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4-Methyl-7-alkynyl coumarin derivatives as potent antimicrobials and antioxidants

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An assortment of previously synthesized 4-methyl-7-alkynyl coumarins **4a-p** was screened for their antimicrobial and antioxidant properties. Some of the compounds exhibited promising antibacterial activities (MIC ranging from 5 to 150 μg/mL) and moderate antifungal activities when compared to the respective standards. The compound **4p** showed comparable antibacterial activity with the standard (ciprofloxacin), whereas the compounds **4b** and **4p** displayed better antifungal activity when compared to the other synthesized compounds. The *in silico* docking studies of the active compound were carried out against the gyrase enzyme and it was acknowledged that **4p** demonstrates ability for considerable hydrogen bonding and hydrophobic interactions which could be the possible reason for its superior activity as compared to the other compounds. The compounds **4f** and **4g** showed comparable antioxidant activity with the standard (butylated hydroxytoluene), which could be ascribed to the presence of electron-donating substituents.

Keywords: Coumarin; Antimicrobial; Antioxidant.

INTRODUCTION

The discovery of different types of microorganisms has explained the major reasons for a variety of infectious diseases responsible for the most complex health issues of this era. Different microorganisms like bacteria, fungi and viruses are identified to cause serious global health hazards [1]. Even if a lot of drugs have been identified as potent antimicrobial agents until now, the rise of multi drug resistance in microorganisms remains as a major global concern [2]. Therefore, the discovery of new drugs with good anti-microbial potency, particularly against the resistant strains, is highly essential for addressing this issue [3]. On the other hand, reactive free radicals and oxygen species present in the biological systems can abstract hydrogen atom from membrane, lipid, protein, DNA etc. and eventually lead to damages of several biological species thereby initiating numerous degenerative diseases [4]. The supply of antioxidants (free radical scavengers) is believed to be beneficial for liquidating this hazard as they possess the specific ability to trap the free radical species. Coumarins belong to an important class of benzopyrones found in green plants and display a broad spectrum of pharmacological activities [5]. The isolation and synthesis of various novel coumarins from natural sources and synthetic laboratories has gained considerable attention nowadays. Several coumarin derivatives are accounted to be potent antibacterial [6], anti-inflammatory [7] and antiviral agents [8] and the various therapeutic applications of coumarin compounds include photo chemotherapy, anti-tumor therapy and anti-HIV therapy [9]. Some of the marketed drugs that contain coumarin core include warfarin, acenocoumarol, carbochromen, etc., and antibiotics such as novobiocin, clorobiocin and coumermycin A1 [10]. Owing to these interesting biological properties, the exploration of natural or synthetic coumarin derivatives for their applicability as drugs has attracted medicinal chemists for decades. On the other hand, alkynes linked with heterocyclic compounds are reported to possess various biological potencies such as neuroprotective, antibacterial and antifungal activities [11,12]. These observations of coumarins and alkynes stimulated us to synthesize a variety of coumarins coupled with terminal alkynes and to examine their pharmacological potential. The synthetic methodology for the palladium-catalyzed copper, amine and ligand-free Sonogashira cross-coupling reaction of 4-methyl-7-nonafluorobutylsulfonyloxy coumarins with different terminal alkynes was previously reported by us [13].

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As a continuation of our ongoing research in the biological evaluation of the previously synthesized molecules [14,15], it has been planned to examine the antimicrobial and antioxidant properties of the formerly prepared 4-methyl-7-alkynyl coumarins. In this paper, we report our results of the evaluation of antimicrobial and antioxidant activity along with the *in silico* docking studies of the aforementioned compounds.

RESULTS AND DISCUSSION

Chemistry

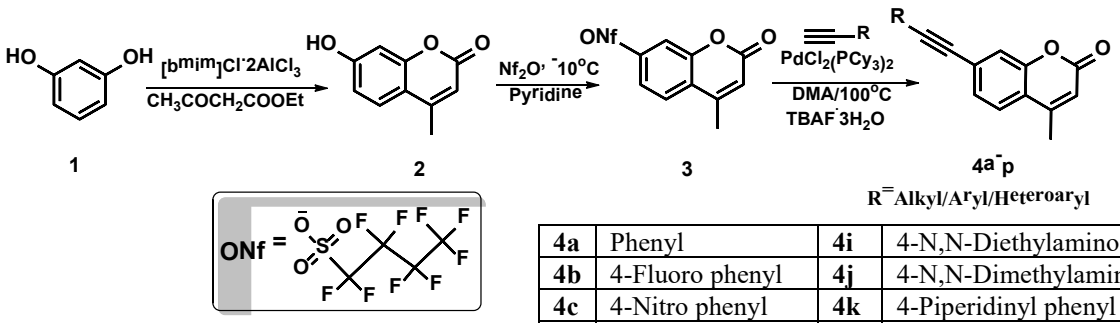
As mentioned earlier, the synthesis of various 4-methyl-7-alkynyl coumarins **4a-p** was achieved by the method previously reported by us [13]. The typical synthetic methodology started from the synthesis of the parent coumarin compound **2** by using the modified Pechmann cyclization reaction (Scheme 1) of resorcinol **1** with ethyl acetoacetate in 1-butyl-3-methylimidazolium chloroaluminate [16]. The obtained hydroxy coumarin **2** was then converted to the corresponding nonaflate **3** by treating it with nonafluorobutane sulfonic anhydride in the presence of pyridine as base at -10 °C. The intermediate **3** was then subjected to Sonogashira coupling with various terminal acetylenes by using $PdCl_2(PCy_3)_2$ as catalyst and $TBAF{\cdot}3H_2O$ as base in DMA at 100 °C under microwave irradiation to pursue a series of 4-methyl-7-alkynyl coumarins **4a-p** of significant pharmacological relevance.

Biology: Antimicrobial activity

The analysis of antibacterial (Table 1) and antifungal activities (Table 3) of the compounds **4a-p** was carried out against two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633), two Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) and three fungi (*Aspergillus flavus* ATCC 9643*, Chrysosporium keratinophilum* ATCC90272 and *Candida albicans* MTCC 227). As apparent from Table 1, some of the compounds exhibited promising antibacterial activity as compared to the standard drug ciprofloxacin. The compounds **4b**, **4c**, **4d**, **4k**, **4m** and **4p** showed good and comparable activity with the standard whilst the compounds **4i**, **4j**, **4n** and **4o** failed to show any activity towards the tested strains. All the other compounds showed moderate to poor antibacterial activity.

The minimum inhibitory concentration (MIC) of the more active compounds was determined by the broth dilution method using nutrient broth (Table 2). The compound **4p** was found to be highly active against all the bacterial strains. The compounds **4k** and **4m** were found to possess superior activity when compared to the compounds **4b**, **4c** and **4d**.

The antifungal activity of the synthesized compounds was studied by taking fluconazole as the standard. Unfortunately, only a few compounds inhibited the growth of most of the tested fungi (Table 3). The compounds **4b** and **4p** showed good activity when compared to the other remaining compounds. Alternatively, all the other compounds failed to show good and comparable activity to that of the standard.



4a	Phenyl	**4i**	4-N,N-Diethylamino phenyl
4b	4-Fluoro phenyl	**4j**	4-N,N-Dimethylamino phenyl
4c	4-Nitro phenyl	**4k**	4-Piperidinyl phenyl
4d	4-Cyano phenyl	**4l**	4,4-Biphenyl
4e	4-Tolyl	**4m**	4,3-Pyridinyl phenyl
4f	4-Hydroxy phenyl	**4n**	2-Hydroxy ethyl
4g	4-Amino phenyl	**4o**	Hydroxy methyl
4h	4-Methoxy phenyl	**4p**	3,5-Difluoro phenyl

Scheme 1. Synthesis of 4-methyl-7-nonafluorobutylsulfonyloxy coumarin intermediate and its Sonogashira coupling with various terminal acetylenes

Table 1. Determination of antibacterial activity of the synthesized organic compounds

Compounds	*Escherichia coli*		*Staphylococcus aureus*		*Pseudomonas aeruginosa*		*Bacillus subtilis*	
Conc. in mg/mL	1	0.5	1	0.5	1	0.5	1	0.5
Control	00		00		00		00	
Ciprofloxacin	22±0.1	17±0.1	21±0.1	18±0.1	21±0.1	18±0.1	23.8±0.1	14.2±0.1
4a	07±0.1	05±0.1	08±0.1	06±0.1	05±0.1	03±0.1	10±0.1	08±0.1
4b	14±0.1	11±0.1	13±0.1	09±0.1	14±0.1	10±0.1	13±0.1	09±0.1
4c	13±0.1	10±0.1	13±0.1	09±0.1	15±0.1	10±0.1	14±0.1	08±0.1
4d	13±0.2	10±0.2	12±0.1	07±0.1	13±0.2	08±0.2	12±0.2	08±0.2
4e	05±0.2	03±0.2	04±0.2	02±0.2	07±0.2	04±0.2	06±0.2	03±0.2
4f	06±0.1	02±0.1	08±0.1	05±0.1	08±0.1	04±0.1	07±0.1	03±0.1
4g	08±0.2	04±0.2	09±0.2	05±0.2	07±0.2	03±0.2	07±0.2	04±0.2
4h	07±0.1	03±0.1	08±0.1	04±0.1	06±0.1	03±0.1	07±0.1	04±0.1
4i	00	00	00	00	00	00	00	00
4j	00	00	00	00	00	00	00	00
4k	15±0.1	11±0.1	12±0.1	09±0.1	14±0.1	10±0.1	13±0.1	08±0.1
4l	06±0.1	03±0.1	08±0.1	05±0.1	07±0.1	04±0.1	06±0.1	03±0.1
4m	13±0.2	11±0.2	11±0.2	07±0.2	14±0.2	10±0.2	14±0.2	09±0.2
4n	00	00	00	00	00	00	00	00
4o	00	00	00	00	00	00	00	00
4p	18±0.1	14±0.1	16±0.1	13±0.1	18±0.1	14±0.1	18±0.1	11±0.1

[a] The experiment was performed in triplicate and the values are expressed as mean ±SD

Table 2. Minimum inhibitory concentration of active compounds[a]

Compounds in μg/mL	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Bacillus subtilis*
4b	50	100	100	50
4c	50	50	100	50
4d	50	100	100	50
4k	10	10	10	25
4m	10	10	10	10
4p	5	10	5	5
Ciprofloxacin	0.6	0.2	0.5	0.4

[a] The experiment was performed in triplicate and the values are expressed as mean ±SD

Table 3. Determination of antifungal activity of the synthesized compounds[a]

Compounds	*Aspergillus flavus*		*Chrysosporium keratinophilum*		*Candida albicans*	
Concn. in mg/mL	1	0.5	1	0.5	1	0.5
Control	00		00		00	
Fluconazole	13±0.1	10±0.1	17±0.1	15±0.1	22±0.1	20±0.1
4a	05±0.2	03±0.2	04±0.2	02±0.2	05±0.2	03±0.2
4b	09±0.1	06±0.1	10±0.1	07±0.1	11±0.1	08±0.1
4c	00	00	00	00	00	00
4d	00	00	00	00	00	00
4e	00	00	00	00	00	00
4f	06±0.2	03±0.2	05±0.1	02±0.1	05±0.1	02±0.1
4g	04±0.1	03±0.1	05±0.1	03±0.1	06±0.2	03±0.1
4h	00	00	00	00	00	00
4i	03±0.1	00	02±0.1	00	03±0.1	01±0.1
4j	00	00	00	00	00	00
4k	02±0.1	00	03±0.1	00	02±0.1	00
4l	05±0.1	03±0.1	04±0.2	02±0.1	06±0.2	04±0.1
4m	05±0.2	02±0.1	03±0.2	01±0.1	04±0.2	02±0.1
4n	06±0.1	02±0.1	05±0.1	02±0.1	04±0.1	01±0.1
4o	00	00	00	00	00	00
4p	10±0.3	07±0.3	08±0.3	06±0.3	12±0.2	07±0.2

[a] The experiment was performed in triplicate and the values are expressed as mean ±SD

Biology: Antimicrobial activity. Structure-activity relationships

The presence of electron withdrawing fluoro groups in **4p** and **4b** is presumed to be the reason for the comparable antimicrobial activity of that compound. The presence of heterocyclic ring having a nitrogen atom was assumed to be beneficial for the superior activity of compounds **4k** and **4m**. The existence of electron withdrawing groups is expected to increase the lipophilicity and thereby enhance the cell permeability of the molecule [17]. In general, it can be summarized that in the present study, the presence of an electron withdrawing group and a heterocyclic group with nitrogen atom in 4-methyl-7-alkynyl coumarins is an essential feature for the antimicrobial potency of the synthesized compounds.

Biology: Antioxidant activity

The DPPH procedure is one of the most common methods for analyzing the concentration of radical scavenging materials as it does not have to be generated prior to analysis [18]. DPPH radical scavenging activity evaluation is a rapid and convenient assay for screening the antioxidant activities of newly synthesized compounds. These observations prompted us to evaluate the radical

scavenging activity (Fig. 1) of the synthesized 4-methyl-7-alkynyl coumarin derivatives as they possess an extended p-conjugated system [19]. The synthesized compounds **4a-p** were evaluated for antioxidant activities by taking butylated hydroxytoluene (BHT) as the standard (Fig. 1). In this assay, the standard BHT showed a strong scavenging activity whereas the compounds **4f** (74.2 %), **4g** (70.8 %), **4h** (61.8 %), **4e** (61.8 %) and **4o** (63.3 %) showed comparable activity (Fig. 1). All the other compounds also exhibited substantial scavenging activity, but demanded higher concentrations of the compounds.

Biology: Antioxidant activity: Structure-activity relationships

The results of antioxidant screening revealed that the presence of electron-donating ring systems attached to the phenyl ring of 4-methyl-7-alkynyl coumarins is an essential characteristic for their radical scavenging activity. The hydrophilic electron-donating groups are expected to assist the stabilization of the oxygen-centered radical and reduce the O–H bond dissociation enthalpy (BDE) thus increasing the radical scavenging activity by hydrogen abstraction [20]. This could be the probable reason for the better activity of compounds **4f**, **4g**, **4h**, **4e** and **4o** to that of the other synthesized molecules.

Biology: Molecular docking studies

Encouraged by the comparable antibacterial activity of some of the synthesized compounds as per the *in vivo* results, it was thought worthy to substantiate those results by performing molecular docking studies or *in silico* studies. The molecular docking study of the most active compound **4p** was carried out against gyrase as it is an essential enzyme in all bacteria but is absent in higher eukaryotes and hence makes it a beautiful antibacterial target [21,22].

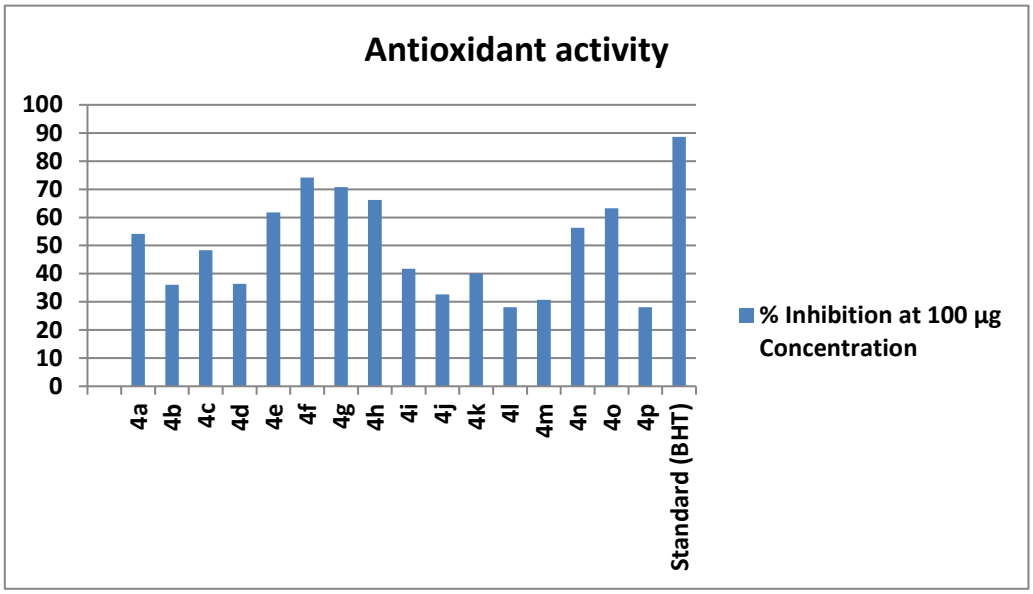


Figure 1. Antioxidant activity of 4-methyl-7-alkynyl coumarins.

Moreover, the mode of antibacterial action of ciprofloxacin is by significantly inhibiting the gyrase enzyme. The comparative docking of receptor gyrase with **4p** and ciprofloxacin exhibited good affinity. The 2D representation of **4p** and the standard ciprofloxacin is depicted in Fig. 2. The compound **4p** demonstrated ability for hydrogen bonding with three amino acids in the receptor active pocket and hydrophobic interactions with four amino acid residues (Fig. 2A). The standard ciprofloxacin (Fig. 2B) represents the hydrophobic contacts with five different residues, later a total of two H-bonds were formed with various amino acids. In all the cases of the 2D representation, ligands are highlighted in blue colour. The set of conserved residues that are commonly involved in interaction with the ligands and ciprofloxacin are encircled with red colour. Furthermore, the extrapolation of binding conformation of **4p** and ciprofloxacin was carried out by 3D protein-ligand interaction analysis. Fig. 2 (C and D) represents the 3D interaction of **4p** and ciprofloxacin, respectively, with gyrase by using the educational version of PyMol. The ligands are represented in green colour, H-bonds with their respective distances are represented in yellow colour and the interacting residues are presented with ball and stick model representation. In the present study, **4p** was identified to be the best antibacterial agent

among all the synthesized compounds which could be attributed to the electron-withdrawing character of fluorine atoms, as well as to the ability of the molecule for considerable hydrogen bonding and hydrophobic interactions.

Experimental: Antibacterial activity

The antibacterial potency of compounds **4a-p** was determined by the well plate method in nutrient agar medium [23]. The compounds, along with the standard ciprofloxacin, were tested against a panel of pathogenic microorganisms like *E. coli*, *S. aureus, B. subtilis* and *P. aeruginosa*. The strains of microorganisms were maintained on nutrient agar medium at 37 °C and the cultures were inoculated in fresh 10-mL nutrient broth to get an initial suspension of approximately 10-100 cfu/mL. All the broths were then statically incubated at the aforementioned temperatures for 18-24 h so that all cells arrive at a stationary phase. Susceptibility of the organisms to the compounds was determined by employing the well plate technique. The bacterial suspensions were diluted tenfold in sterilized distilled water and 0.1 mL from the appropriate dilution was spread plated on nutrient agar in order to give a population of about 10^6 cfu/plate. A 6 mm diameter well was then carefully punched using a sterile cork borer and 30 µL of test solutions of different concentrations were added into each labeled well. The same procedure was replicated for different microorganisms and each experiment was carried out in triplicate. After the incubation, the inhibition zone was measured and the values for control were deduced to get the actual values.

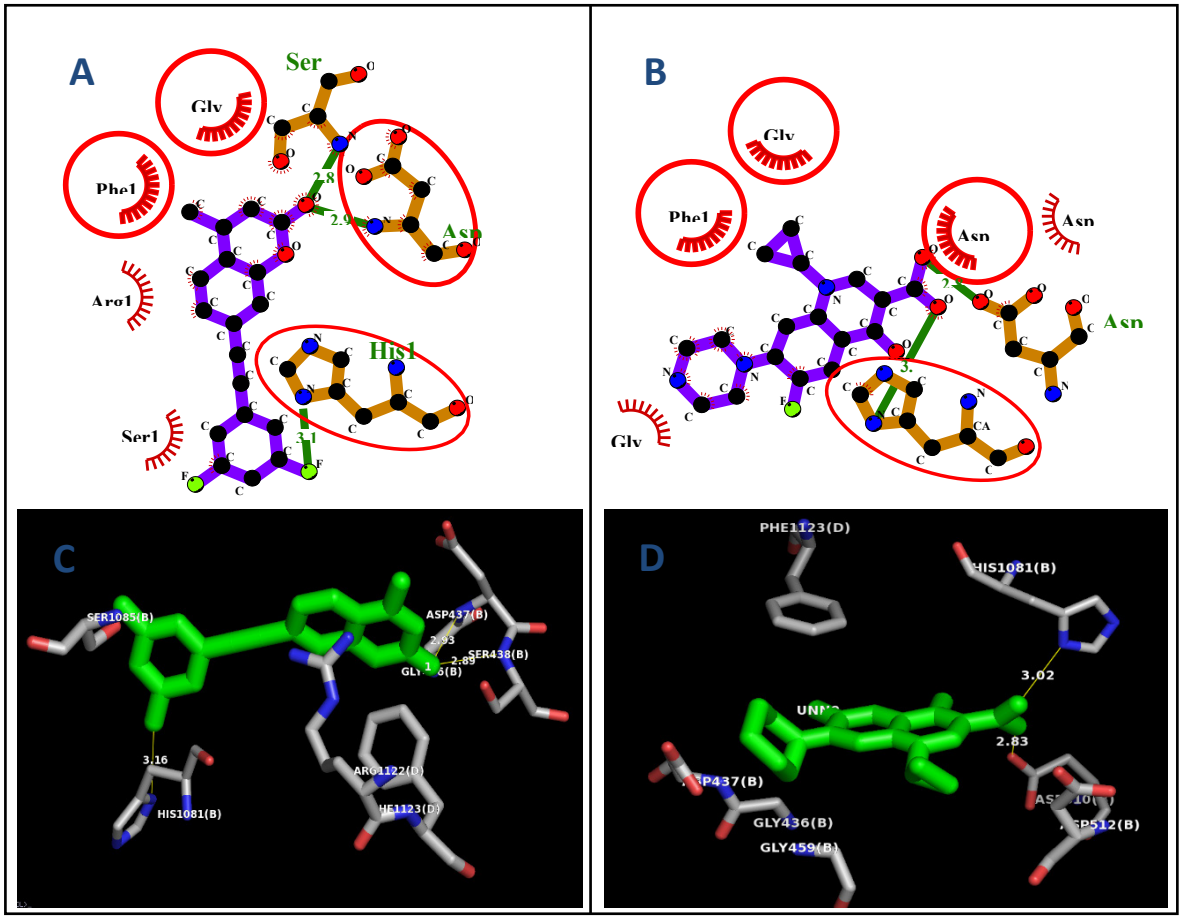


Figure 2. 2D and 3D representation of the interaction of **4p** and ciprofloxacin with 2XCT (gyrase)

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of the active compounds was determined by the broth dilution method using nutrient broth. The MIC value, representing the lowest concentration that completely inhibited the formation of visible growth, was estimated after 18 h of incubation at 37 °C.

Antifungal activity

Antifungal studies of the synthesized compounds **4a-p** were performed by the well plate method against *A. flavus*, *C. keratinophilum* and *C. albicans*.

Normal saline was taken to make a suspension of spores of fungal strains for lawning [24] and a loopful of a particular fungal strain was transferred to 3 mL of saline to get a suspension of the corresponding species. 20 mL of agar medium was poured into each Petri dish, excess of suspension was decanted and plates were dried in an incubator at 37 °C for 1 h. The borer was punched carefully using sterile cork. The wells were made on these seeded agar plates and various concentrations of the test compounds in DMSO were added to each labeled well. A control in DMSO was also prepared for the plates in the same way. The Petri dishes were prepared in triplicate and maintained at 25 °C for 72 h and antifungal activity was evaluated by measuring the diameter of the inhibition zone. The activity of each compound was compared along with fluconazole as the standard.

Antioxidant activity

The usual colorimetric DPPH• scavenging capacity assay was performed according to a previously described laboratory protocol [25]. 100 µL (100 µg concentration) samples of the compounds in methanol were added to 3 mL of 0.004 % w/v DPPH• solution and each test tube was made up to a final volume of 4 mL. BHT was used as a reference standard and was dissolved in methanol to get the same concentration as that of the remaining extracts. Each mixture was vortexed for some time and left to stand in the dark for 10 min at ambient temperature. The absorbance of each reaction mixture at 517 nm was measured against a blank of methanol by using a UV-visible spectrometer (Shimadzu UV-1800). The level of DPPH• remaining in each reaction was calculated as:

$$\% \text{ Scavenging activity} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100$$

The inhibition curve was plotted for triplicate experiments and represented as percentage of mean inhibition ± standard deviation.

In silico studies

An entirely in-house extended drug discovery informatics system OSIRIS was used to perform ADMET based calculations. It is a Java based library layer that provides reusable cheminformatics functionality and was used to calculate the toxicity risks and overall drug score *in silico* [26]. The structures of the synthesized molecules and of the standards were drawn in ChemBioDraw tool (ChemBioOffice Ultra 14.0 suite) assigned with proper 2D orientation and the structure of each was checked for drawing error. Energy of each molecule was reduced using ChemBio3D (ChemBioOffice Ultra 14.0 suite). The energy-minimized ligand molecules were then utilized as input for AutoDockVina, to carry out the docking simulation [27]. The protein databank (PDB) coordinate file entitled '2XCT.pdb' was employed as receptor (protein) molecule which is a structure of *S. aureus* gyrase in complex with ciprofloxacin and DNA [28]. All the water molecules were removed from the receptor and SPDBV DeepView was used to automatically rebuild the missing side chains in the receptor. The Graphical User Interface program 'MGL Tools' was used to set the grid box for docking simulations and the grid was set in such a way that it surrounded the region of interest (active site) in the macromolecule. In the present study, the active site was chosen based on the amino acid residues of 2XCT, which are involved in binding with ciprofloxacin. Therefore, the grid was centered at the region including the two amino acid residues (Arg 458 and Gly 459) and the four nitrogenous bases from DNA. that is guanine (G), adenine (A), thymine (T) or cytosine (C), as evidenced by Bax *et al*. in 2010 [29]. This surrounded the active site. The grid box volume was set to 8, 14 and 14 Å for x, y and z dimensions, respectively, and the grid center was set to 3.194, 43.143 and 69.977 for x, y and z center respectively, that covered the two amino acid residues and four nitrogenous bases in the considered active pocket. The AutoGrid 4.0 Program was used to produce grid maps. The docking algorithm provided with AutoDockVina was used to search for the best docked conformation between the ligand and the protein. During the docking procedure, a maximum of 10 conformers was considered for each ligand. All the AutoDock docking runs were carried out in Core i7 Intel processor CPU with 8 GB DDR3l RAM. AutoDockVina was compiled and run under Windows 8.0 professional operating system and LigPlot+ [30] and PyMol [31] were used to deduce the pictorial representation of the interaction between ligands and target protein.

CONCLUSION

In summary, we have evaluated the antimicrobial and antioxidant activity of an array of previously synthesized 4-methyl-7-alkynyl coumarins by the

well plate method. The compound **4p** exhibited comparable antibacterial activity with ciprofloxacin against all the tested bacteria. The *in silico* docking studies of the more active antibacterial agents were carried out against the gyrase enzyme and revealed that **4p** demonstrates ability for significant hydrogen bonding and hydrophobic interactions, which could be a plausible reason for its improved potency along with the presence of electron-withdrawing fluoro groups. The antifungal activity of the compounds was found to be moderate. Nevertheless, the compounds **4b** and **4p** showed better antifungal activity when compared to the other remaining compounds. The compounds **4f** and **4g** showed comparable antioxidant activity with the standard BHT, presumably due to the presence of electron-donating substituents. The present study paved the way for understanding of the biological profile of 4-methyl-7-alkynyl coumarin analogues and further derivatization and lead optimization are currently in progress in our laboratory.

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