DESIGN, SYNTHESIS AND DOCKING STUDIES OF NOVEL SPIROAZETIDINONE SUBSTITUTED STEROIDAL DERIVATIVES POSSESSING POTENT DIVERSIFIED PHARMACOLOGICAL PROPERTIES

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Steroidal spiroazetidinone derivatives (**3, 10-12**) were obtained by the multi-step reactions of ketosteroids. It involved Staudinger ketene-imine [2+2] cycloaddition reaction of steroidal iminophenylcholest-5-enes (**2, 7-9**). The structural assignment of the products was confirmed on the basis of IR, ^{1}H NMR, ^{13}C NMR, MS and analytical data. The synthesized compounds were screened for *in vitro* antimicrobial activity against different bacterial and fungal strains by agar diffusion method and *in vitro* antioxidant activity by using DPPH method. Docking studies were performed to investigate the hypothetical binding mode of the steroidal spiroazetidinones. The results suggest that steroidal bearing a core spiroazetidinone scaffold would be potent phramacological agents..

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INTRODUCTION

β-Lactam or azetidin-2-one is the crucial structural skeletons of important and widely applied classes of antibiotics such as penicillin, cephalosporin, carbapenem.[1] Naturally occurring as well as synthetic monolactams, such as nocardicins and tabtoxin, are also known for their unique antibacterial activities.[2,3] In addition, this scaffold has found new pharmaceutical applications other than its use as antibiotics, such as LHRH antagonists, cholesterol absorption inhibitors and anticancer agents.[4-7] Some azetidin-2-one derivatives have also been recognized as inhibitors of human leukocytase elastase.[8] The powerful activity of penicillin and the clinically more useful cephalosporin led medicinal chemists to investigate the chemistry of the azetidinone ring system.[9] The activity of 2-azetidinone antibiotics has been traditionally correlated to the chemical reactivity of the carbonyl group toward nucleophilic attack because these drugs act as acylating agents of their target enzymes, namely the serine D,D-peptidases (called PBPs, for Penicillin Binding Proteins) involved in the final biosynthesis step of peptidoglycane, a main constituent of the bacterial cell wall.[10,11] Spiroazetidinone have received much attention because they are relatively unexplored compared with mono- and bicyclic *β*-lactams.[12] They are also found in biologically active natural compounds such as chartelline[13] and behave as *β*-turn mimetics which is one of the major molecular recognition events for receptor-ligand interactions.[14] Besides their importance as the key structural component of antibiotics, *β*-lactams have been attracting considerable interest in organic synthesis as versatile synthetic intermediates and *chiral* synthons.[15] Although most penicillin and cephalosporin-like compounds have been obtained by biosynthesis, by chemical modification of intermediates produced *via* biosynthesis or by chemical synthesis,[16,17] there is still need for the effective synthesis of optically pure, novel *β*-lactams, for bioassay of antibacterial activity because of the growing resistance of bacteria against antimicrobial drugs. Steroids are representative of a rich structural molecular diversity and ability to interact with various biological targets and pathways. Most of the steroid based pharmaceuticals are semi-synthetic compounds and prepared by connecting a special functionality to the core of a steroid skeleton.[18] In view of the aforementioned facts and in continuation of our programme on the synthesis of heterosteroids,[19] we report herein synthesis of steroidal derivatives associated with spiroazetidinone moiety to the cholesteryl skeleton. The newly synthesized steroidal spiroazetidinone (**3, 10-12**) have been characterized on the basis of IR, ^{1}H NMR, ^{13}C NMR, MS and analytical data as well as evaluated for their *in vitro* antimicrobial and antioxidant activities. Furthermore, docking studies have also been performed.

EXPERIMENTAL

Material and methods

Chemicals were purchased from Merck and Sigma-Aldrich as 'synthesis grade' and solvents were purified prior to use. Melting points are recorded in degrees Celsius on a Kofler apparatus. IR spectra (KBr disks) were recorded on Interspec 2020 FT-IR Spectrometer Spectro Lab. ^{1}H and ^{13}C NMR spectra were run in $CDCl_3$ on a JEOL Eclipse (400 MHz) instrument with TMS as internal standard and values are given in ppm (δ) and coupling constants are given in Hz.

Mass spectra were recorded on a JEOL SX 102/DA-6000 Mass Spectrometer. Elemental analyses for C, H and N were within ±0.4% of the theoretical values. The progress of all reactions was monitored by thin layer chromatography (TLC) plates with 0.5 mm layer of silica gel G, light petroleum ether refers to a fraction of b.p. 60-80 ˚C and exposed to iodine vapors to check the purity as well as the progress of reaction. Column chromatography was performed on silica gel. Sodium sulfate (anhydrous) was used as a drying agent.

General method for the synthesis of steroidal iminophenylcholest-5-enes (2, 7-9)

Cholest-5-ene-3-one (1 mmol) was fused with aniline (1 mmol) for 30 min and then 25 mL of absolute ethanol was added to the reaction mixture. The reaction mixture was refluxed for 6-8 h and then cooled to room temperature. The separated solid was filtered, washed with water and recrystallized from benzene to give iminophenylcholest-5-ene derivatives (**2, 7-9**).

General method for the synthesis of steroidal spiroazetidinone (3, 10-12)

Steroidal iminophenylcholest-5-ene (**2, 7-9**) was dissolved in 30 ml of dichloromethane and to this solution, chloroacetyl chloride and few drops of triethylamine were added. Then the reaction mixture was refluxed for 3-3.5 h. The progress as well as completion of reaction was monitored by TLC. After completion of the reaction, the solvent were removed under reduced pressure. The crude product obtained was purified over silica gel column (light petroleum ether-ether; 10:1). Recrystallization from methanol afforded products (**3, 10-12**).

3*β*-Cholest-5-en-3-spiro-1′-phenyl-3′-chloro-azetidin-2′-one (3)

Yellow powder (75 %). M.p: 174-176 ˚C; IR (KBr, cm^{-1}): 3080, 1583, 1395 (C-H, aromatic), 1763 (C=O), 1618 (C=C), 1375 (C-N), 742 (C-Cl); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52-7.82 (m, 5H, aromatic), 5.81 (dd, 1H, C_6-H, J=5.6, 8.4Hz), 4.61 (s, 1H, C′$_3$-H), 2.20 (s, 2H, C_4- H_2), 1.22 (s, 3H, C_{10}-CH_3), 0.74 (s, 3H, C_{13}-CH_3), 0.97 and 0.98 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.2, 141.2, 138.4, 129.6, 128.4, 127.3, 126.3, 121.2, 119.4, 70.4, 56.6, 54.1, 50.2, 46.6, 42.7, 39.3, 38.5, 37.3, 35.2, 33.2, 32.4, 315, 28.4, 27.4, 26.3, 25.2, 24.4, 23.6, 22..5, 21.2, 20.4, 19.4, 16.4, 11.4; Anal. Calcd for $C_{35}H_{50}ClNO$; C, 78.39; H, 9.40; N, 2.61 found; C, 78.43; H, 9.42; N, 2.65; MS: *m/z* 535/537 [$M^{+\cdot}$].

3*β*-Acetoxycholest-5-en-7-spiro-1′-phenyl-3′-chloro-azetidin-2′-one (10)

White powder (77%). M.p: 172-174 ˚C; IR (KBr, cm^{-1}): 3075, 1588, 1401 (C-H aromatic), 1735 ($OCOCH_3$), 1755 (C=O), 1622 (C=C), 1374 (C-N), 744 (C-Cl); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.54-7.82 (m, 5H, aromatic), 5.61 (s, 1H, C_6-H), 4.82 (m, 1H, $C_3\alpha$-H, $W_{1/2}$=15 Hz, axial), 4.62 (s, 1H, C′$_3$-H), 2.02 (s, 3H, $OCOCH_3$), 1.10 (C_{10}-CH_3), 0.71 (C_{13}-CH_3), 0.93 and 0.81 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.3, 164.4, 141.6, 138.5, 129.7, 128.6, 127.2, 126.3, 121.2, 119.4, 76.4, 59.5, 54.2, 50.4, 46.2, 42.6, 39.2, 38.1, 37.2, 36.3, 35.3, 33.2, 32.1, 31.4, 29.4, 28.4, 27.2, 26.4, 24.1, 23.3, 22.2, 21.1, 20.4, 19.2, 18.1, 16.3, 11.1; Anal. Calcd for $C_{37}H_{52}ClNO_3$; C, 74.78; H, 8.82; N, 2.36 found; C, 74.75; H, 8.83; N, 2.39; MS: *m/z* 594/596 [$M^{+\cdot}$].

3*β*-Chlorocholest-5-en-7-spiro-1′-phenyl-3′-chloro-azetidin-2′-one (11)

White powder (76 %). M.p: 170-172 ˚C; IR (KBr, cm^{-1}): 3078, 1583, 1403 (C-H, aromatic), 1760 (C=O), 1624 (C=C), 1378 (C-N), 745, 752 (2×C-Cl); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.53-7.82 (m, 5H, aromatic), 5.41 (s, 1H, C_6-H), 4.64 (s, 1H, C′$_3$-H), 3.91 (m, 1H, $C_3\alpha$-H, $W_{1/2}$=14 Hz, axial), 1.10 (C_{10}-CH_3), 0.71 (C_{13}-CH_3), 0.93 and 0.81 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.2, 141.3, 138.4, 129.2, 128.3, 127.2, 126.4, 121.1, 119.2, 74.3, 62.3, 54.2, 50.2, 46.3, 42.2, 39.2, 38.4, 37.2, 36.6, 35.2, 33.2, 32.4, 31.1, 28.1, 27.2, 26.4, 24.1, 23.2, 22.6, 21.1, 20.2, 19.3, 18.5, 16.4, 11.3; Anal. Calcd for $C_{35}H_{49}Cl_2NO$; C, 73.66; H, 8.65; N, 2.45 found; C, 73.67; H, 8.67; N, 2.46; MS: *m/z* 569/571 [$M^{+\cdot}$].

Cholest-5-en-7-spiro-1′-phenyl-3′-chloro-azetidin-2′-one (12)

Yellow powder (74%). M.p: 170-173 ˚C; IR (KBr, cm^{-1}): 3074, 1587, 1401 (C-H aromatic), 1761 (C=O), 1625 (C=C), 1377 (C-N), 748 (C-Cl); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52-7.82 (m, 5H, aromatic), 5.30 (s, 1H, C_6-H), 4.65 (s, 1H, C′$_3$-H), 1.10 (C_{10}- CH_3), 0.71 (C_{13}-CH_3), 0.93 and 0.81 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.3, 141.2, 138.5, 129.3, 128.4, 127.2, 126.4, 121.2, 119.1, 72.2, 56.5, 54.3, 50.2, 46.1, 42.3, 39.4, 38.1, 37.2, 36.1, 35.3, 33.5, 32.1, 31.2, 28.4, 27.4, 26.5, 24.6, 23.5, 22.6, 21.2, 20.1, 19.2, 18.3, 16.4, 11.2; Anal. Calcd for $C_{35}H_{50}ClNO$; C, 78.39; H, 9.40; N, 2.61 found: C, 78.40; H, 9.43; N, 2.62; MS: *m/z* 535/537 [$M^{+\cdot}$].

Rule of Five

The physicochemical parameters including octanol partition coefficients (CLogP), Mw (molecular weight), HBD (number of hydrogen bond donors), HBA (number of hydrogen bond acceptors) and TPSA (topological polar surface area) were calculated using ChemBioOffice 2008.

Antimicrobial activity

In the context of our studies, the synthesized compounds were screened *in vitro* for antibacterial activities against the culture of *Streptococcus pyogenes* (ATCC-29213), *Staphylococcus epidermidis* (ATCC-29887), *Pseudomonas aeruginosa* (ATCC-27853), *Kliebsiella pneumonia* (Clinical isolate) and *Escherichia coli* (ATCC-25922) by disk diffusion method.[20] The minimum inhibitory concentration (MIC) was evaluated by the macrodilution test using standard inoculums of 1-2×10^7 c.f.u.mL^{-1} (0.5 McFarland standards). Serial dilutions of the synthesized compounds, previously dissolved in dimethyl sulfoxide (DMSO) were prepared to final concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 $mgmL^{-1}$. To each tube was added 100 mL of 24 h old inoculums. The tests use DMSO and Ciprofloxacin as

negative and positive controls. To obtain the MBC, 0.1 mL volume was taken from each tube and spread on agar plates. The number of c.f.u. was counted after 18-24 h of incubation at 35 ˚C. The *in vitro* antifungal activities were carried out against *Candida albicans, Penicillium marneffei, Aspergillus fumigates* and *Trichophyton mentagrophytes* by agar diffusion method.[20] The minimum inhibitory concentration (MIC) was determined by broth dilution technique as in antibacterial activity. The Inhibition zones of compounds were compared with Fluconazole used as standard drug. The nutrient broth which contained logarithmic serially two fold diluted amount of test compound and controls was inoculated with approximately 1.6-6× 10^4 c.f.u. mL^{-1}. The cultures were incubated for 48 h and the growth was monitored. To obtain minimum fungicidal concentration (MFC), 0.1 mL volume was taken from each tube and spread on agar plates. The number of c.f.u. was counted as the lowest drug concentration at which 99% of the inoculums were killed.

Effect of acid/ alkaline treatment on the antimicrobial activity

To examine the effect of acid/ alkaline treatment on the antimicrobial activity, the synthesized compounds were exposed to acid (pH 3.0) or alkali (pH 12.0) at room temperature for 30 min, and then were neutralized by adding 1M NaOH or 1M HNO_3. After getting the pH back to 7, these treated compounds at a concentration of 5 mgL^{-1} were added to microbial suspension at approximately 10^6 c.f.u.mL^{-1}. The mixture was incubated aerobically at 35 ˚C for 5 h.[21]

Antimicrobial activities under different temperatures

To estimate the effect of incubation temperature on the antimicrobial activity, a mixture containing fresh LB medium, synthesized compounds and microbial suspension were combined to create a final concentration at 5 mgL^{-1} and microbial cells at 10^6 c.f.u. mL^{-1}. The mixtures were aerobically cultured at 4, 23 and 35 ˚C for 5 h. The unexposed microbial under the same temperature were used as control.[21]

Antioxidant activity

The synthesized compounds were tested for their antioxidant property by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method.[22] Drug stock solution 1 $mgmL^{-1}$ was diluted to final concentration of 2, 4, 6, 8, 10 and 12 $mgmL^{-1}$ in methanol. Methanolic DPPH solution (1 mL, 0.3 mmol) was added to 3.0 mL of drug solution of different concentrations. The tube was kept at an ambient temperature for 30 min and the absorbance was measured at 517 nm. The scavenging activity was calculated by following formula:

$$\% \text{ Inhibition} = 100 \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}$$

where

A_{control} is the absorbance of the L-ascorbic acid (standard) and

A_{sample} is the absorbance of different compounds.

The methanolic DPPH solution (1 mL, 0.3 mM) was used as control.

Docking study

Protein and ligand preparation

The three dimensional structures of the targets were downloaded from protein data bank. Hydrogen atom and MMFF partial charge were added to the enzyme. Potential steric clashes and added hydrogen atoms were relaxed by using the minimization procedure. The minimization was performed by using a CHARMm force field[23] with dependent dielectric implicit solvent model along and conjugates gradient method. This process was carried out until the average absolute derivative of co-ordinates with respect to energy fell below the 0.1 kcal $A^{\circ-1}$. The two dimensional structures of ligands were prepared by using the ChemDraw Ultra 11.0 software integrated with Cambridgesoft Software (Cambridgesoft Corporation).[24] Further refinement of compounds was performed by using energy minimization protocol with cvff force field.

Molecular docking

GOLD (Genetic Optimisation for Ligand Docking) 5.0 was used for docking of the compounds dataset against the selected targets in present study. Docking annealing parameters for van der Walls and hydrogen bonding were set to 5.0 and 2.5, respectively. The parameters used for genetic algorithm were population size 100, selection pressure 1.2, number of operations 1,00,000, number of islands 5, niche size 2, migrate 10, mutate 100 and cross-over 100. Interaction analyses were performed by using Ligplot.[25] Figures of the complexes was prepared by using discovery studio visualizer.

RESULTS AND DISCUSSION

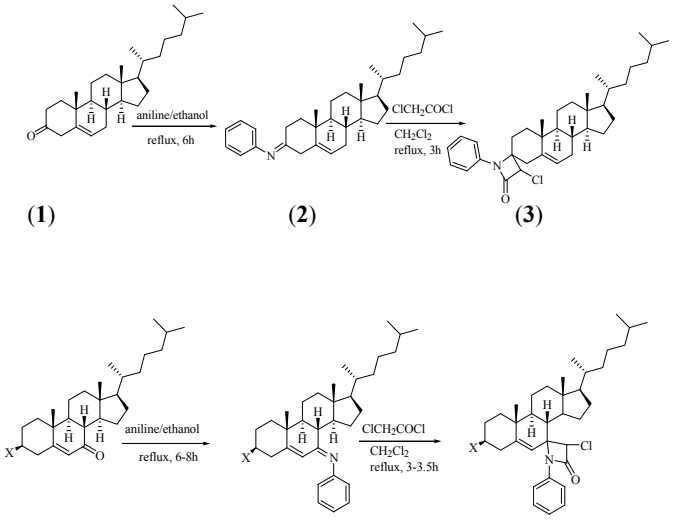
Steroidal iminophenylcholest-5-enes (**2, 7-9**) prepared by condensing steroidal unsaturated ketones (**1, 4-6**) with aniline which were used as starting material for the preparation of steroidal spiroazetidinone. There are only few reports available on the synthesis of spiroazetidinone at C4,[26] especially the Staudinger reactions with *α*-heteroatom-substituted imines due to their instability caused by the bridgehead C4 heteroatom.[27] In our present work, we have investigated the synthesis of steroidal spiroazetidinone *via* Staudinger ketene-imine [2+2] cycloaddition reaction using steroidal cholest-5-ene substituted exocyclic imines (**2**, **7-9**).[28] To search for optimum reaction conditions and establish a reproducible procedure, 3*β*-acetoxy-7-iminophenylcholest-5-ene (**7**) and chloroacetyl chloride were selected as model substrates. Table **1** lists several organic solvents in order to optimize the reaction conditions.

Table 1.Screening of different solvents for reaction between 3*β* acetoxyiminophenylcholest-5-ene (**7**) with chloroacetyl chloride

Entry	Solvent	Yield % [a,b]
1	$CHCl_3$	41
2	THF	30
3	DMF	32
4	CH_2Cl_2	42
5	Toulene	21
6	Benzene	28

[a]completion of reaction was monitored by TLC. [b]Isolated overall yield.

In order to investigate the scope of this reaction, a variety of different steroidal compounds were subjected to this reaction (**Scheme 1**). All the reactions proceeded smoothly and the reaction was completed within 3-3.5 h to afford the products **3, 10-12** in excellent yields (74-77%) (**Scheme 1**).



(**1**) (**2**) (**3**)

X	**X**	**X**
OAc (**4**)	OAc (**7**)	OAc (**10**)
Cl (**5**)	Cl (**8**)	Cl (**11**)
H (**6**)	H (**9**)	H (**12**)

Scheme 1. Synthesis of steroidal spiroazetidinone derivatives.

The Staudinger [2+2] cycloaddition reaction of steroidal iminophenylcholest-5-enes (**2, 7-9**) with chloroacetyl chloride afforded steroidal spiroazetidinone derivatives (**3, 10-12**). The structures of the compounds were established by means of their IR, ^{1}H NMR, ^{13}C NMR, MS and analytical data. The selected diagnostic bands in IR spectra of synthesized products provided useful information for determining structures of the synthesized compounds. The absorption bands at 3074-3080 cm^{-1} are attributed to the aromatic ring in the products. All the compounds **3, 10-12** exhibited absorption bands at 1755-1763 cm^{-1} due to amide group while the bands in the range of 1618-1625, 1374-1376 and 742-748 cm^{-1} can be ascribed to C=C, C-N and C-Cl, respectively. The formation of steroidal spiroazetidinone was further confirmed with the ^{1}H NMR spectra. The ^{1}H NMR spectra of the compounds, besides the expected signals for cholestane moiety, exhibited multiplet signals at 7.50-7.82 for aromatic hydrogen and one singlet for one proton at δ 4.61-4.64 for C′$_3$-H. ^{13}C NMR spectra, besides the characteristic signals for the cholestane nucleus showed δ 160-165 corresponding to the carbonyl group. The signals obtained at δ 70.4-76.4 confirm the presence of C-Cl bond. The mass spectral data of compounds **3, 10-12** showed molecular ion peaks [$M^{+\cdot}$] at m/z 535/537, 594/596, 569/571 and 535/537, respectively.

Rule of Five

High oral bioavailability is an important factor for the development of therapeutic agents. Good intestinal absorption, molecular flexibility, low polar surface area and total hydrogen bond are important predictors of good oral bioavailability.[29] Lipinski used these molecular properties in formulating his 'Rule of Five'. The rule states that most molecules with good membrane permeability have log $P \leq 5$, $M_W \leq 500$, the number of hydrogen bond acceptors ≤ 10 and the number of hydrogen bond donors ≤ 5. This rule is widely used as a filter for drug-like properties .[30] Table **2** lists the values of these properties for steroidal spiroazetidinone derivatives (**3, 10-12**).

All of the compounds fulfill three out of the criteria of Lipinski rule and we can say it is adhere to 'Lipinski's Rule of Five'.[31] The result suggested that these compounds can be used as templates for a drug discovery effort.

Antimicrobial activity

In the context of our studies, the synthesized compounds were screened for their *in vitro* antimicrobial activities against Gram-positive and Gram-negative bacterial strains and were found to possess activities against the microorganisms listed in Tables **3** and **4**.

The investigation of antibacterial screening data revealed that all of the synthesized compounds showed moderate to good bacterial inhibition. All of the compounds exhibit excellent activities against Gram-positive bacteria. On the other hand, all the compounds possess moderate to excellent antimicrobial activities against Gram-negative strains. In general, we can say that the antibacterial activity in the case of Gram-positive bacteria were a little higher compare to Gram-negative bacteria which can be explained by the presence of an extra layer of protection by lipopolysaccharide in addition to the peptidoglycan membrane in Gram-negative bacteria.[32]Among the synthesized compounds it was clear that compound **3** showed very good antibacterial activity nearly equivalent to that of standard drug Ciprofloxacin.

The investigation of antifungal screening data (Tables **5** and **6**) indicates that all of the synthesized compounds showed moderate to good activity.

Among the screened compounds, compounds **3** and **12** showed good inhibition against *Aspergillus fumigatus* and *Candida albicans*. MFC of most of the compounds was found to be two to four folds higher than their corresponding MIC results.

Table 2. Calculated physicochemical properties of steroidal spiroazetidinones **3, 10-12**.

Compound	Mw	HBD	HBA	TPSA	no.violations
3	536.23	0	2	20.31	2
10	594.27	0	4	46.61	2
11	570.68	0	2	20.31	2
12	536.23	0	2	20.31	2

Table 3. Antibacterial activity of (zones of inhibition) of steroidal spiroazetidinones **3, 10-12**

Compound	Diammeter of zone of inhibition (mm)				
	Gram positive bacteria		Gram negative bacteria		
	S. Pyogenes	*MRSA**	*P.aeruginosa*	*K. pneumoniae*	*E. coli*
3	14.5±0.1	13.2±0.5	15.1±0.3	12.2±0.4	15.1±0.2
10	15.1±0.2	16.8±0.2	14.9±0.4	16.1±0.6	18.9±0.4
11	17.8±0.3	16.9±0.4	19.2±0.3	17.8±0.3	17.3±0.6
12	13.9±0.4	12.9±0.4	11.2±0.5	14.8±0.2	15.3±0.3
Standard	23.0±0.2	22±0.2	32.0±0.3	19.0±0.2	27.0±0.2
DMSO	-	-	-	-	-

Positive control (standard); Ciprofloxacin and negative control (DMSO) measured by the Halo Zone Test (Unit, mm); * Methicillin resistant *Staphylococcus aureus* (MRSA +Ve).

Table 4. MIC and MBC results of steroidal spiroazetidinones **3, 10-12** against bacterial strains.

Compound	Gram positive bacteria				Gram negative bacteria					
	S.pyogenes		*MRSA**		*P.aeruginosa*		*K.pneumonia*		*E.coli*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
3	50	100	50	100	50	100	50	>100	50	>100
10	50	100	50	>100	50	50	100	>100	100	>100
11	50	100	25	50	50	100	50	>100	50	>100
12	50	100	25	50	50	100	50	>100	50	>100
Standard	12.5	12.5	6.25	12.5	12.5	25	6.25	25	6.25	12.5

(Standard); Ciprofloxacin; MIC (μg mL^{-1}) = minimum inhibitory concentration, i.e the lowest concentration of the compound to inhibit the growth of bacteria completely; MBC(μg mL^{-1}) = minimum bacterial concentration, i.e., the lowest concentration of the compound for killing the bacteria completely; * Methicillin resistant *Staphylococcus aureus* (MRSA +Ve).

Table 5. Antifungal activity of steroidal spiroazetidinones **3, 10-12**

Compound	Diameter of zone of inhibition (mm)			
	CA	AF	TM	PM
3	26.8±0.2	23.7±0.3	23.6±0.6	14.5±0.4
10	20.9±0.4	13.8±0.3	14.9±0.4	12.8±0.5
11	21.8±0.2	20.6±0.5	23.6±0.8	13.5±0.9
12	27.8±0.2	22.7±0.3	18.6±0.6	16.5±0.4
Standard	30.0±0.2	27.0±0.2	24.0±0.3	20.0±0.5
DMSO	-	-	-	-

CA; *Candida albicans* ,AF; *Aspergillus fumigates,*TM ; *Trichophyton mentagrophytes,* PM; *Pencillium marneffei*. Positive control (standard) ; Fluconazole and negative control (DMSO)

Table 6. MIC and MFC of steroidal spiroazetidinones **3, 10-12** against fungal strains

Compound	CA		AF		TM		PM	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
3	25	100	25	100	50	100	25	100
10	50	100	50	100	100	>100	100	>100
11	50	100	50	100	100	>100	50	100
12	25	50	25	100	50	100	25	100
Standard	6.25	25	12.5	12.5	6.25	25	12.5	25

(Standard); Fluconazole, CA; *Candida albicans,* AF; *Aspergillus fumigates*,TM; *Trichophyton menotagrophytes,* PM; *Pencillium marneffei.* MIC (μg/ml) = minimum inhibitory concentration, i.e the lowest concentration of the compound to inhibit the growth of fungus completely; MFC (μg/ml) = minimum fungicidal concentration, i.e., the lowest concentration of the compound for killing the fungus completely.

Table 7. Antioxidant activity of steroidal spiroazetidinones **3, 10-12**

Compound	% Inhibition			
	25 µg mL^{-1}	50 µg mL^{-1}	75 µg mL^{-1}	100 µg mL^{-1}
3	16.8±0.2	23.7±0.3	19.6±0.6	24.5±0.4
10	10.9±0.4	13.8±0.3	14.9±0.4	22.8±0.5
11	11.8±0.1	13.6±0.5	18.6±0.8	20.5±0.9
12	17.8±0.2	18.7±0.3	20.6±0.2	26.5±0.4
Standard	36.0±0.3	37.0±0.2	44.0±0.3	50.0±0.5

1. Values represent the mean ± standard error mean (SEM) of three experiment. 2. (–): No inhibition, standard: ascorbic acid.

Table 8. The ligand molecules with number of molecular with *DNA gyrase* protein.

Compound	GOLD fitness score	No of interactions			Xscore, kcal mol^{-1}
		Hydrogen bond	Lipophilic	Non-bonded	
Ciprofloxacin	45.84	NIL	6; Asn 41, Ser 42, Gln 45, Arg 71, Ile 73, Thr 137	23	-7.61
3	15.47	NIL	9; Ile 38, Asn 41, Glu 45, Val 66, Arg 71, Ile 73, Pro 74, Thr 137, Ile 139	22	-8.19
10	14.67	NIL	11; Ile 38, Asn 41, Glu 45, Ala 48, Val 66, Asp 68, Arg 71, Ile 73, Pro 74, Thr 137, Ile 139	28	-7.25
11	15.53	NIL	9; Ile 38, Asn 41, Glu 45, Ala 48, Arg 71, Ile 73, Pro 74, Thr 137, Ile 139	19	-7.38
12	12.22	NIL	12; Ile 38, Asn 41, Asp 44,Glu 45, Ala 48, Val 66, Asp 68, Arg 71, Ile 73, Pro 74, Thr 137, Ile 139	28	- 7.07

Table 9. The ligand molecules with number of molecular interactions with *CYP 51*.

Compound	GOLD fitness score	No of Interactions			Xscore, kcal mol^{-1}
		Hydrogen bond	Lipophilic	Non-Bonded	
Fluconazole	50.06	1; His 101	5; Lys 97, Ala 256, Phe 387, Cys 394, Val 395	23	-8.06
3	18.75	NIL	8; Gln 72, Ala 73, Tyr 76, Phe 83, Arg 96, Lys 97, Leu 100, Leu 321,	21	-7.20
10	25.84	NIL	13; Ala 73, Tyr 76, Pro 93, Lys 97, His 101, Ala 256, Leu 321, Pro 386, Phe 387, His 392, Arg 393, Cys 394, Val 395	41	-7.61
11	21.84	NIL	11; Gln 72, Ala 73, Tyr 76, Phe 78, Met 79, Lys 97, Phe 255, Ala 256, His 259, Leu 321, Leu 324,	40	-7.77
12	15.03	NIL	13; Gln 72, Ala 73, Tyr 76, Phe 78, Met 79, Lys 97, Phe 255, Ala 256, His 259, Leu 321, Leu 324, His 392, Val 434	45	-6.86

Effect of acid/alkaline treatment on the antimicrobial activity

The alkali treatment showed no significant changes on the antimicrobial activity. While the antimicrobial activity of the synthesized compounds treated with acid solution at a pH of 3 showed decrease in the antimicrobial activity, down to below 10% at 5 mgL^{-1}. This may be explained in terms of possible damage on the surface functional groups at low-pH environment, leading to the aggregations of the compounds. The aggregates would have more difficulty in penetrating the peptidoglycan layer of bacterium.[33]

Antimicrobial activities under different temperatures

Microbes grow at various conditions and most pathogens grow at human body temperatures. Thus, we studied the effect of various temperatures on the antimicrobial activities of our synthesized compounds. The antimicrobial activity of the synthesized compounds was proportional to the incubation temperature. At 4 ˚C, no obvious effect was achieved. On the other hand, at the maximum temperature tested, 35 ˚C, the antimicrobial activity was found to be most effective. It is possible that at higher temperature, the

cell membrane were leaky allowing entry of the synthesized compounds which resulted in higher mortality. This will be in agreement with studies that state that transport into the plasma membrane is essential for inhibiting microbial growth.[34]

Antioxidant activity

The *in vitro* antioxidant activity and scavenging effects of steroidal spiroazetidinone derivatives **3**, **10-12** were evaluated by using different reactive species assay containing DPPH radical scavenging activity. The free radical scavenging activity of all the synthesized compounds **3**, **10-12** were evaluated through their ability to quench the DPPH• using ascorbic acid as reference. Among the synthesized compounds, compounds **3** and **12** exhibited good antioxidant properties. All the synthesized compounds were less potent than the reference ascorbic acid. From the results it can be interpreted that the aromatic ring showed marked effects on the antioxidant property of these compounds.[35] The potencies for the antioxidant activity of the synthesized compounds **3**, **10-12** to the reference drug are shown in Table **7**.

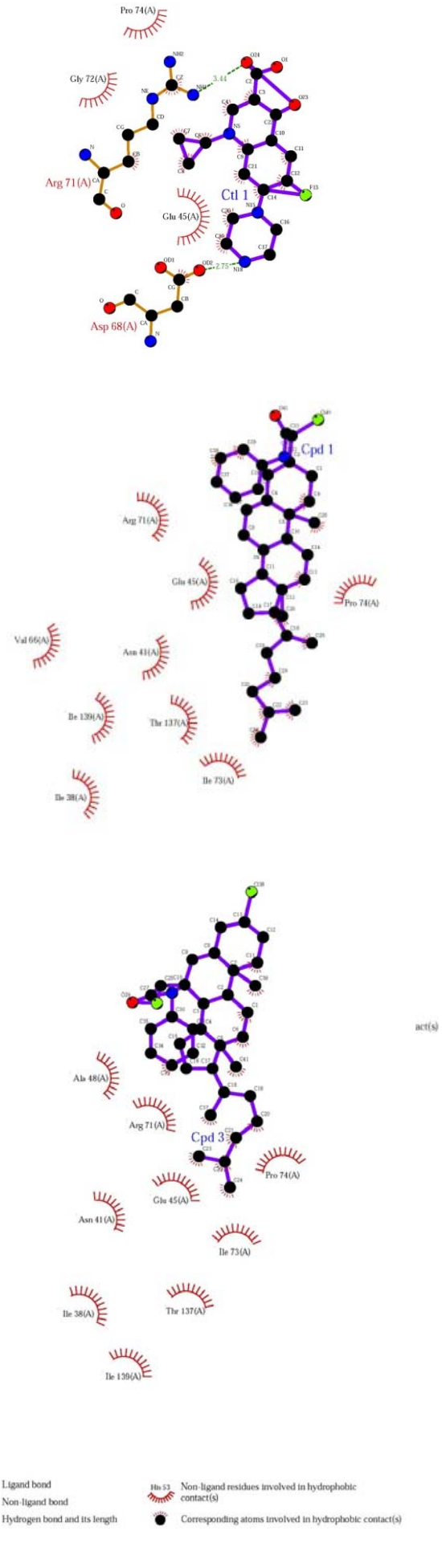
Docking study

To get a better comprehension of the high microbial inhibitory potency of the newly synthesized compounds at a molecular level and shed light on the structural determinants for their activity, docking investigations were carried out through the automated docking program GOLD 5.0 against selected targets in present study.

Molecular docking with the active site of DNA gyrase (PDB ID: 3U2D)

DNA gyrase member of the type II family of topoisomerases that control the topological state of DNA in cells.[36] DNA gyrase couples ATP hydrolysis by the GyrB subunit to supercoiling of DNA, which is required for maintenance of DNA topology during the replication process. It is an essential enzyme across bacterial species and inhibition results in disruption of DNA synthesis and subsequently, cell death. DNA gyrase has long been known as an attractive target for antibacterial drugs.[37] In order to know the mode of action of steroidal spiroazetidinones (**3**, **10-12**) docking studies were carried out. All of the compounds had no hydrogen bonds with the DNA gyrase but have hydrophobic interactions Table **8**.

Among the synthesized compounds **3**, **10-12**, compound **11** showed good molecular interactions with 15.53 GOLD fitness score. It was evident from the figures that for each compound, the binding site and bonding interactions was found varied. It was interesting to observe that even though the core structure of all the compounds was same, the degree of interaction and binding location were found to be almost similar. The binding sites of the compounds were found to be in close proximity to the binding site of Ciprofloxacin. The variation in the bioactivity is mainly attributed to the difference in their binding site. The results show that the overall trend of the interaction energies of all the derivatives is in good agreement with the *in vitro* antibacterial activity.



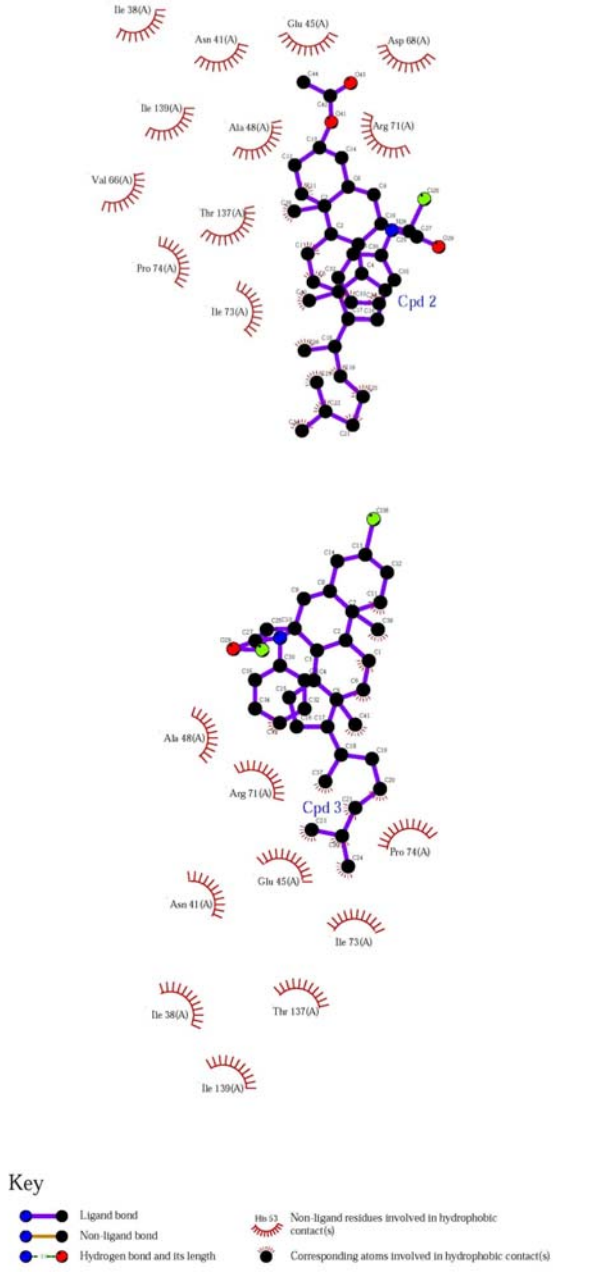
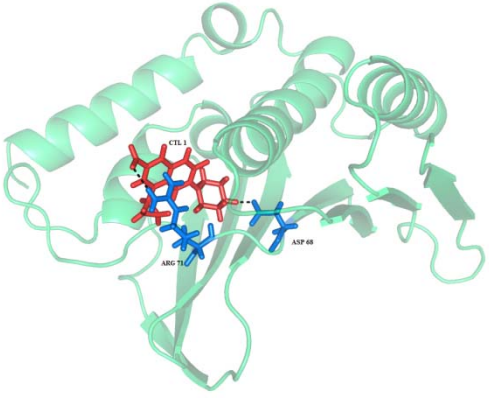


Figure 1. **(a)** (Docked) Ciprofloxacin and steroidal spiroazetidinones (**3**, **10-12**) docked in binding site of DNA gyrase protein. The protein is in cartoon representation and the ligand molecules are in sticks colored;



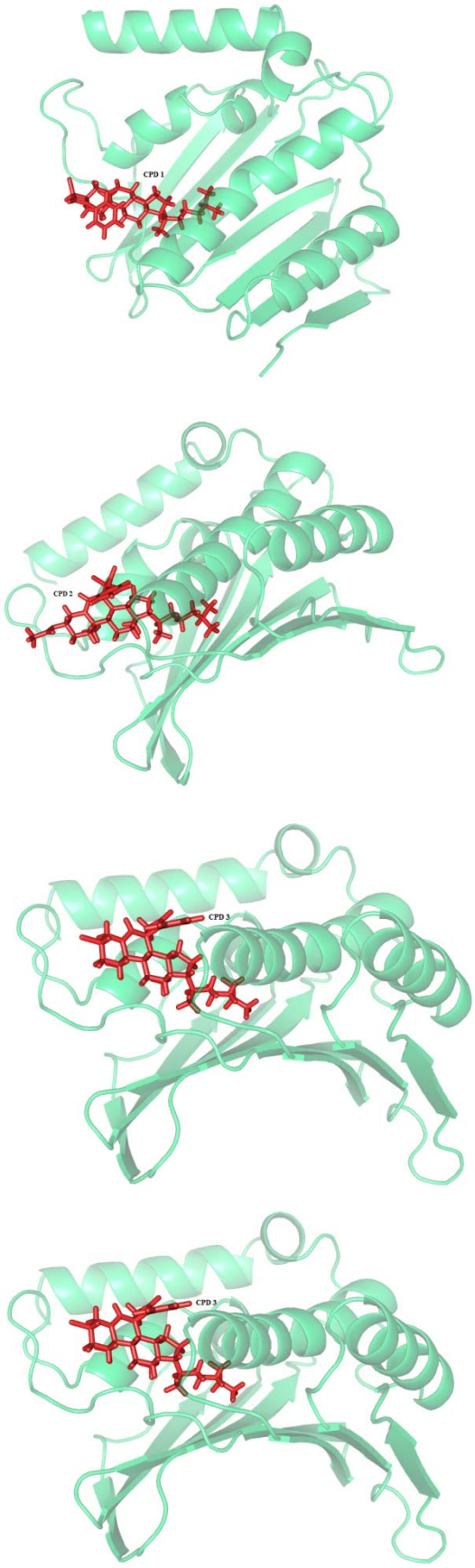


Figure 1 (b). (Ligplot) The interaction plot of Ciprofloxacin and steroidal spiroazetidinones (**3**, **10-12**) with the binding site residues of DNA gyrase protein. The hydrogen bonds and hydrophobic interactions are shown which are holding the ligand within the binding site.

Molecular docking with the active site of CYP51 (PDB ID: 1E9X)

CYP51 is one of the key enzymes of sterol biosynthesis in different biological kingdoms, such as fungi, higher plants and mammals.[38]As an essential enzyme in the fungal life cycle, CYP51 has been a primary target for antifungal agents.[39] Selective inhibition of CYP51 causes depletion of ergosterol and accumulation of lanosterol and other 14-methyl sterols, resulting in inhibition of the growth of fungal cells.[40] Due to the importance of CYP51 in antifungal drug studies, we have decided to study the binding of this protein with our synthesized compounds (Table **9**).

The synthesized compounds showed strong molecular interactions within active site of CYP51. Compounds **3**, **10-12** form stable complexes with 18.75, 25.84, 21.56 and 15.03 GOLD fitness score which are reasonably good as compared to Fluconazole having 50.06 fitness score.

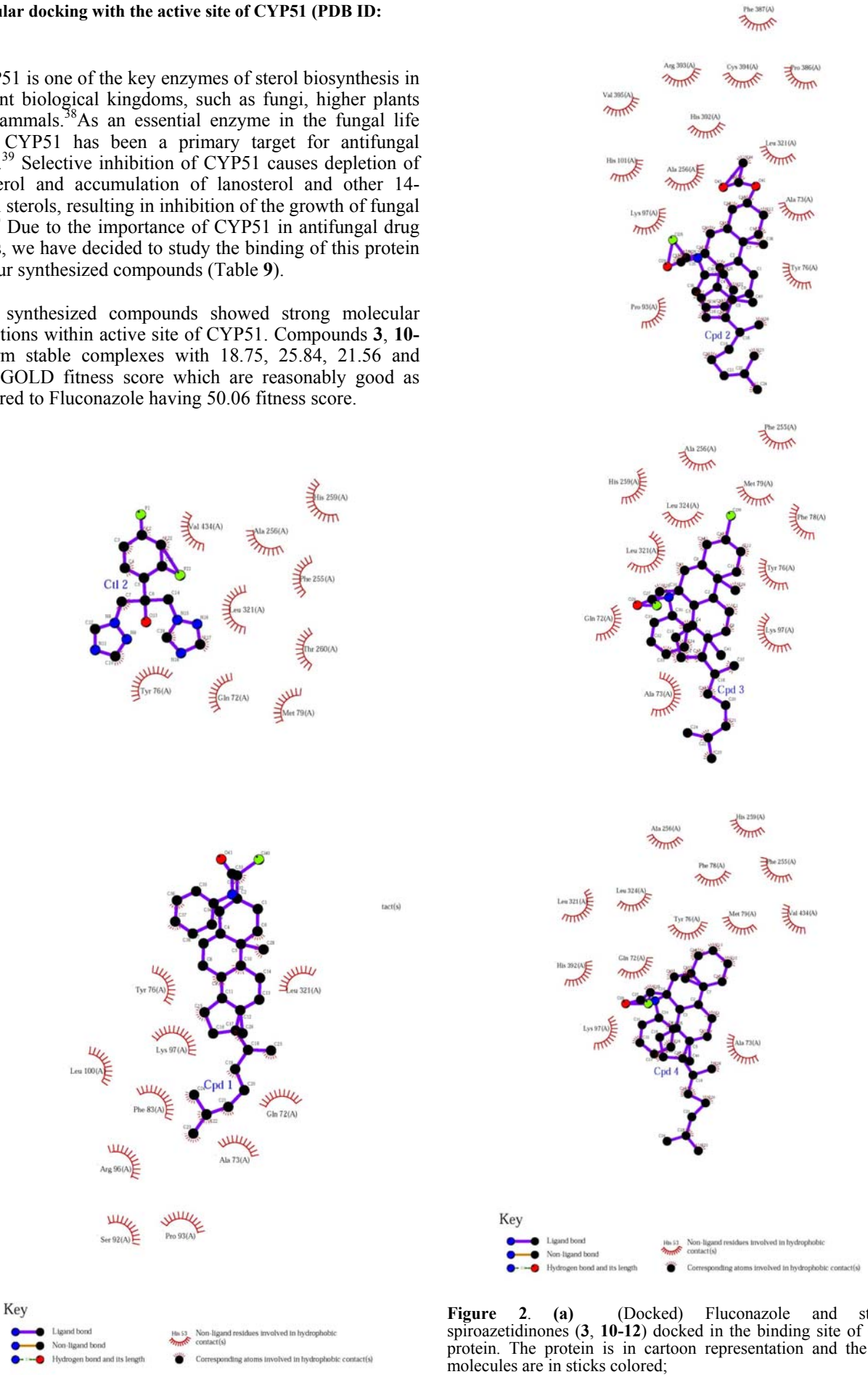
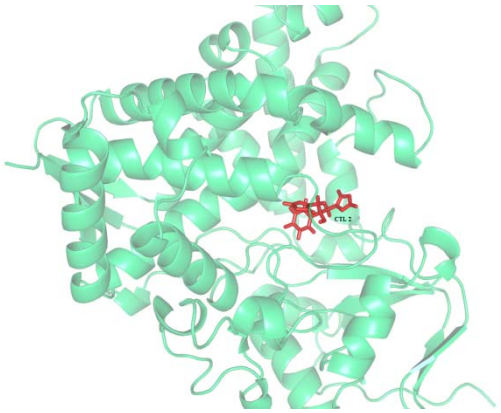
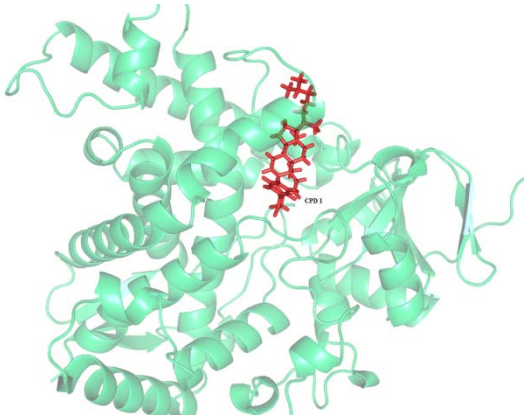
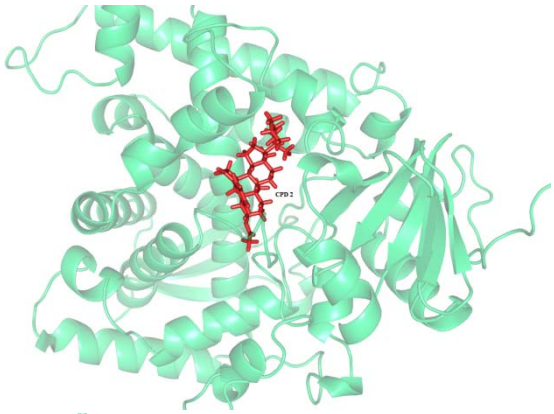
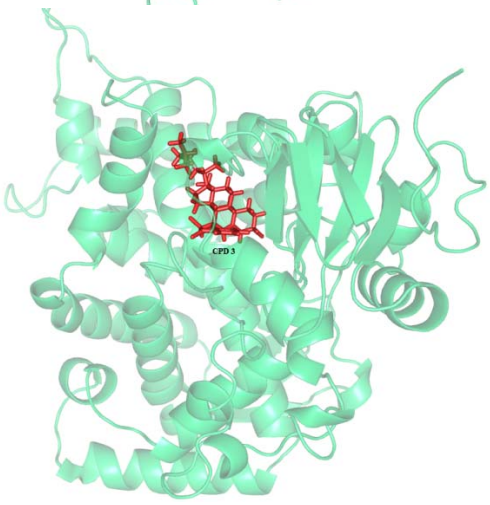


Figure 2. **(a)** (Docked) Fluconazole and steroidal spiroazetidinones (**3**, **10-12**) docked in the binding site of CYP51 protein. The protein is in cartoon representation and the ligand molecules are in sticks colored;









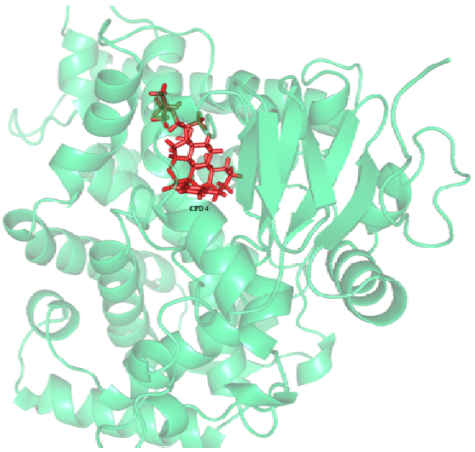


Figure 2.(b) (Ligplot) The interaction plot of Fluconazole and steroidal spiroazetidinones (**3**, **10-12**) with the binding site residues of CYP51 protein. The hydrogen bonds and hydrophobic interactions are shown which are holding the ligand within the binding site.

The result of docking suggests that the appropriate length of the substituents on the derivatives is important for antifungal activities. The binding mode of the docked molecules suggested that the compounds **3**, **10-12** with hydrogen bonding donor or acceptor substituents. The good correlation between antifungal activity and docking score indicates that a suitable substituent has a great impact on the antifungal activity.

CONCLUSIONS

In conclusion, we have reported the synthesis of steroidal spiroazetidinones (**3**, **10-12**) *via* Staudinger ketene-imine [2+2] cycloaddition reaction in excellent yields. The biological evaluation of steroidal spiroazetidinones (**3**, **10-12**) as antimicrobial and antioxidant agent has been achieved. The results from the docking studies were found to be in good agreement with the results from pharmacological studies. The results suggest that steroidal spiroazetidinone derivatives (**3**, **10-12**) would be promising compounds that may lead for the further development of potent biologically active agents.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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