of each compound was used for testing. The cups each of 9mm diameter were made by scooping out medium with a sterilized cork borer in a petri dish which was streaked with the organisms. The solutions of each test compound (0.05 and 0.1ml) were added separately in the cups and petri dishes were subsequently incubated. Gentamycin used as standard reference drugs (200µg/ml) and Dimethyl Sulphoxide as a control which did not reveal any inhibition. The inhibition zone that appeared after 24hrs, around the well in each plate was measured as a zone of inhibition in mm. The activity of each compound was compared with Gentamycin as standard. Experiments were carried out in triplicates and the standard deviation was calculated. The results are summarized in table 1.

Antifungal activity

The newly synthesized compounds **3(a-h)** were screened for *in-vitro* antifungal activity against four fungal strains, *Aspergillus oryzae* (*A. oryzae*), *Aspergillus nizer* (*A. nizer*), *Aspergillus terreus* (*A. terreus*) and *Aspergillus flavus* (*A. flavus*). For antifungal the test cultures were grown separately in Sabouards Dextrose broth (SDB) (Hi Media, India) at RT for 48hrs. After checking the purity, 100µL of test cultures were spread on Sabouards Dextrose Agar plates (SDA), using cork bore 6mm diameter wells were made on plates. The test compounds were dissolved in DMSO. Each well was filled with 100µL volume of test compound. The DMSO was used as negative control while *fluconazol* (1000µg/mL) as positive control. Inoculated plates were kept at RT for 48hr. Each plate was then observed for zone of inhibition. Zone of inhibition produced by each compound was measured in mm. The activity of each compound was compared with *fluconazol* as standard. The results are summarized in table 2.

Molecular docking studies

In order to understand binding mode and to identify the structural features of the active molecules from 2,6-bis(6-substituted-2H-chromen-2-one)-4-(2,5-disubstituted-1H-indol)pyridine derivatives, we performed molecular docking studies with the *S. aureus* Enoyl-ACP-reductase (SaFabI) (PDB code 4CVO). Bacterial fatty acid synthesis was found to be an attractive target in recent studies. Bacterial fatty acid synthesis process divided into forms Fatty acid synthesis-I(FAS-I) and Fatty acid synthesis-II(FAS-II). Various enzymes who take part in the synthesis of bacterial fatty acids was found an attractive drug target and more importantly in bacterial fatty acid synthesis-II was found a unique pathway present in bacterial cells. The bacterial enzyme enoyl-ACP-reductase (FabI) also known as ENR was found responsible for the reduction step of each elongation cycle. The complex X-ray crystal structures of crystal structure of the N-terminal colied-coil domain of human DNA excision repair protein ERCC-6 was retrieved from the RCSB protein data bank (http://www.rcsb.org/pdb) and used for the docking study.

In the previous studies of synthesis and biological evolution of novel coumarins derivatives

owning piperazine skeleton based derivatives has been reported for inhibition of Enoyl-ACP-reductase (SaFabI) which made a firm basis for selection of this enzyme as the potential target. The molecular docking study will act as key indicator to understand the basis of molecular inhibition Enoyl-ACP-reductase (SaFabI) activity. It also helps in correlation of *in-vitro* activity of 2,6-bis (6-substituted-2H-chromen-2-one)-4-(2,5-disubstituted-1H-indol) pyridine derivatives.

The computational (theoretical) predictions data from the molecular docking study were found to be replicating the results of experimental antimicrobial activity. All the newly synthesized compounds **3a, 3b, 3d, 3e, 3f, 3h** and **standards** used in *in-vitro* study were successfully docked into the active site of target enzyme SaFabI and it has observed that they have varying degrees of affinity to the active site residues.

The detail molecular interactions study in between active site amino acid residue and components of molecule was carried out to understand the thermodynamic stability of different 2,6-bis(6-substituted-2H-chromen-2-one)-4-(2,5-disubstituted-1H-indol) pyridine derivatives, which also provide insight information about binding modes observed within the active site cavity. The theoretical predication detail values of the molecular docking study are presented in table 3.

Antioxidant activities

Scavenging effect on stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

A rapid, simple and inexpensive method to measure antioxidant capacity of substances involves the use of the DPPH free radical. It is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors. Antioxidants tested on DPPH were also found extremely effective in cell systems. This simple test further provides information on the ability of a compound to donate electrons during antioxidant action. The radical scavenging mechanism is based on the transfer of acidic H-atom from the compound to DPPH radical to form DPPH-H.

The free radical scavenging activity of **3(a-h)** was carried out in the presence of the stable free DPPH radical following (Godipurge et al., 2016) reported earlier by our group, using butylatedhydroxy anisole (BHA) and Ascorbic acid(AA) as standards. The radical scavenging activity (RSA) for methanolic solutions of **3(a-h)** at concentrations 25, 50, 75 and 100µg/mL containing freshly prepared DPPH solution (0.004%w/v) was carried out and compared with those of standards BHA and AA. The absorbance of the solution was measured at 517nm with a spectrophotometer. The RSA were expressed as the inhibition percentage and were calculated using the formula.

$$\text{RSA}\ (\%) = \frac{A - B}{A} X\ 100$$

Where A is absorbance of the control and B is absorbance of the synthesized compounds.

Total antioxidant capacity

The total antioxidant capacity of the synthesized compounds was evaluated by the phosphomolybdenum method according to the procedure described by (Godipurge et al., 2016). The assay is based on the reduction of Mo(VI) to Mo(V) by synthesized compounds and subsequent formation of green phosphate/Mo(V) complex at acid pH. A 0.3ml of solution of **3(a-h)** was combined with 3mL of reagent solution (0.6M sulfuric acid, 28mL sodium phosphate and 4mL ammonium molybdate). The absorbance of the reaction mixture was measured at 695nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3mL) was used as the blank. The antioxidant activity is expressed as the number of gram equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic (25, 50, 75 and 100µg/ml) with methanol.

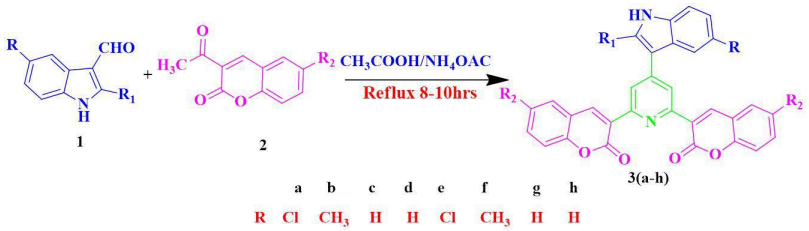
Ferric reducing antioxidant power activity

The total reducing power of the synthesized compounds was determined according to the method described (Godipurge et al., 2016). Volumes of 25-100µg/mL of different concentrations of **3(a-h)** solutions (in DMSO 1mL) were mixed with phosphate buffer solution 2.5mL (0.2M, pH = 6.6) and 2.5mL (1%) potassium ferriccyanide $[K_3[Fe(CN)_6]]$ in test tubes. The mixture was placed in a water bath at 50^{0}C for 20min. Then, 2.5mL of (10%) trichloroacetic acid was added to the mixture and mixed thoroughly. A volume of 2.5mL of this mixture was then added to 2.5mL of distilled water and 0.5mL $FeCl_3$ (0.1%) solution and allowed to stand for 10min. Then, the absorbance of this mixture was measured at 700nm using a UV-VIS spectrophotometer, the higher the absorbance of the reaction mixture, the greater the reducing power. (BHA) and (AA) was used as a positive control.

Results and discussion

Chemistry

We report here in, a simple and convenient one-pot, three-component synthesis of 2,6-bis (6-substituted-2H-chromen-2-one)-4-(2,5-disubstituted-1H-indol)pyridine derivatives **3(a–h)** by the reaction of 2,5-disubstituted-1H-indol-3-carboxaldehyde **1(a–d)** with 3-acetyl coumarin (**2a**) or 3-acetyl-6-bromocoumarin (**2e**) and ammonium acetate were dissolved in acetic acid and the reaction mixture was refluxed for about 8-10hrs in an oil bath at the temperature of 110 -115^{0}C. After completion of the reaction, the reaction mixture was kept at room temperature overnight, and poured into ice cold water. The solid separated was filtered and washed with sodium bicarbonate solution and recrystallized using ethanol to get pure compounds (Scheme).



	a	b	c	d	e	f	g	h
R	Cl	CH_3	H	H	Cl	CH_3	H	H
R_1	Ph	Ph	Ph	H	Ph	Ph	Ph	H
R_2	H	H	H	H	Br	Br	Br	Br

Scheme: Synthesis of various derivatives

The IR spectrum of **(3a)** exhibited characteristic absorption peaks at 3424 (NH of indole), 3002(Ar-H), 1654(C=O), 1437 (C=N), 1315(-O-) and 708(C-Cl). The ^{1}H-NMR spectrum of **(3a)** exhibited a multiplet between δ 7.28 - 9.24 (m, 21H, Ar-H) accounting for aromatic protons and the down field signal at δ 10.09 (s, 1H, NH) attributed to indole (NH). The mass peak for **(3a)** at (m/z) = 591(M^+)(100%), 593 (M^++2)(33%) supports the formation of **(3a**). The IR spectrum of **(3e)** showed absorption peaks at 3413 (NH of indole), 3020(Ar-H), 1660(C=O), 1477 (C=N), 1320(-O-), 739(C-Br) and 704(C-Cl). The ^{1}H-NMR spectrum of **(3e)** exhibited a multiplet between δ 7.28 - 8.47 (m, 18H, Ar-H) accounting for aromatic protons and down field signal at δ 10.09 (s, 1H, NH) attributed to indole (NH).The mass peak for **(4a)** at (m/z) =747 749 (M^+), 751 (M^++2) and 753 (M^++4) supports the formation of compound **(3e)**.

Biological studies

Antibacterial activity

The results of antibacterial activities revealed that the majority of the synthesized compounds showed varying degree of inhibition against tested microorganisms. Compared with the standard Gentamycin, the antibacterial potency of compounds **3a** was found to be the most active with, *S. aureus*, **3b** with *S. aureus, E. coli*, **3e** with *E. coli, E. fecalis* and **3h** with *S. typhi* at 100µg/ml (table 1). According to the Structure-Activity Relationship (SAR), it is clear that initially, compounds showed considerable activity due to the presence of electron withdrawing -Cl and -Br group. Among the synthesized compounds with -Cl substituents on phenyl ring of indole (Biradar et al, 2009; Biradar et al, 2011)(as reported) and -Br substituent's on coumarin is more active than the other analogues. The reason would be more lipophilic nature of indole and coumarin moiety along with the presence of electronegative groups like halogens.

Table 1: *In-vitro* Antibacterial activity of compounds 3(a-h)

Compounds	Conc. (µg/ml)	Zone of inhibition in mm(mean ± SD)				
		E.*coli*	E.*fecalis*	*Shegella*	S.*aureus*	S.*typhi*
3a	25	10±0.57	11±0.28	10±0.57	15±0.57	12±1
	50	14±0.57	15±0.28	14±0.57	18±0.28	15±1
	100	19±0.57	20±0.28	16±0.28	21±0.55	18±1
3b	25	12±0.28	10±0.28	10±0.5	10±0.57	12±0.28
	50	18±0.28	15±0.28	11±0.5	16±0.57	18±0.28
	100	**22±1**	19±0.28	16±0.28	**22±0.28**	20±0.5
3c	25	05±0.28	10±0.11	10±1	-	08±0.57
	50	10±0.28	15±0.1	11±0.57	08±0.5	11±1
	100	14±0.57	18±0.28	14±0.57	10±0.28	16±1
3d	25	07±0.5	10±0.25	09±0.57	08±0.57	10±0.57
	50	10±0.57	18±0.1	11±0.57	10±1	12±0.57
	100	14±0.28	20±0.25	16±0.28	10±0.57	14±0.57
3e	25	11±0.28	11±0.57	10±0.28	10±0.28	-
	50	18±0.28	15±1.1	15±0.5	16±0.28	08±0.5
	100	**21±0.5**	**21±0.5**	19±05	18±0.28	10±0.57
3f	25	-	11±0.26	10±0.28	-	10±1
	50	11±0.28	15±0.57	14±0.28	09±0.57	12±0.57
	100	16±0.5	20±0.52	19±0.28	10±0.57	16±0.57
3g	25	08±0.28	08±1	09±0.28	09±0.57	06±0.28
	50	11±0.28	11±0.28	18±0.5	15±0.28	11±0.28
	100	14±0.28	17±0.5	20±0.5	17±0.28	13±0.5
3h	25	11±0.5	10±0.57	11±0.57	11±0.57	18±0.57
	50	15±0.28	11±0.57	15±0.57	15±0.57	19±0.5
	100	18±0.28	16±0.57	16±0.28	18±0.57	**21±0.28**
Gentamycine	25	16±0.28	19±0.28	18±0.11	21±0.57	18±0.5
	50	18±0.46	20±0.57	20±0.11	22±0.28	20±0.28
	100	24±0.28	22±0.57	23±0.11	24±0.28	23±0.5

-Not shown any zone of inhibition, each value represents mean ± SD (n=3)

Antifungal activity

The antifungal screening revealed that some of the tested compounds showed good inhibition against various tested fungal strains (table 2). Initially, compound (**3d**) showed considerable activity with *A. nizer*. It is to be noted that the hydrogen substituent present on the phenyl ring of indole was found to have the strongest influence on the activity than the standard *fluconazol*. Compounds **(3h)** have also

Table 2: *In-vitro* Antifungal activity of compounds 3(a-h)

Compounds	Conc. (µg/ml)	Zone of inhibition in mm(mean ± SD)			
		A. *oryzae*	A. *terreus*	A. *nizer*	A. *flavus*
3a	25	6±0.57	5±0.76	8±0.28	8±0.57
	50	10±0.76	9±0.2	10±0.25	10±0.28
	100	14±1	13±0.25	14±0.25	15±0.28
3b	25	-	-	8±0.28	5±0.36
	50	10±0.5	11±0.28	11±0.5	11±0.41
	100	16±0.57	17±0.28	12±0.28	16±0.28
3c	25	6±1	8±1	10±0.25	10±0.5
	50	12±0.57	11±0.5	12±0.28	12±0.57
	100	16±1	12±0.25	14±0.28	16±0.11
3d	25	7±0.28	10±0.57	10±0.25	-
	50	10±0.5	15±0.5	11±0.36	9±0.11
	100	13±0.28	17±0.28	**17±0.25**	14±0.1
3e	25	6±0.76	6±0.57	8±0.2	-
	50	9±0.28	9±0.28	11±0.28	11±0.25
	100	10±0.5	14±0.28	13±0.28	14±0.28
3f	25	6±1	-	9±0.28	9±0.57
	50	10±0.5	6±0.36	11±0.25	11±0.28
	100	15±0.28	14±0.11	14±0.28	14±0.2
3g	25	8±0.11	4±0.2	8±0.25	8±0.25
	50	10±0.28	11±0.28	11±0.28	12±0.28
	100	13±0.57	14±0.28	13±0.25	14±0.2
3h	25	-	11±0.28	11±0.5	-
	50	8±0.11	11±0.28	11±0.2	8±0.57
	100	15±0.25	16±0.28	**17±0.25**	10±0.25
Fluconazole	25	16±0.28	15±0.11	15±0.57	15±0.28
	50	18±0.28	18±0.36	16±0.4	16±0.25
	100	20±0.36	20±0.25	19±0.28	18±0.25

-Not shown any zone of inhibition, each value represents mean ± SD (n=3)

exhibited good activity because of -Br functional group at *C-6* position on coumerin ring. From the studies, the analogues holding electron donating methyl group (Biradar et al, 2009; Biradar et al, 2011)(as reported) were not demanded for enhanced activity against all fungal strains. This might be the reason for decreases the activity in compounds **(3b)** and **(3f)** compared to other analogues. It is concluded antifungal investigation demonstrated that the halogen and hydrogen substituent's was the source for the significant increases in the activity.

To compare the binding affinity of the newly synthesized compounds, they were subjected for molecular docking using Tripos SYBYL X 2. 2.1 program. The newly synthesized compounds **3a, 3b, 3d, 3e, 3f, 3h** and **standards** were analyzed for their mechanism of antimicrobial action

To represent the details of docking score following terms is used as **total score** as total docking score, **crash score** as degree of in appropriate penetration by the ligand into the protein and of interpenetration between ligand atoms that are separated by rotatable bonds of compounds and **polar score** gives an idea about the contribution of the polar non-hydrogen bonding interactions to the total score is shown in (table 3). The value of total score (inhibition constant -log (ki)) provides an indication of how potent an inhibitor is. Higher the value for total score (inhibition constant -log (ki)), more is the potency of inhibitor.

Table 3. Molecular docking and Biological activity of compounds 3(a-h) and Standard

Compounds	Total Score (-logki)	Crash Score	Polar Score
3a	**7.1025**	**-0.874**	**3.5931**
3b	**8.2484**	**-1.0921**	**1.164**
3d	6.3945	-0.8021	3.019
3e	4.2911	-1.3231	0.0544
3f	5.1609	-1.0395	1.8306
3h	5.9964	-1.3994	4.1198
Gentamycine	**6.6555**	**-3.4744**	**2.625**

The total score which indication of how much the potent an inhibitor is very high for molecule **3b** which indicate that it is most active molecules among the six docked molecules. The compound binds and inhibit with a total docking score of 8.2484, crash score of -1.0921 which indicate degree of inappropriate penetration by the ligand into the protein and of interpenetration between ligand atoms that are separated by rotatable bonds and polar score of 1.164 contributions of the polar non-hydrogen bonding interactions to the total score.

The detail analysis of the binding interactions and binding pose of **3b** showed that it is stabilized within the active site of SaFabI through an extensive network of favorable non covalent interactions such as Pi interaction, Pi-Pi T shaped interaction, alkyl interaction, and Pi-alkyl interactions. The active site amino acid Tyr157 interacts with benzopyranone carbonyl oxygen atom to forms conventional hydrogen bond with distance of 2.05Å. Amino acid Ile193 interact non-covalently with a phenyl ring to form Pi-donor hydrogen bond of distance 2.57 Å. Pi-sulfur interactions of polar amino acid MET99 with benzopyranone ring Pi electrons of distance 5.99Å. The active site amino acid residues such as MET168, Ile2071, Pro192, Phe204, Ala198, Ala95, Val201, Leu102 and Arg103, form Pi-Pi T-shaped stacked, Pi-sigma and Pi-alkyl interactions with indole, pyridine, benzopyranone and phenyl ring with various distances shown in **figure 1a**.

Compound 3a also showed very good total docking score, crash score and polar score 7.1025, -0.874 and 3.5931 respectively give an idea about its high potency. Analysis of molecular/binding interactions and binding poses gives an information about how it occupy in the active site of target enzyme SaFabI and it from various kinds of non covalent interactions conventional hydrogen bond, Pi-donor hydrogen bond, Pi-sigma interaction, Pi-Pi T interaction,

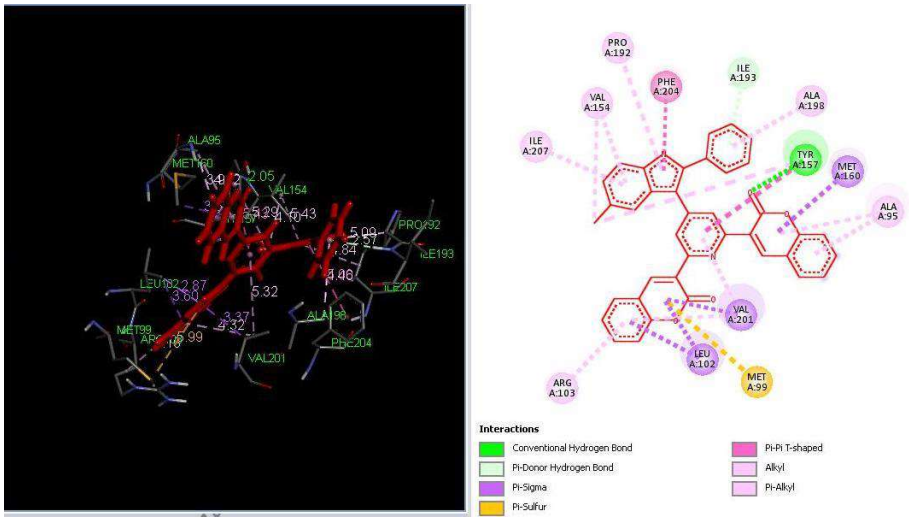


Figure1a: Binding Pose and molecular interactions of **3b** into the active site of SaFabI

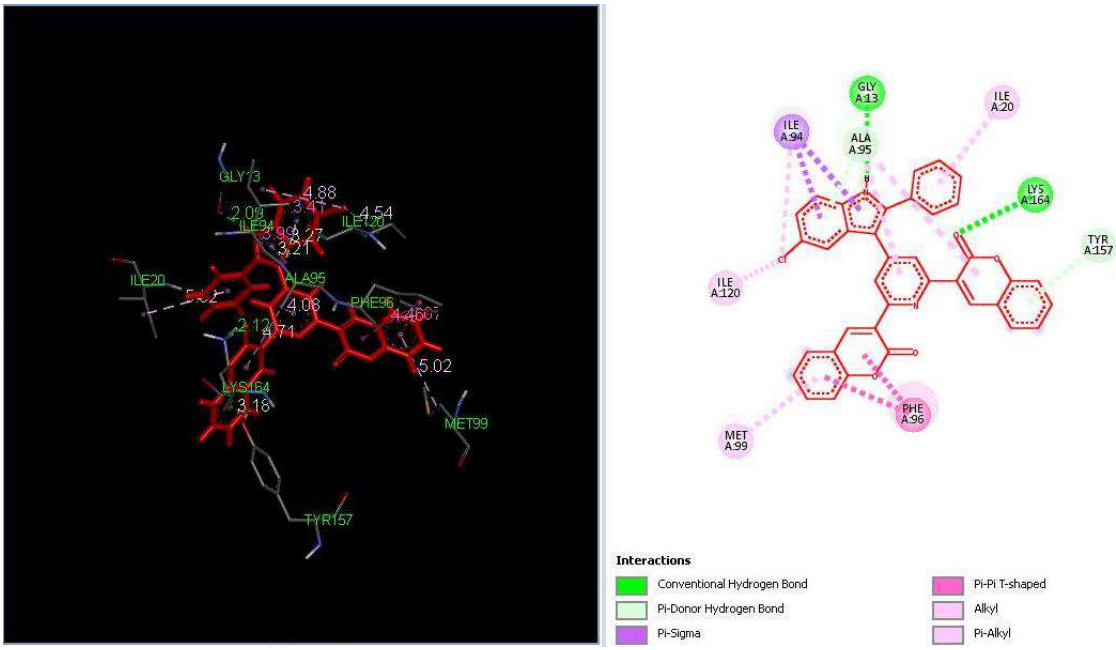


Figure 1b. Binding Pose and molecular interactions of **3a** into the active site of SaFabI

alkyl interaction, amide Pi stacked and Pi-alkyl interactions. Polar amino acids Lys164 interact with benzopyranone carbonyl oxygen atom to form a conventional hydrogen bond with distance of 2.12Å. Aliphatic acid amino acid Gly13 interacts with indole nitrogen atom to form a conventional hydrogen bond interaction of distance 2.09Å. Amino acid Ala95 interact with phenyl ring of indole interaction of distance 3.21Å while Tyr157 interact with phenyl ring of benzopyranone with distance of 3.18Å both forms forms Pi-donor interactions. Active site amino acids such as Ile120, Met99, Phe96, Ile94 and Ile20 forms Pi-Pi stacked, Pi-Pi T shaped, amide Pi-stacked and Pi-alkyl and alkyl of various distances shown in **figure 1b.**

Molecular docking study and analysis of **3a** and **3b** compounds revealed that presence of a similar binding mode and binding interactions as observed for **3a, 3b** and **standards** such as **Gentamycin** but among them **3b** is most active and others are moderately potent derivatives of pyridine. Observational analysis and theoretical predication of molecular docking study shown that various basic features of synthesized compounds and pharmacophore scaffold have potential to inhibit survival of both gram positive and negative, may act as potential leads in antimicrobial drug discovery.

Scavenging effect on stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The newly synthesized compounds were tested for DPPH free RSA with (BHA) and (AA) as standards. The investigation of DPPH radical scavenging activity revealed that compounds with -CH_3, -H and -Br substituent's (Biradar et al, 2009; Biradar et al, 2011)(as reported) like **3b, 3c** and **3h** have exhibited excellent RSA at 25, 75 and 100µg/ml and the results are given in **figure 2.**

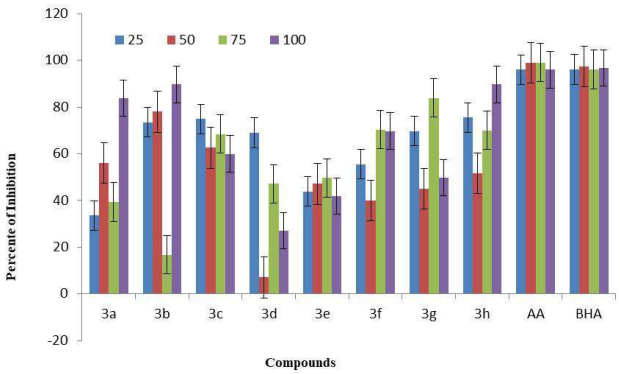


Figure 2. DPPH free radical scavenging activity

Total antioxidant capacity

The total antioxidant capacity of the synthesized compounds was evaluated by the phosphomolybdenum method. The antioxidant activity is expressed as the number of gram equivalent of ascorbic acid. The investigation of total antioxidant capacity of the synthesized compounds revealed that compounds with -Cl and -CH_3 substituents (Biradar et al, 2009; Biradar et al, 2011)(as reported) like **3a** and **3b** have exhibited excellent total antioxidant capacity activity at 25, 75 and 100µg/ml respectively. The absorbance of synthesized compounds as shown in **figure 3.**

Ferric reducing antioxidant power activity

The total reducing power of the synthesized compounds was determined according to the method (Biradar et al, 2009; Biradar et al, 2011). (BHA) and (AA) were used as a positive control. The ferric reducing antioxidant power activity is expressed as the reductive ability. The investigation of ferric reducing antioxidant power of the

synthesized compounds revealed that compounds with -Cl and -H (Biradar et al, 2011) (as reported) like **3a, 3c** and **3d** have displayed excellent ferric reducing antioxidant power at 25, 75 and 100µg/ml respectively. The absorbance of synthesized compounds as shown in **figure 4.**

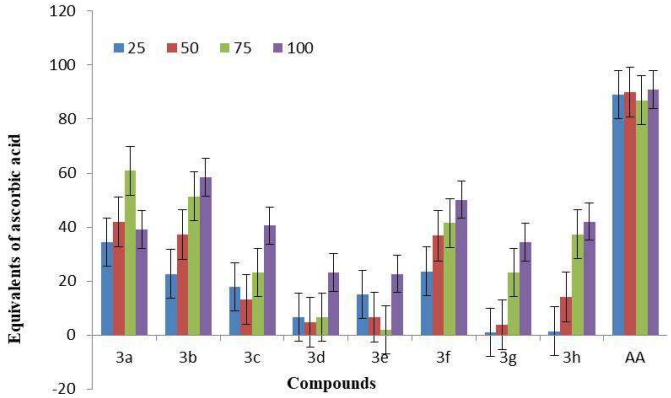


Figure 3. Total antioxidant capacity

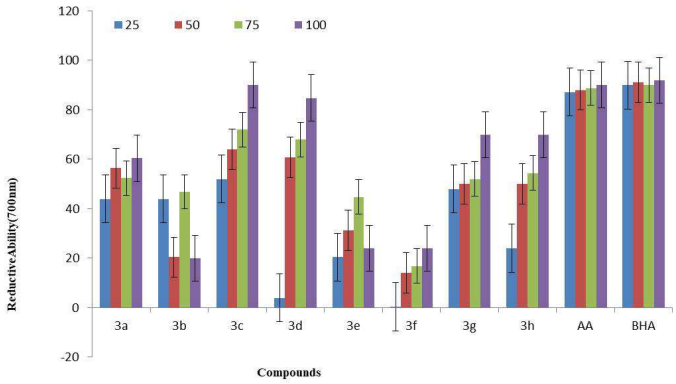


Figure 4. Ferric reducing antioxidant power activity

Conclusion

In conclusion, we have designed an efficient, convenient one-pot, three-component synthetic route for the synthesis of some novel biologically active 2,6-bis (6-substituted-2H-chromen-2-one)-4-(2,5-disubstituted-1H-indol)pyridine derivatives linked to substituted coumarins and indole substitutions like chloro, methyl and hydrogen. Compounds **3(a-h)** have exhibited promising antimicrobial and antioxidant activities. Results revealed that the compounds containing the halogen or electron withdrawing substituent's either on the coumarin or phenyl ring of indole exhibited potent antimicrobial and antioxidant activities. This observation suggests that di-substitution in the target compounds by halogens enhanced the antimicrobial and antioxidant activities.

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