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Synthesis, characterization of steroidal heterocyclic compounds, DNA condensation and molecular docking studies and their *in vitro* anticancer and acetylcholinesterase inhibition activities

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Abstract

A facile, convenient and efficient approach for the synthesis of new series of steroidal heterocyclic compounds (**4-12**) by reacting a mixture of compounds (**1e-3e**) with o-aminothiophenol/ o-aminophenol/ o-phenylenediamine is reported. The structural assignment of products is confirmed on the basis of IR, ^{1}H NMR, ^{13}C NMR, MS and analytical data. The compounds obey the Lipinski's 'Rule of Five' analysis based on computational prediction and pharmacokinetic properties. The anticancer activity has been tested *in vitro* against three cancer cell lines **Hep3 B** (human hepatocellular carcinoma), **MCF 7** (human breast adenocarcinoma), **HeLa** (human cervical carcinoma) and one non-cancer normal cell i.e. **PBMCs** (peripheral blood mononuclear cell) by MTT assay. In addition, the synthesized compounds are also tested for their *in vitro* antioxidant activity by various reported methods in which compounds **10-12** exhibited good antioxidant activity. Nonenzymatic degradation of DNA has been investigated. The acetylcholinesterase (AChE) inhibitor activities of the steroidal derivatives are also evaluated using Ellman's method. Moreover, the application of compounds **6** as DNA gene transporter is evaluated by DNA condensation and ascertained by employing TEM and AFM, which illustrate that the compound **6** induces the condensation of CT-DNA. Molecular docking studies further characterize the interaction of the synthesized compounds with DNA.

Keywords: Anticancer, Antioxidant, DNA condensation, AChE inhibitor, Molecular docking.

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Introduction

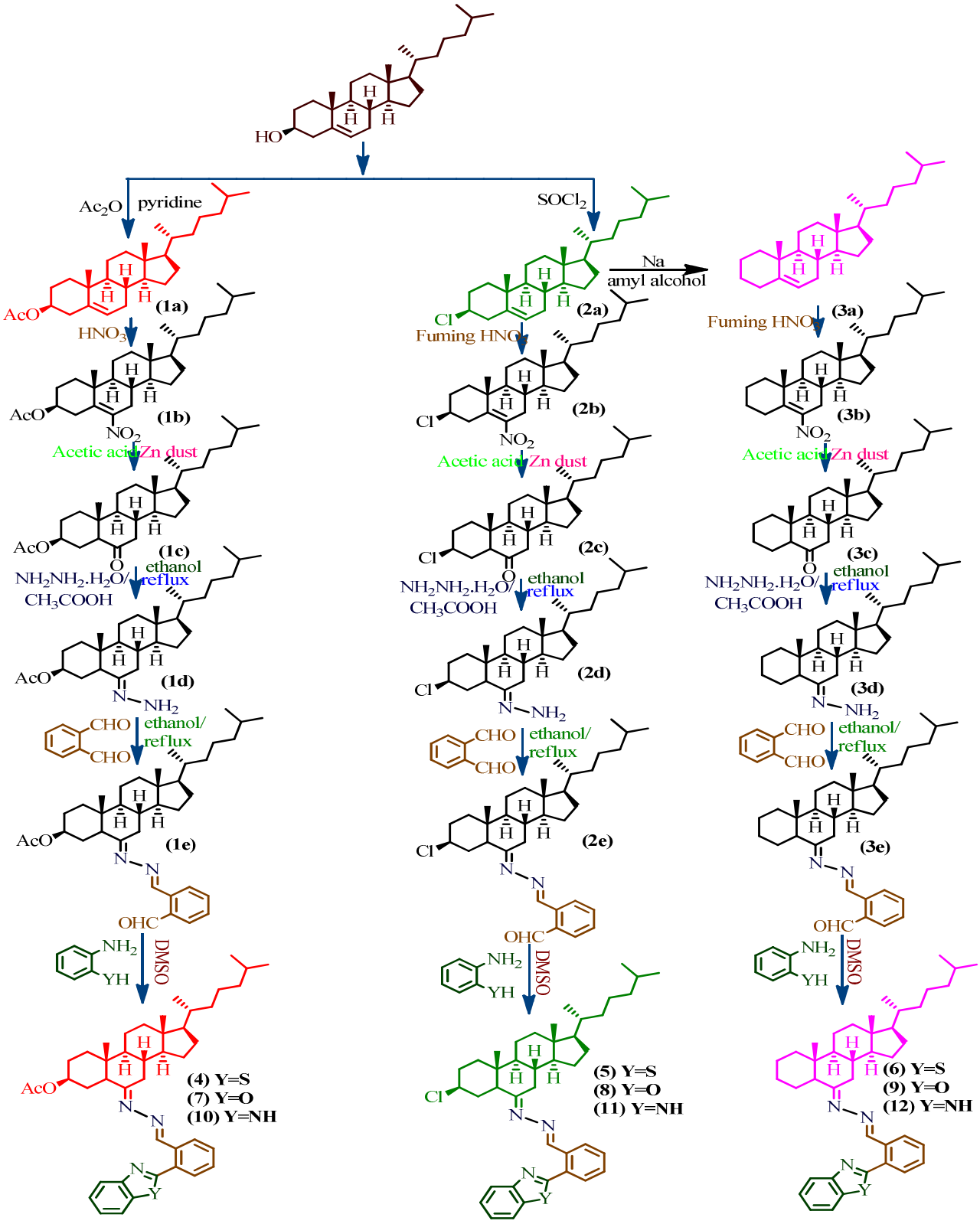
Steroidal compounds have drawn attention not only by unusual and interesting chemical structures, but also due to their widespread application as anti-inflammatory, diuretic, anabolic, contraceptive, antiandrogenic, progestational and anticancer agents.[1] In the global drug market, steroid drugs rank second only after antibiotics.[2] Most of steroidal drugs in use today are semi-synthetic compounds and widely used in traditional medicines by the modification of the steroid ring system and side chains.[3-6] Steroids regulate a wide variety of physiological and developmental processes in vertebrates and are used as the building block in the construction of larger molecules with sufficient rigidity and with suitable substitution for the further modifying of their molecular properties. The major advantage of steroids is their rigid skeleton, variability of substitution and their similarity to naturally occurring structures.[7] The synthesis and physiological activity of hetero-steroids have received a lot of attention over the decades by medicinal chemists.[8,9] The interesting structural and stereochemical features of the steroid nucleus provide additional fascination to the researchers, and thereby the introduction of heteroatom, heterocycle or replacement of one or more carbon atoms in the steroidal skeleton has been envisaged to discover new chemical entities with a potential to afford some promising drugs of the future and brings notable modifications of its biological activity.[10-12] The incorporation of a heterocyclic ring and/or a heteroatom in the steroid backbone affects the chemical properties of a steroid, the development of new types of activity mainly depending on the position of heterocyclic ring on the steroid nucleus and often results in useful alterations in its biological activities. As a result, researchers are on a continuous search to design and produce better hetero-steroids.[13,14] Based on all these facts and in continuation of our earlier studies of hetero-steroidal compounds,[15] herein we report the synthesis of steroidal heterocyclic

compounds (**4-12**) which are also investigated for their antioxidant, anticancer, AChE inhibition, DNA gene transporter and Molecular docking studies.

Results and discussion

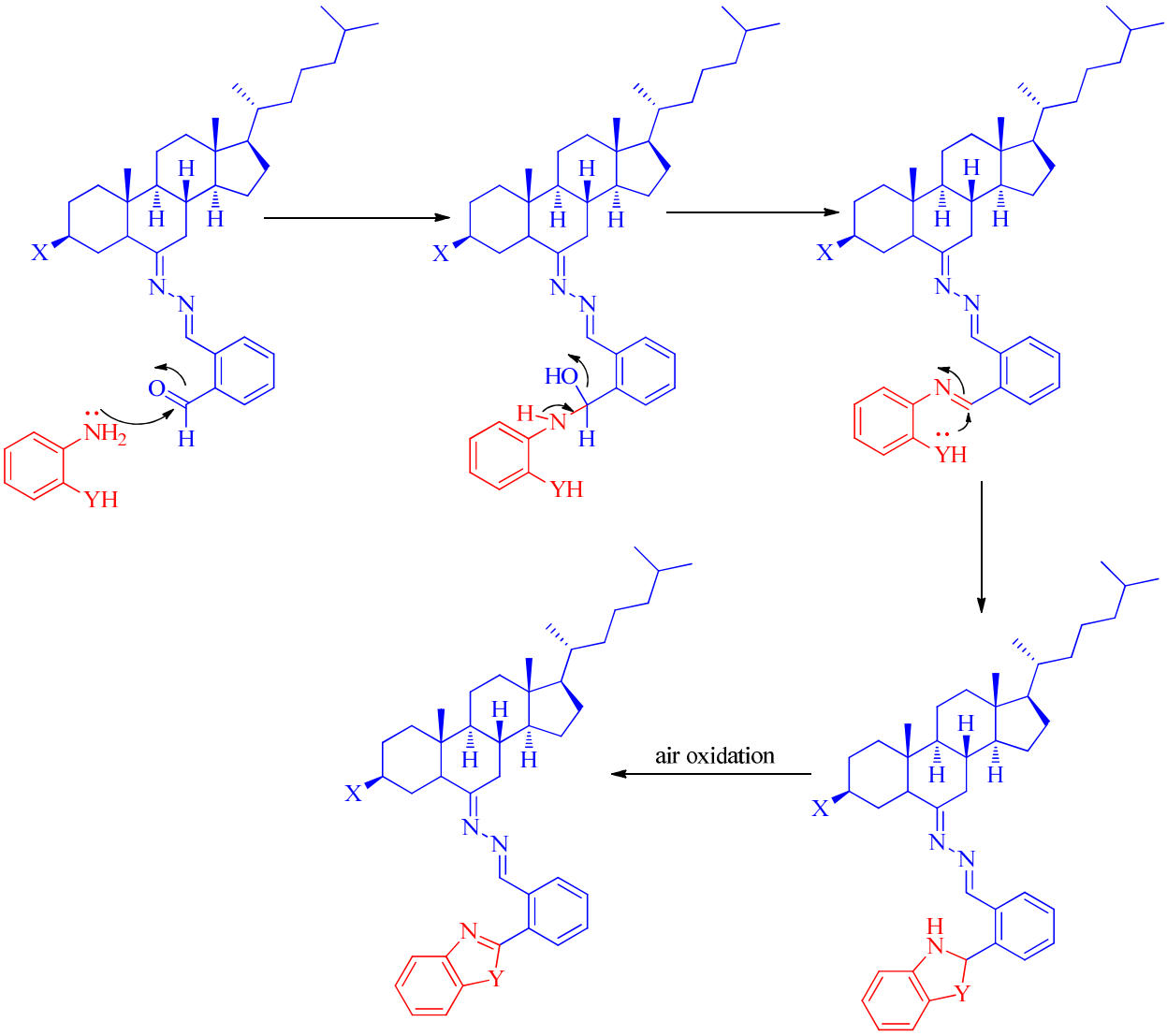
Chemistry

Development of highly functional molecules from simple building blocks has always attracted the curiosity of synthetic chemists. So we herein report a convenient route for the synthesis of steroidal heterocyclic compounds **4-12**. All the compounds (**Scheme 1**) were prepared by refluxing compounds **1e-3e** with o-aminothiophenol/ o-aminophenol/ o-phenylenediamine in DMSO.[16]



Scheme 1. Schematic pathway for the formation of steroidal compounds (**4-12**).

The structures of the products were established by means of their IR, ^{1}H NMR, ^{13}C NMR, MS and analytical data. The selected diagnostic bands in the IR spectra of synthesized compounds provide useful information for determining their structures. The absorption bands at 1627-1637 cm^{-1} (C=N), 682-687 cm^{-1} (C-S-C), 1050-1061 cm^{-1} (C-O) and 1310-1329 cm^{-1} (C-N) confirmed the formation of thiazole, oxazole and imidazole ring systems in products (**4-12)**. The absorption bands at 1560-1569 cm^{-1}, 1387-1397 cm^{-1}, 3081-3089 cm^{-1} are attributed to the aromatic ring in the products **4-12** and the bands in the range 3315-3342 cm^{-1} confirm the presence of NH in compounds **10-12**. In their ^{1}H NMR study the downfield singlet at δ 5.3-4.4 was ascribed to NH of imidazole ring. In ^{13}C NMR spectra, the signals at δ 159.2-156.4, 153.5-150.2, 149.2-145.6, 160.5-137.6 confirm the presence of N=C-S, N=C-O, N=C-NH and C=N groups in the products. A conceivable reaction mechanism for the synthesis of compounds **4-12** is represented in Scheme **2**. Finally the presence of distinct molecular ion peak [$M^{+\cdot}$] at *m/z*: 679, 655/657, 621, 663, 639/641, 605, 662, 638/640, 604 and 574, 550/552, 516 in the MS spectra also proved the formation of the compounds (**4-12** and **1e-3e**, respectively). This strategy can also be applied to further modifications on the steroidal substituted heterocyclic systems.



Scheme 2. A provisional mechanism for the synthesis of steroidal compounds (**4-12**).

Pharmacology

Rule of Five and bioactivity score

The use of Lipinski's rule as a filter to choose the reasonable scaffolds for biological activity is well known.[17] The rule states that most molecules with good membrane permeability have log P ≤ 5, molecular weight ≤ 500, number of hydrogen bond acceptors ≤ 10, number of hydrogen bond donors ≤ 5 and polar surface area less than 140 Å^2. The synthesized compounds showed

two violations of Lipinski rules due to a calculated Clog P value above the limit of 5 and the molecular weight above 500 (**Table 1**).

Table 1. Calculated physicochemical properties of steroidal heterocyclic derivatives (**4-12**).

Comp.	Mw	ClogP	HBD	HBA	TPSA	No violations
4	680.01	9.59	0	5	63.92	2
5	656.42	9.69	0	3	37.62	2
6	621.97	9.72	0	3	37.62	2
7	663.94	9.45	0	6	77.06	2
8	640.35	9.57	0	4	50.76	2
9	605.91	9.60	0	4	50.76	2
10	662.96	9.42	1	6	79.71	2
11	639.37	9.54	1	4	53.41	2
12	604.92	9.57	1	4	53.41	2

On the basis of the above results, we can say the compounds obeyed the Lipinski's 'Rule of Five' analysis based on computational prediction of molecular and pharmacokinetic properties,[18] it was found that the synthesized compounds have good oral absorption. The exceptions to the Lipinski's rule are recognized and involve anticancer drugs such as Doxorubicin.[19] The physico-chemical properties of the synthesized compounds are reasonable starting points for a drug discovery effort. The bioactivity scores of the synthesized compounds were also calculated for six criteria, GPCR ligand activity, ion channel modulation, kinase inhibition activity, protease inhibitor, enzyme inhibitor and nuclear receptor ligand activity (**Table 2**).

Table 2. Bioactivity score of steroidal heterocyclic derivatives (**4-12**).

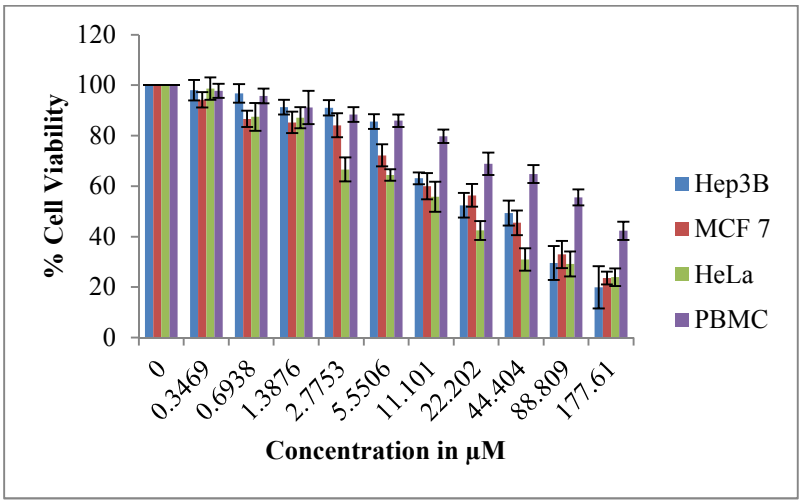
Comp.	GPCR ligand	Ion channel	Kinase inhibitor	Protease inhibitor	Nuclear Receptor ligand	Enzyme inhibitor
4	-0.65	-1.48	-1.34	-0.46	-0.90	-0.67
5	-0.37	-1.14	-0.99	-0.38	-0.58	-0.40
6	-0.33	-0.97	-0.84	-0.32	-0.43	-0.30
7	-0.66	-1.48	-1.41	-0.55	-0.79	-0.69
8	-0.39	-1.14	-1.06	-0.47	-0.46	-0.42
9	-0.35	-0.97	-0.91	-0.42	-0.31	-0.32
10	-0.48	-1.29	-1.20	-0.35	-0.81	-0.57
11	-0.20	-0.93	-0.84	-0.27	-0.49	-0.29
12	-0.16	-0.76	-0.68	-0.21	-0.34	-0.18

As a general rule, larger is the bioactivity score, higher is the probability that investigated compound will be active. For organic molecules if the bioactivity score is (>0.00), then the compound is active, but if it is between -0.50 to 0.00 then the compound is moderately active[20] and if the compound (<-0.50), then it is inactive compound.[21] The results of the present study in Table **2,** demonstrated that the some investigated compounds are biologically moderately active molecules and some are inactive molecules.

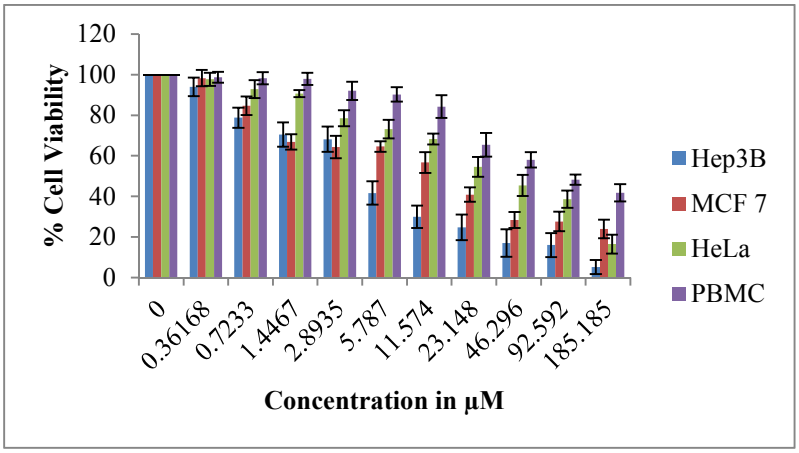
Anticancer activity

The *in vitro* anticancer screening of steroidal heterocyclic compounds (**4-12**) was done using human cancer cell lines **Hep3 B**, **MCF 7**, **HeLa** and non-cancer **PBMCs** cells. Doxorubicin (**Dox**) and 5-Fluorouracil (**5-Fu**) were used as cytotoxic drugs of reference. A period of 48 h of drug exposure was chosen to test cytotoxicity. The cytotoxicity (IC_{50}) is the concentration in 'μM' required for 50% inhibition of cell growth as compared to that of untreated control. The growth inhibitory effect of compounds (**4-12**) towards the cancer cells was measured by means of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in which the cell viability was measured with the purple formazan that was metabolized from MTT by mitochondrial dehydrogenase, which is active only in live cells. IC_{50} of the synthesized steroidal

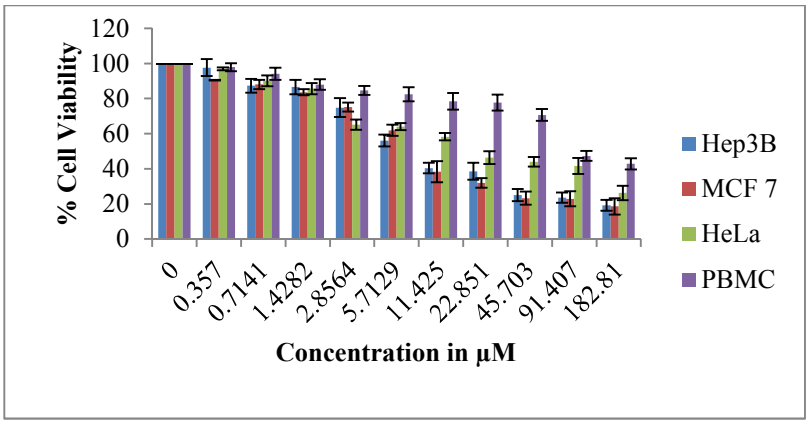
heterocyclic compounds **4-12** against cancer cell lines as well as normal cells are detailed in Table **3**, whereas the bar graph of dose-dependent effects of the compounds (**4-12**) as displayed in Figure **1**.



Compound 4



Compound 5



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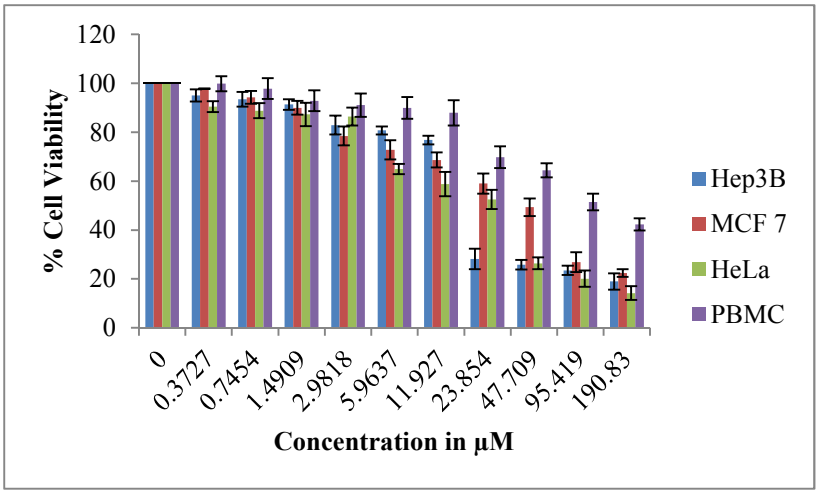

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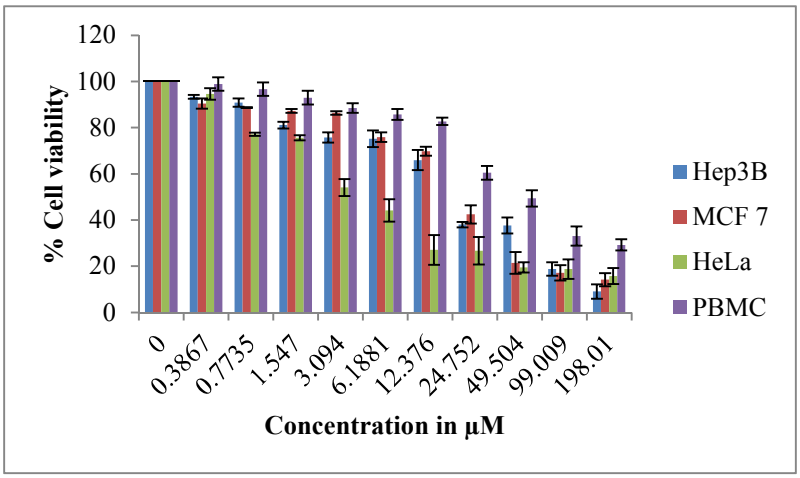
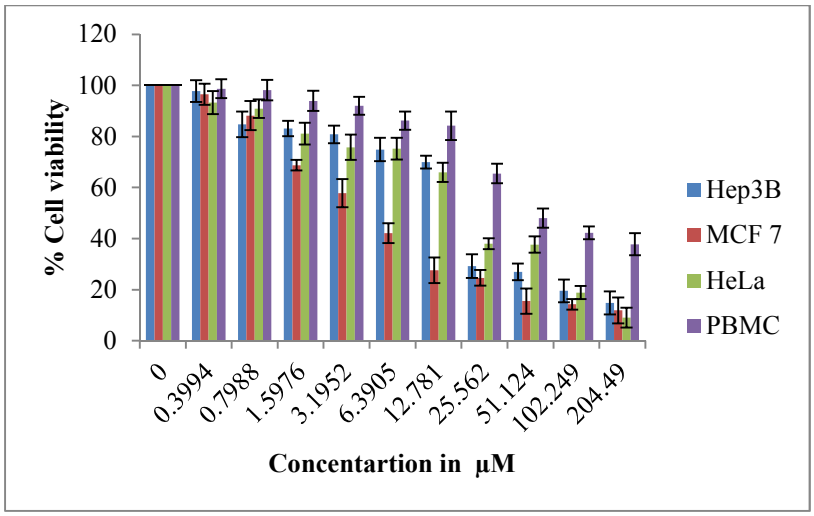
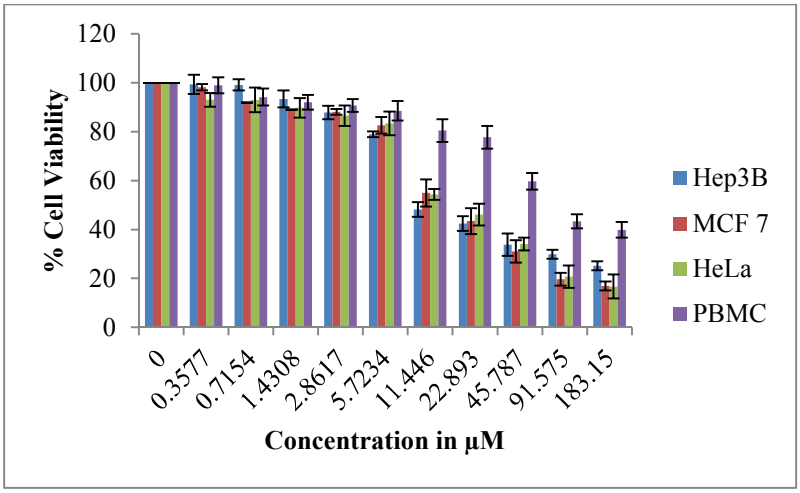


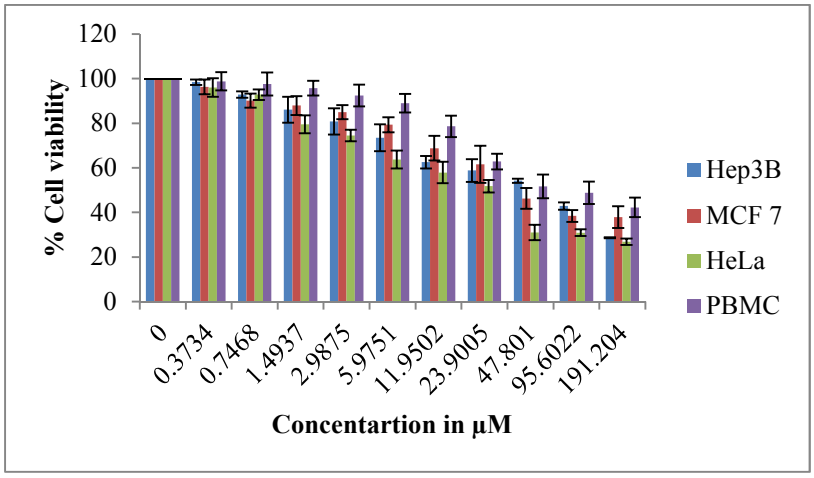
Compound 8



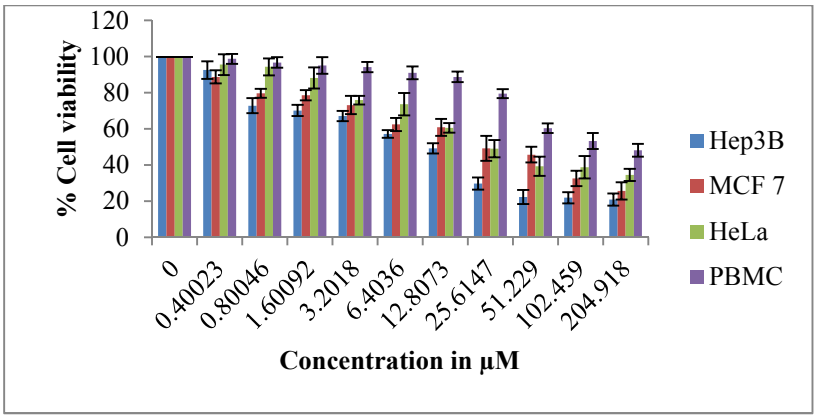
Compound 9



Compound 10



Compound 11



Compound 12

Figure 1. Dose-dependent effects of steroidal heterocyclic compounds (**4-12**) on cell viability of **HeLa**, **Hep3 B**, **MCF 7** cancer cell lines and **PBMCs** cell. Data shown are mean ± standard error of at least three independent experiments.

A number of correlations can be made from the data given in the Table **3**. It is evident from the IC_{50} values, that all the compounds showed moderate to good activity while compounds **5**, **6**, **8**, **9** and **12** elicited a marked inhibitory activity (IC_{50}< 19 µM) against all three cell lines.

Table 3. The cytotoxicity data of steroidal heterocyclic derivatives (**4-12**).

	IC_{50} (µmol L^{-1})			
Comp.	Hep3 B	MCF 7	HeLa	PBMC
4	16.94±1.6	21.70±1.5	12.20±1.3	51.31±2.1
5	09.03±1.3	09.83±2.1	19.40±1.5	53.32±1.3
6	12.70±1.6	15.06±2.2	12.30±1.4	52.22±1.5
7	21.20±1.2	23.60±2.4	24.40±2.1	54.12±2.4
8	21.18±1.4	18.21±.1.7	11.11±1.5	50.17±1.8
9	19.12±1.7	08.26±1.6	18.80±1.3	51.11±1.7
10	19.05±1.6	21.20±2.5	22.30±3.1	56.38±1.5
11	29.19±2.5	24.17±2.4	22.13±1.7	52.24±1.1
12	11.80±2.3	17.80±1.6	20.05±2.3	54.21±1.9
Dox[a]	04.16±1.6	06.18±2.1	06.80±2.5	-
5-Fu[b]	01.95±2.5	04.52±1.3	02.78±1.1	-

Standard drugs used for reference.
[a] Doxorubicin.
[b] 5-Fluorouracil.

All the compounds were found to be nontoxic to normal cells (IC_{50}>50 µM). It is noteworthy point that compound **6** was found to be active against all the three cancer cell lines. Compound **8**

was specific to **HeLa** cells and compound **9** showed selectivity towards **MCF 7** cells while compound **12** was found to be effective against **Hep3 B** cells. It might be concluded that substituents at 3*β*-position as well as heterocyclic moiety (S, O, NH) played a key role in determining activity. It is manifested from the data that chloro derivative (compound **5**) with S in the heterocyclic ring showed marked inhibitory activity against **Hep3 B** and **MCF 7** cell lines (IC_{50}: 09.03 μM; **Hep3 B**, 09.83 μM; **MCF 7**) while delta-5 derivative containing oxygen was most active against **MCF 7** (compound **9**, IC_{50}: 08.26 μM). This might be attributed to their differences in either polarity which changes their lipophilicity or the conformation which alters the target protein binding properties present within the cell or on the cell membrane. Although the exact reason of such kind of behaviour of compounds is unknown and there is no definite trend in structure activity relationship. Yet it gives useful information regarding SARS.

Antioxidant activity

By DPPH assay

The *in vitro* antioxidant activity and scavenging effects of steroidal compounds **4-12** were evaluated by using different reactive species assay containing DPPH radical scavenging activity. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability.[22] DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[23] The reduction capability of DPPH radicals was determined by a decrease in their absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants, because of reaction between antioxidant molecules and radical, progresses, which result in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow. It has been documented that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic

compounds (e.g., hydroquinone, pyrogallol, gallic acid), reduce and decolorize 1, 1-diphenylpicrylhydrazyl (DPPH) by their hydrogen donating capabilities. The free radical scavenging activity of all the synthesized compounds **4-12** were evaluated through their ability to quench the **DPPH.** using ascorbic acid as a reference. The potencies for the antioxidant activity of compounds **4-12** to the reference compound are shown in Table **4** and Figure **2**.

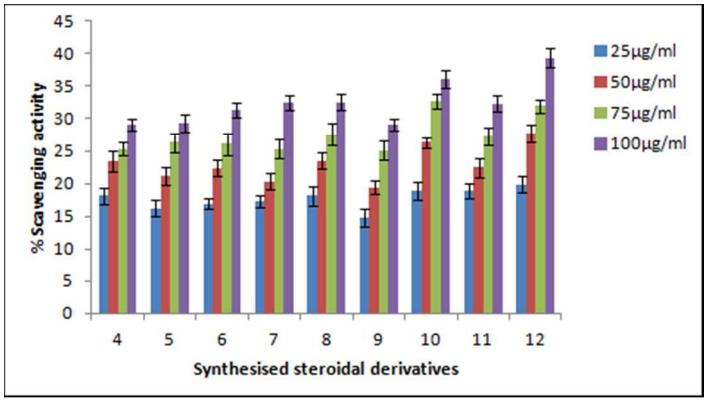


Figure 2. Concentration dependent antioxidant activities of steroidal heterocyclic compounds (**4-12**). Data shown are mean ± standard error of at least three independent experiments.

In general, all the synthesized compounds were less potent than ascorbic acid as the reference. Among these compounds, **10, 11** and **12** exhibited a slightly better antioxidant activity, with the strongest being observed in compound **10** and **12**.

Table 4. The DPPH antioxidant activity of compounds[a] (**4-12**).

	% inhibition			
Comp.	25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
4	18.12±1.20	23.41±1.60	25.32±1.10	29.14±0.91
5	16.23±1.20	21.14±1.40	26.31±1.50	29.34±1.30
6	16.87±0.81	22.34±1.20	26.13±1.70	31.42±1.10
7	17.23±0.90	20.33±1.20	25.31±1.50	32.56±1.10
8	18.13±1.40	23.52±1.30	27.56±1.70	32.54±1.20
9	14.74±1.40	19.45±1.10	25.12±1.50	29.13±0.91
10	18.84±1.30	26.34±0.79	32.76±1.10	36.13±1.30
11	18.92±1.20	22.45±1.50	27.24±1.30	32.34±1.20
12	19.89±1.30	27.67±1.40	31.94±1.10	39.34±1.50
Standard control[b]	38.23±1.20	39.00±1.10	47.23±1.30	55.23±1.50
	-	-	-	-

[a]Values represent the mean ± standard error mean (SEM) of three experiments.

[b]No inhibition, standard: ascorbic acid.

By Ferric reducing antioxidant power (FRAP) assay

The FRAP assay mainly depends on the reducing capacity of Fe^{3+} to Fe^{2+} conversion and serves as a significant indicator of its potential antioxidant activity. The antioxidant activities have been attributed to various reactions, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continuous proton abstraction and radical scavenging activity.[24] It has been also proved that the potential antioxidants through *in vitro* ferric reducing antioxidant power assay increased the total antioxidant capacity of blood plasma.[25] In addition, the reducing capacity measures the ease of the compounds in donating electrons that terminates the oxidation chain reaction by reducing the oxidized intermediates into the stable form.[26] The reducing power scavenging activity of all the synthesized compounds **4-12** were evaluated through their ability by radical quenching (H transfer) using trolox as a reference and the free radical scavenging power of compounds increased with an increasing concentration. The reducing power scavenging activity of the compounds **4-12** to the reference compound are shown in Table **5** and Figure **3**.

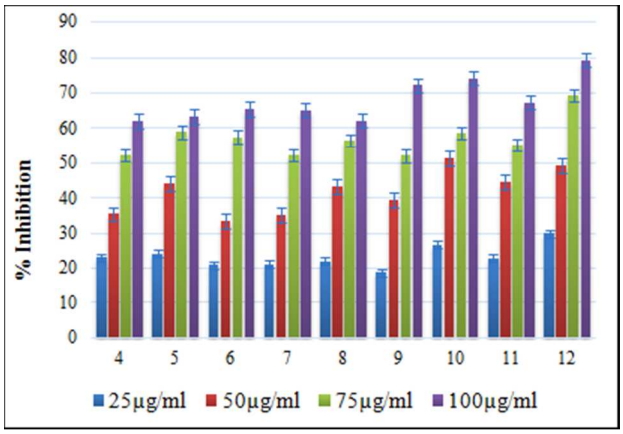


Figure 3. Concentration dependant FRAP assay of steroidal heterocyclic compounds (**4-12**). Data shown are mean ± standard error of at least three independent experiments.

Among the tested compounds, many compounds showed significant reducing power capacities. But compounds **10-12** showed good reducing power capacities while all other compounds exhibited moderate activity in comparison with the standard trolox. The good reducing power capacity of these compounds might be due to the presence of imidazole functionalities.

Table 5. The reducing power scavenging antioxidant activity of compounds[a] (**4-12**).

	% inhibition			
Comp.	25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
4	23.12±3.20	35.41±2.60	52.32±3.10	62.14±3.91
5	24.23±2.20	44.14±3.40	58.91±2.50	63.34±3.30
6	20.87±2.81	33.54±2.20	57.53±3.70	65.56±4.10
7	21.23±1.90	35.33±2.20	52.31±3.50	65.16±4.10
8	22.13±2.40	43.32±2.30	56.56±2.70	62.24±3.90
9	18.74±2.40	39.45±2.10	52.12±3.50	72.13±3.91
10	26.84±1.30	51.34±2.79	58.76±3.10	74.13±3.30
11	22.92±2.20	44.45±3.50	55.24±2.30	67.34±3.20
12	29.89±2.30	49.45±2.40	69.34±3.10	79.34±3.50
Standard	39.23±1.20	51.01±1.10	77.23±1.30	85.23±1.50
control[b]	-	-	-	-

[a]Values represent the mean ± standard error mean (SEM) of three experiments.
[b]No inhibition, standard: trolox.

By ABTS assay

The ABTS radical cation decolorization assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and chain-breaking antioxidants.[27,28] ABTS is another widely used synthetic radical for both the polar and non-polar samples. The $ABTS^{\bullet+}$ scavenging abilities of the compounds **4-12** were also evaluated and reported in Table **6**.

Table 6. The radical scavenging antioxidant activity of compounds[a] (**4-12**).

Comp.	% inhibition 25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
4	18.32±1.2	29.51±1.9	39.32±2.8	54.14±2.81
5	19.23±2.4	34.14±2.7	48.91±2.9	58.34±3.80
6	20.87±2.7	31.54±2.4	41.52±3.3	55.56±3.20
7	20.23±1.9	27.33±2.6	42.33±3.4	55.17±3.90
8	22.13±2.4	29.32±2.5	36.56±2.4	53.24±3.60
9	19.84±2.7	29.47±2.5	39.42±3.5	56.13±3.30
10	23.14±3.3	41.24±2.7	51.16±3.3	64.43±3.50
11	21.94±2.3	34.45±3.1	45.54±3.3	61.53±3.40
12	23.29±2.5	49.67±3.3	57.14±2.9	68.24±3.10
Standard	37.25±1.2	52.01±1.1	61.24±1.3	75.23±1.50
control[b]	-	-	-	-

[a]Values represent the mean ± standard error mean (SEM) of three experiments.
[b]No inhibition, standard: ascorbic acid.

The radical has a relatively stable blue-green color, which equates to absorbance at 734 nm. Antioxidant compounds reduce the intensity of this color to a degree that is proportionate to their antioxidant concentration or activity. Figure **4**, depicts the scavenging activities of the compounds **4-12** against ABTS radicals.

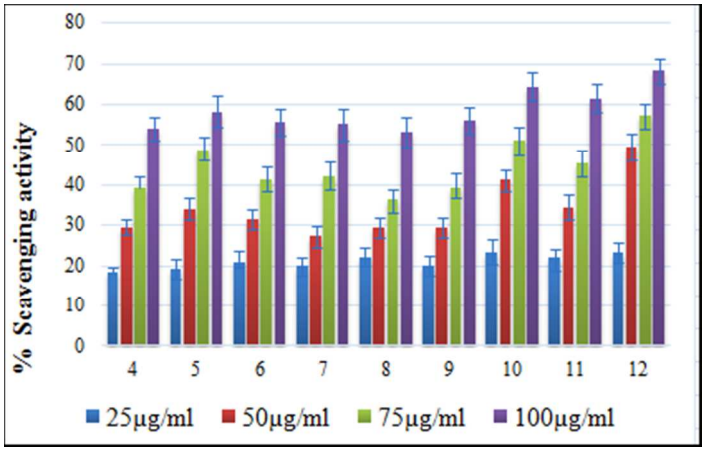


Figure 4. Concentration dependant ABTS assay of steroidal heterocyclic compounds (**4-12**). Data shown are mean ± standard error of at least three independent experiments.

These data demonstrate that scavenging activity increased as the concentration increased. The compounds **10-12** had a maximum scavenging activity among all in the order of **12 > 10 > 11**. It was interesting to note that this order is in concordance with the DPPH and FRAP assay.

Fluorescence Microscopy

HeLa cells treated with compounds **4-12** were observed under Fluorescence Microscope and microscopic examination of gross morphology of the treated and untreated cancer cells are shown in Figure **5**. These images clearly designated reduction in cancer cell count as well as cytotoxicity potential of above mentioned steroids, in the order of **8 > 4 > 6 > 9 > 5 > 12 > 11 > 10 > 7** and arrow indicates apoptosis of cells.

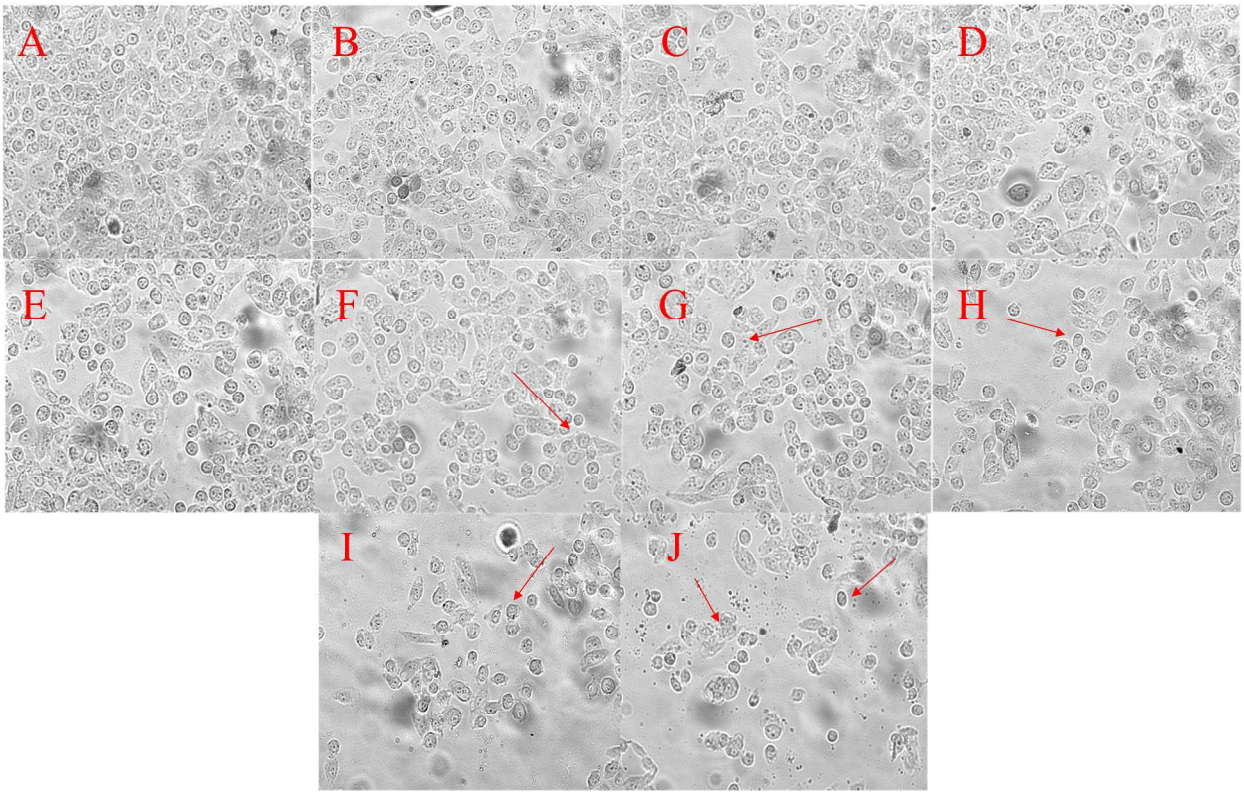
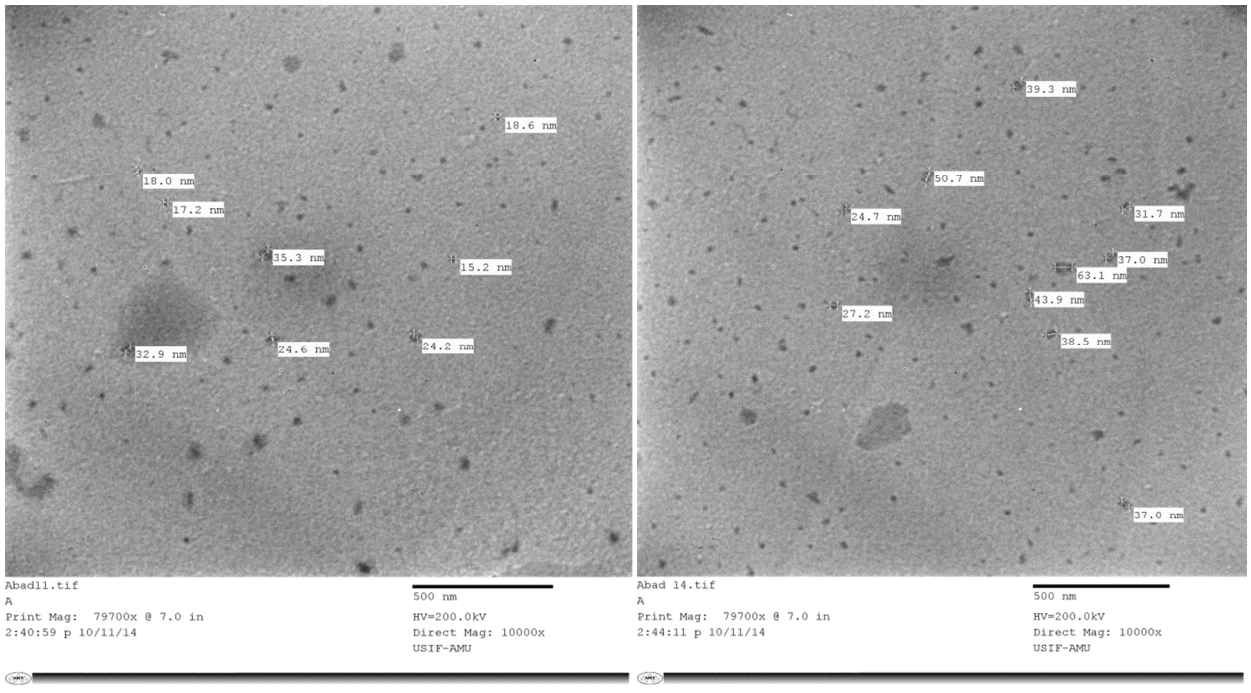


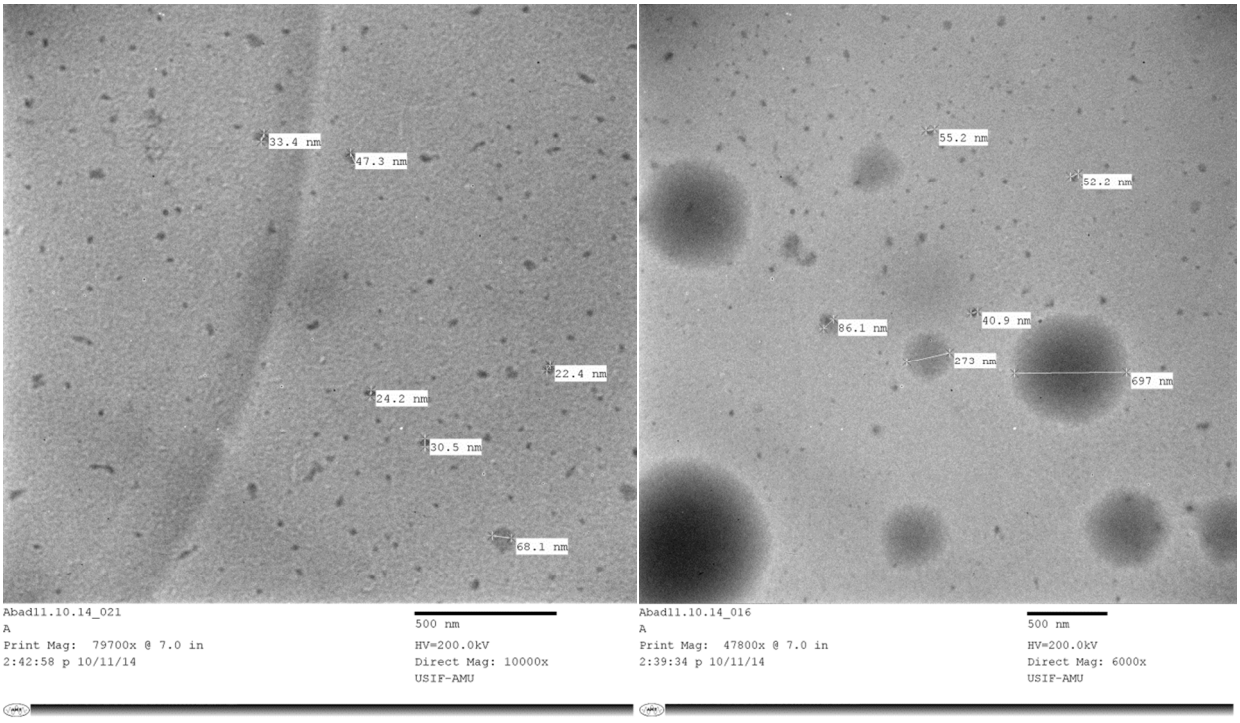
Figure 5. Micrographs showing effect of steroidal compounds **4-12** on **HeLa** Cells. Micrographs captured after 48 h of incubation in bright field under Fluorescence Microscope. **A** = Untreated control, **B** = Treated with compound **7**, **C**= Treated with compound **10, D**= Treated with compound **11, E**= Treated with compound **12, F**= Treated with compound **5, G**= Treated with compound **9, H**= Treated with compound **6, I**= Treated with compound **4** and **J**= Treated with compound **8.**

DNA condensation

DNA condensation is an essential process to transport a therapeutic gene to its target. It includes the collapse of extended DNA chain into compact, orderly particles containing one or more molecule.[29] Steroidal compounds are able to recognize and bind to single or double-stranded DNA with high affinity and selectivity due to structural hydrophobicity.[30] In this study, DNA condensation to compound **6** was determined by employing various visualization techniques *viz* transmission electron microscopy (TEM) and atomic force microscopy (AFM).[31] These techniques have been used here to analyze the morphology of the steroidal-DNA complex condensates. The TEM images of the compound are given in Figure **6**.



(a) **(b)**



(c) **(d)**

Figure 6. The TEM images of the compound **6** before CT-DNA condensates (a) and after CT-DNA condensates (b), (c) and (d).

The shape of the particles is irregular and the particles are of variable diameter ranging from 15.2 to 697 nm. The particles size increases when CT-DNA condensed to it indicating that the compound **6** can condense fragments of CT-DNA to compact solid DNA drug condensate as shown in Figure **6**(**a-d**). Several numbers of drugs are unable to be effective due to the lack of information of their structural and morphological details.[32] To obtain the morphological and structural information of the compound and the condensates, tapping mode AFM experiments were performed using commercially etched silicon tips as AFM probes. Two and three dimensional AFM images of the compound and the condensate materials are shown in Figures **7**.

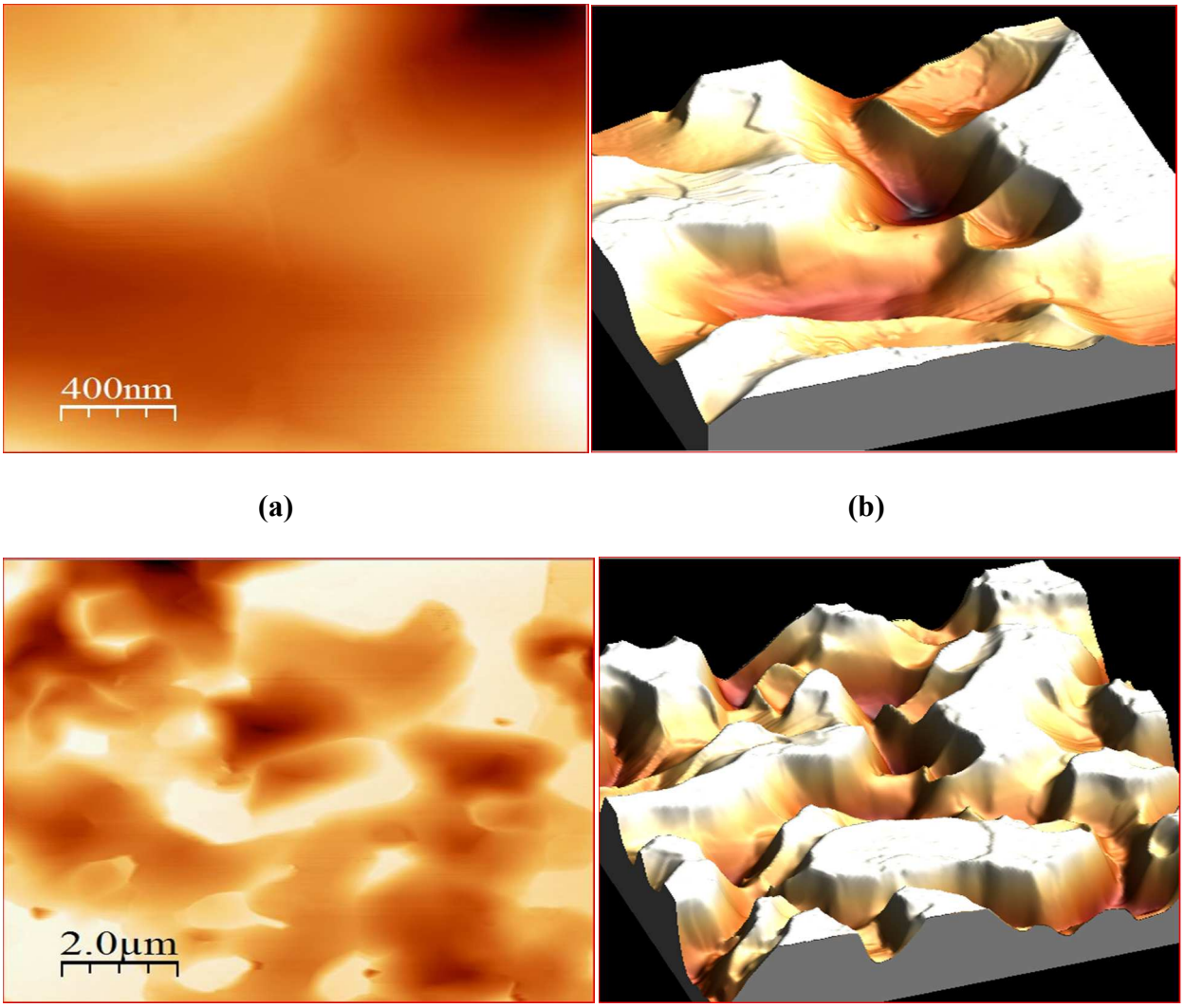


Figure 7. The AFM images of the compound **6** before CT-DNA condensates (a) 2D (Two dimensions) and (b) 3D (Three dimensions) and after CT-DNA condensates (c) 2D (Two dimensions) and (d) 3D (Three dimensions).

The change in the morphological structure of compound **6** and its condensates with CT-DNA clearly validate that the complex is facilitating the DNA condensation.

Nonenzymatic DNA damage

The mechanisms governing the anticancer actions of steroid derivatives are not fully known. A number of pathways have been studied regarding the cytotoxic activity of steroids.[33] Our

experiments suggested that the cell death may be due to cleavage or fragmentation of DNA of cancer cells and the active species responsible for this are ROS (·OH) which is confirmed from the *in vitro* reaction of different concentrations of compound **6** with copper in presence of thiobarbituric acid (**Figure 8**).

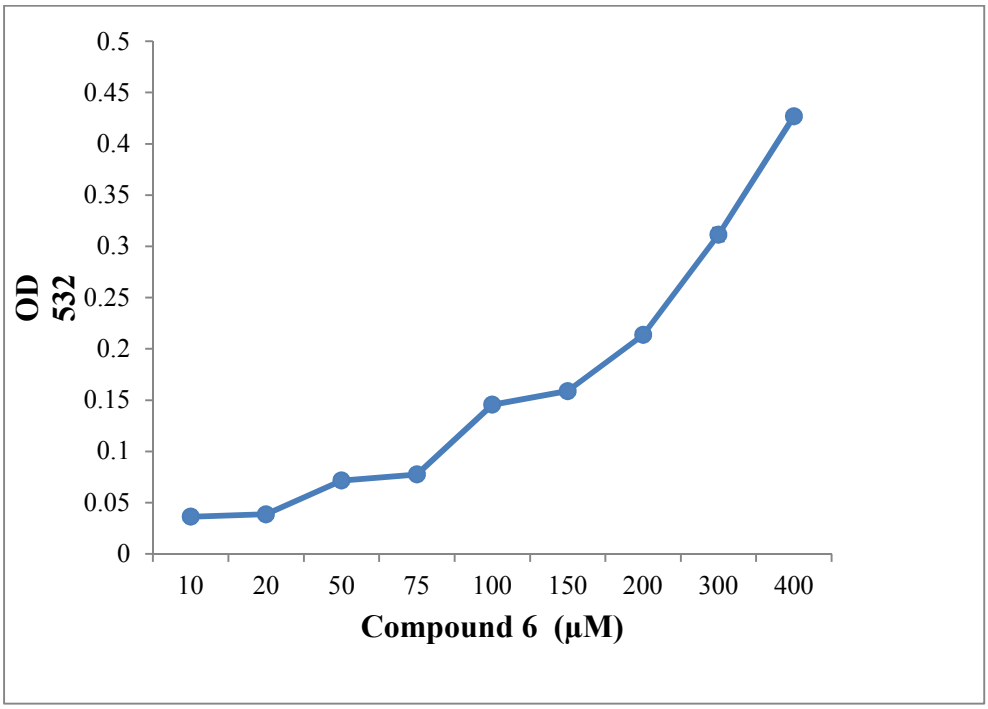
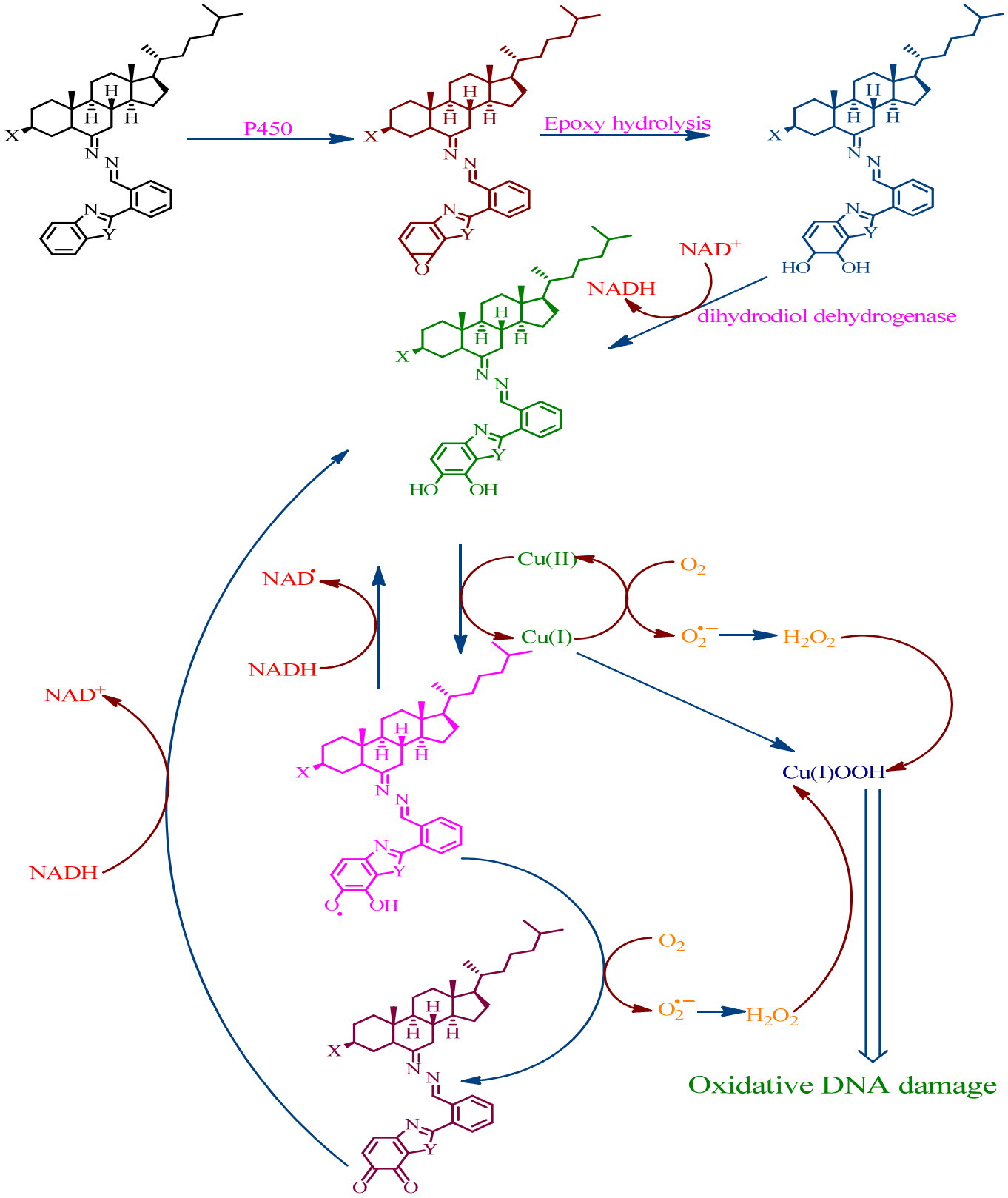


Figure 8. Determination of hydroxyl radical production of compound **6** by assay thiobarbituric acid.

The results confirmed the relatively higher rate of formation of hydroxyl radicals and correlated with the rate of DNA degredation by the compound **6**. The proposed mechanism of DNA damage is believed to occur via redox cycling (**Scheme 3**). In the DNA cleavage reactions mediated by various antioxidants in the presence of Cu (II), it has been well known that Cu (II) is reduced to Cu (I) by the antioxidant which is an essential intermediate in the DNA cleavage reactions.[34-36] It is also generally understood that DNA cleavage by various antioxidants and Cu (II) is the result of the generation of hydroxyl radicals. Cu (II) is reduced to Cu (I) and the re-

oxidation of Cu (I) to Cu (II) by molecular oxygen gives rise to superoxide anion which in turn leads to the formation of H_2O_2.[37] Studies have also shown that Cu (II) has a high affinity to DNA, preferentially binding to guanine residues at the N7 position.[38] This Cu (II)-DNA interaction has been shown to promote DNA oxidation and the resulting damage enhanced by packaging of DNA as a nucleosome.[39]



Scheme 3. Proposed mechanism of oxidative DNA damage of steroidal compounds.

In vitro acetylcholinesterase inhibition activity

The anti-cholinesterase effects of the compounds (**4-12**) were determined by modified Ellman's spectrophotometric method using AChE from Electrophorus electricus with Tacrine as reference compound. All the compounds were carefully measured and the results are shown in Table **7**. The results were collected from at least three independent measurements. From the results obtained, compound **4, 7** and **10** (IC_{50} = 0.31 ± 0.10, 0.37 ± 0.02 and 0.39 ± 0.03, respectively) exhibited significant inhibition on AChE among all the compounds. The improved activity of the acetoxy derivatives in comparison to all synthesized compounds can be explained on the basis of its skeleton and electronic properties at 3*β*-position of the cholestane ring. The presence of acetoxy group at C-3 of cholestane skeleton increases activity due to the formation of additional non-classical bonds with amino acid residues of the protein and easily performs as guest relation with receptor protein (host). The synthesized compounds were found to be fairly active with respect to the reference drug, Tacrine.

Table 7. *In vitro anti*-AChE activities data of the synthesized compounds (**4-12)** and reference drug (Tacrine).

Comp.	AChE inhibitor IC_{50} (μM)[a]
4	0.31 ± 0.10
5	0.52 ± 0.30
6	0.53 ± 0.01
7	0.37 ± 0.02
8	0.49 ± 0.02
9	0.59 ± 0.10
10	0.39 ± 0.03
11	0.48 ± 0.02
12	0.56 ± 0.20
Tacrine	0.29 ± 0.06

[a]The values are mean of three independent experiments ± SD.

Molecular docking

Molecular docking is one of the important computational chemistry techniques that are routinely used for drug discovery processess. Molecular modelling studies of nucleic acids and their

complexes can provide valuable information that is not available by other experimental techniques such as X-ray crystallography or NMR spectroscopic data. The conformational diversity of DNA allows for large deformations upon binding of ligands to it. Drug-DNA interactions are of high pharmaceutical interest since the mode of action of various drugs is directly associated with their binding to DNA. A reliable prediction of drug-DNA binding at the atomic level by molecular docking methods provides the basis for the design of new drug compounds.[40,41] Targeting the minor groove of DNA by small molecules has long been considered as an important tool in molecular recognition of specific DNA sequence.[42,43] Molecular modeling allows the flexibility within the ligand to be modeled that can utilize more detailed molecular mechanics to calculate the relative binding energy of the ligand-receptor complex in the context of the putative active site. In our experiment, structure of compounds (**4-12**) was made flexible to attain different conformations in order to predict the best fit orientation of compounds with DNA duplex of sequence d(CGCGAATTCGCG)$_2$ dodecamer (PDB ID:1BNA) which provides an energetically favorable docked pose. Table **8** shows the minimized orientation of minor groove interaction of compounds **4-12** with DNA and the resulting relative binding energy of the docked pose. The minimized conformation of only compound **10** sitting in the groove of the sequence d(CGCGAATTCGCG)$_2$ show hydrogen bonding with the NH of the imidazole ring in the docked pose of the dodecamer. While all other compounds do not show any possible hydrogen bonding with the base pairs of the DNA dodecamer. Generally, the more negative the relative binding energy, the stronger is the interaction between molecule and DNA and more stable is the complex formed. Thus the complex formed between compound **7** and DNA with relative binding energy of -8.338 kcal/mol might be having highest stability.[44-47] The relative binding energies of compound **10** and **4** with DNA are almost equal suggesting that both

are forming equally stable complex with DNA and the presence of hydrogen bonds between compound **10** and DNA might not be affecting the stability of the complex. Irrespective of the absence of any net positive charge on compounds **4-12**, negative values of the relative binding energies indicated a higher binding potential of these compounds with DNA. Hence, these docking results provide valuable information about the mode of interaction of our compounds with DNA and the conformation constraints for adduct formation.

Table 8. Molecular docked models of steroidal compounds (**4-12**).

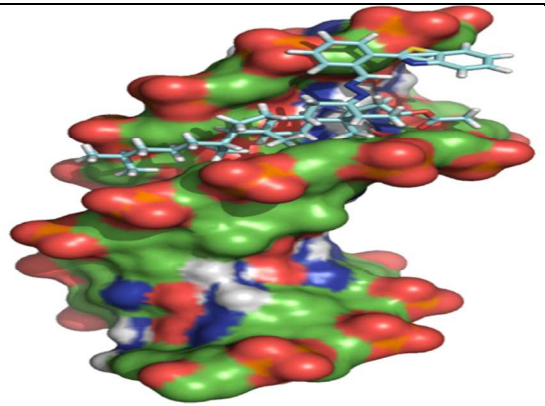
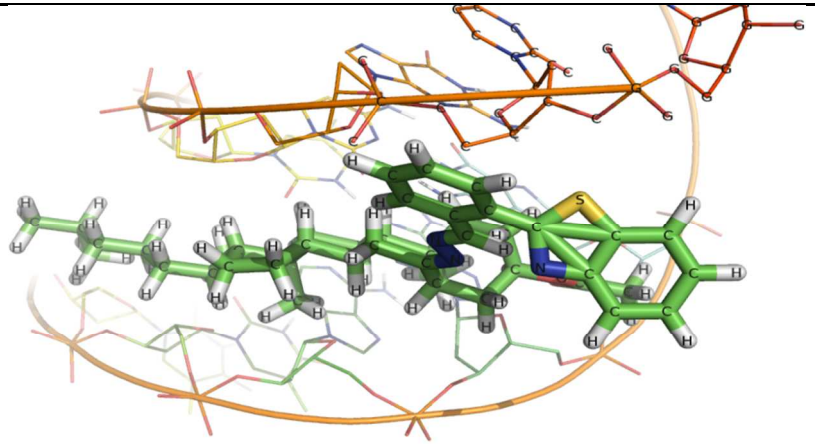
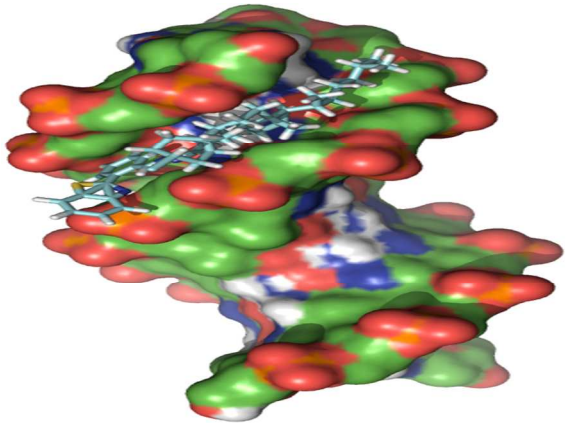
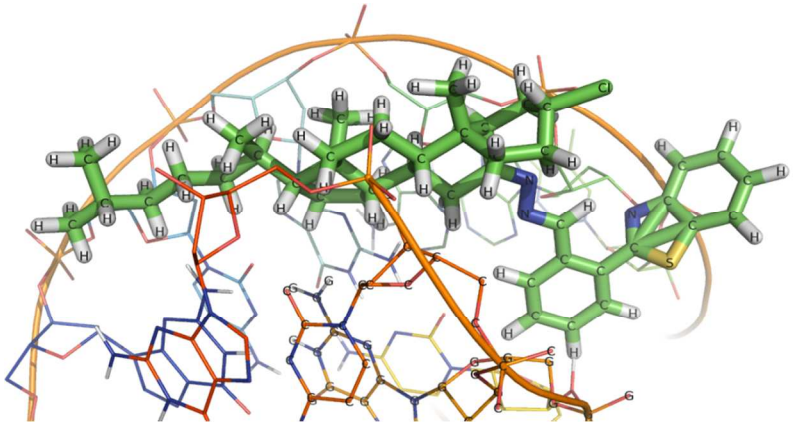
Product	Docked pose	Relative binding energy (kcal/mol)	Stereoview of the docked pose Showing possible H-bonding
4	 **4a**	-8.276	 **4b**
5	 **5a**	-7.953	 **5b**

Table 8. *(continued)*

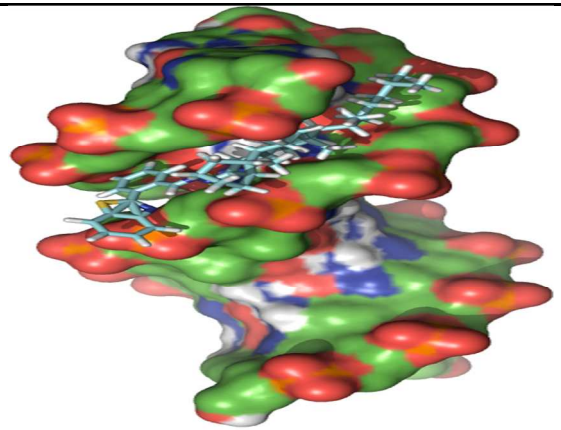
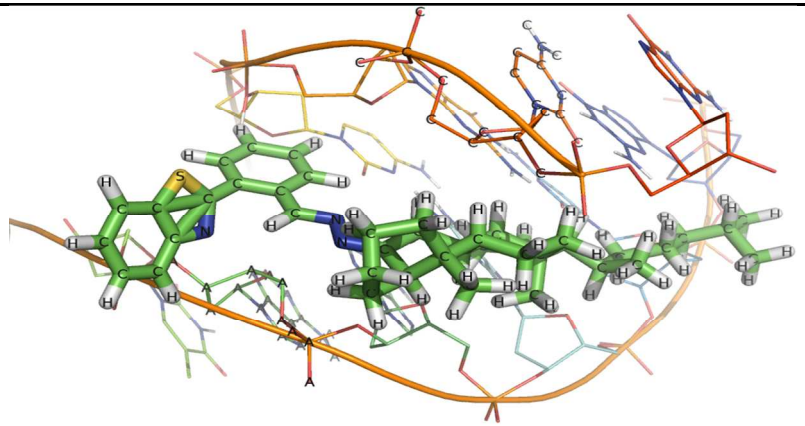
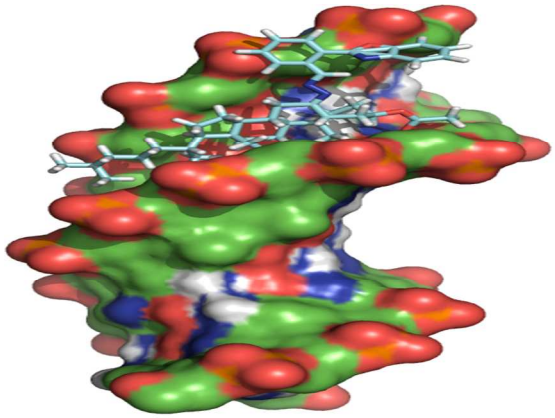
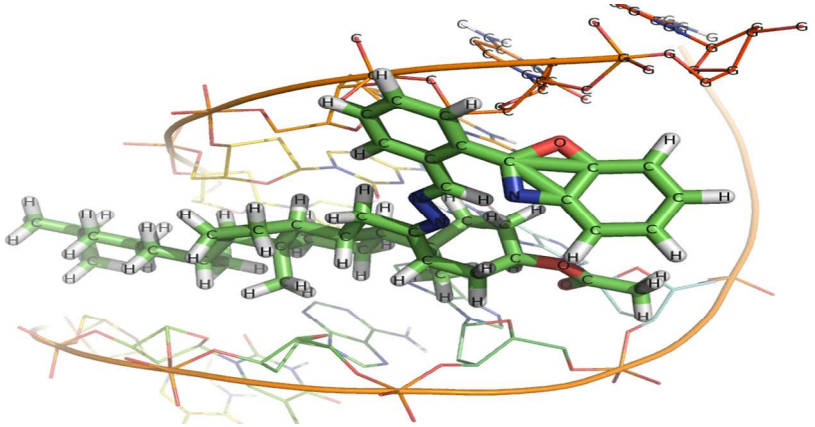
Product	Docked pose	Relative binding energy (kcal/mol)	Stereoview of the docked pose Showing possible H-bonding
6	 **6a**	-7.925	 **6b**
7	 **7a**	-8.338	 **7b**

Table 8. *(continued)*

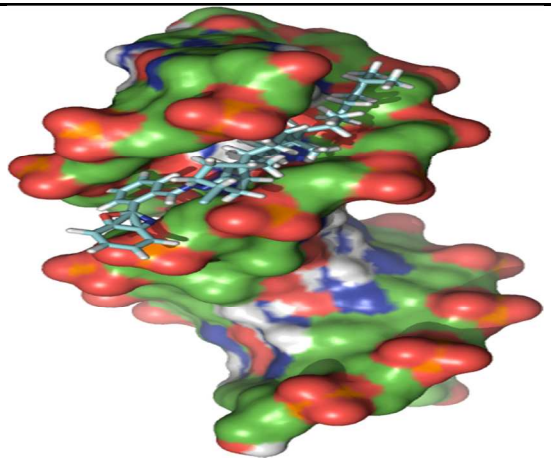
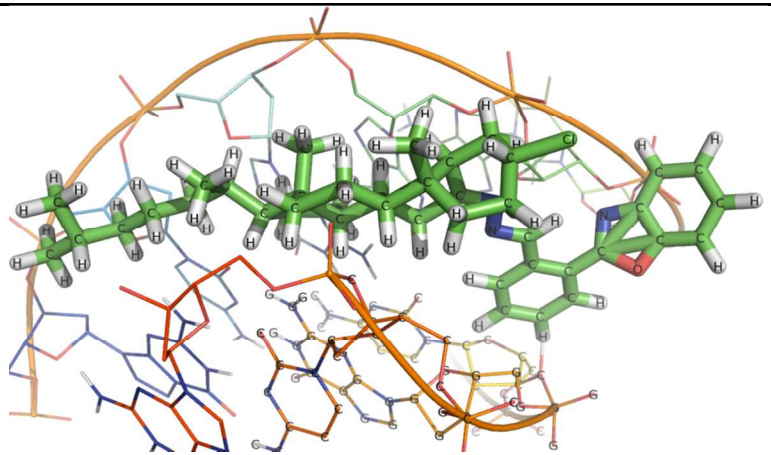
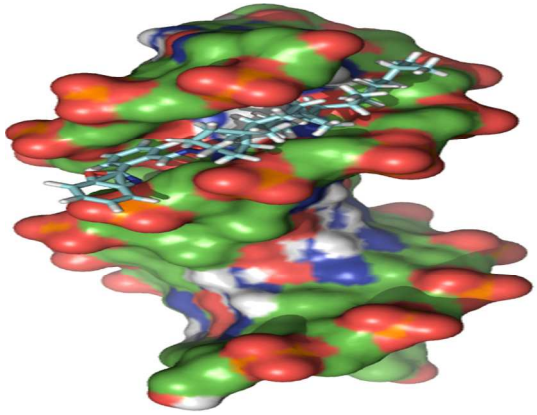
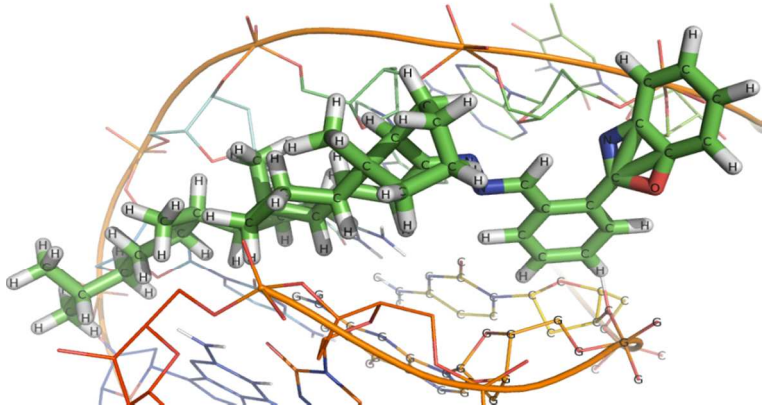
Product	Docked pose	Relative binding energy (kcal/mol)	Stereoview of the docked pose Showing possible H-bonding
8	 **8a**	-7.890	 **8b**
9	 **9a**	-7.930	 **9b**

Table 8. *(continued)*

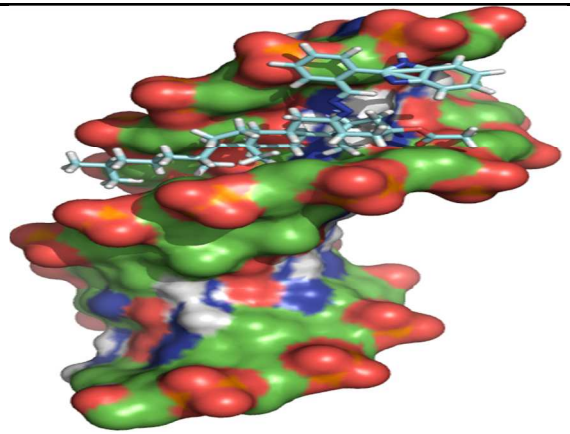
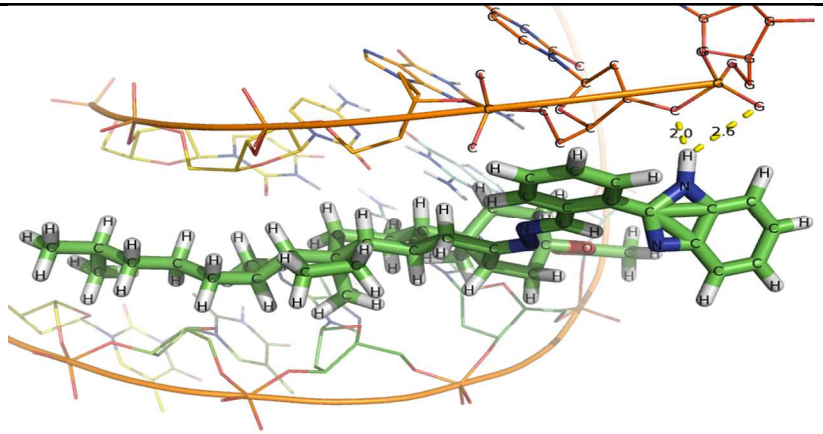
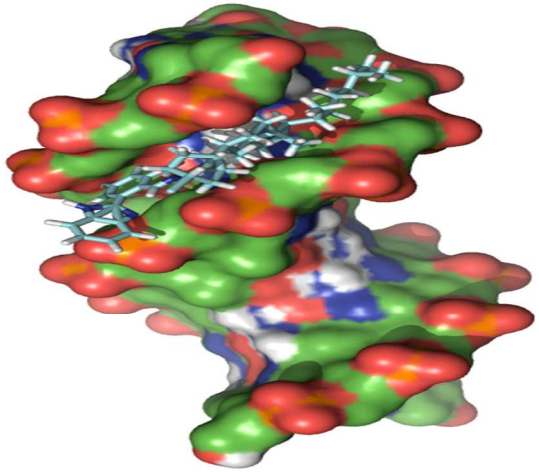
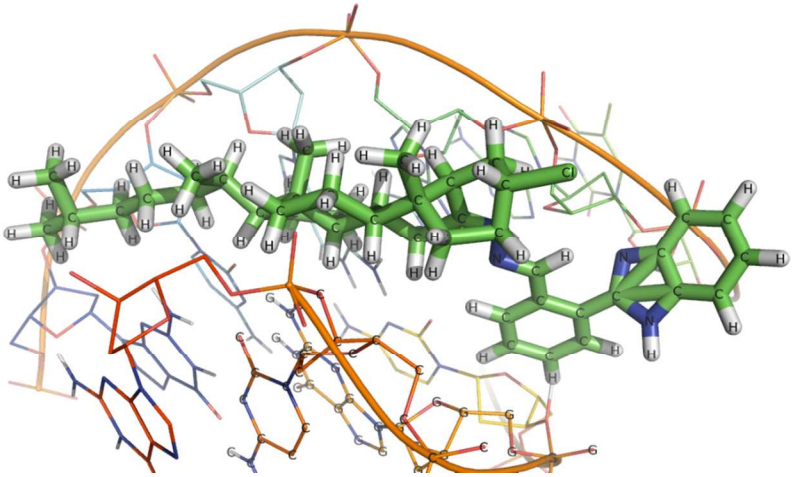
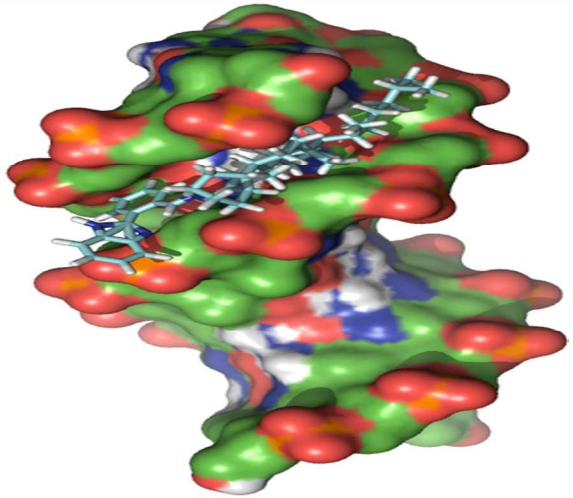
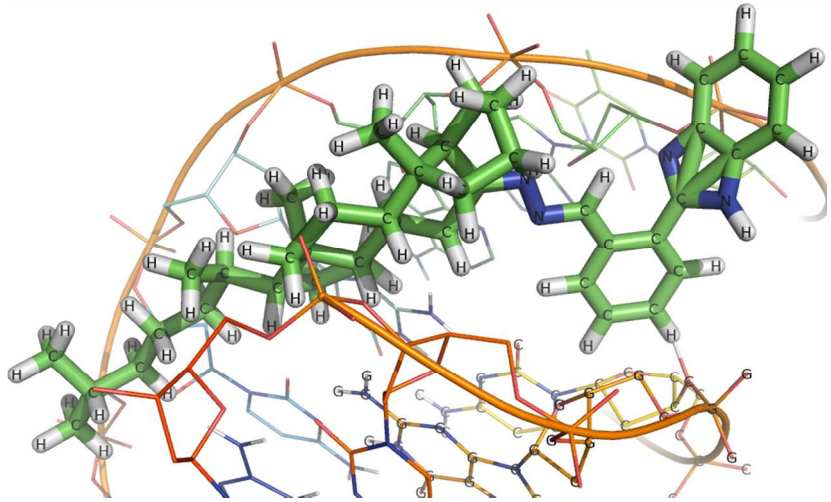
Product	Docked pose	Relative binding energy (kcal/mol)	Stereoview of the docked pose Showing possible H-bonding
10	 **10a**	-8.277	 **10b**
11	 **11a**	-8.026	 **11b**

Table 8. *(continued)*

Product	Docked pose	Relative binding energy (kcal/mol)	Stereoview of the docked pose Showing possible H-bonding
12	 **12a**	-8.140	 **12b**

The S-, O- and N- termini of the compounds are shown as yellow, red and blue sticks, respectively.
Docked pose showing the minor groove interaction of compounds **4-12** with DNA dodecamer duplex of sequence d(CGCGAATTCGCG)$_2$ (PDB ID:1BNA) and the resulting relative binding energy of the docked DNA-compounds (**4-12**) complex.

Experimental

Materials and methods

All glass apparatus were oven-dried prior to use. Chemicals and solvents used in this study were of ACS grade and used directly without additional steps of purification. Melting points were determined on a Biogen digital auto melting point apparatus. The IR spectra were recorded on KBr pellets with Perkin Elmer FT-IR Spectrometer spectrum Two and values are given in cm^{-1}. ^{1}H and ^{13}C NMR spectra were run in $CDCl_3$ on a Bruker Avance II 400 NMR Spectrometer (operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C NMR) with TMS as internal standard and values are given in parts per million (ppm) (δ). Mass spectra were recorded on a JEOL D-300 Mass spectrometer. Elemental analyses were recorded on Perkin Elmer 2400 CHN Elemental Analyzer. Thin layer chromatography (TLC) plates were coated with silica gel G and exposed to iodine vapours to check the homogeneity as well as the progress of reaction. Sodium sulfate (anhydrous) was used as a drying agent.

*General method for the synthesis of steroidal ketones (**1c-3c**)*

3β-acetoxy-5α-cholestan-6-one,[48] 3β-chloro-5α-cholestan-6-one,[49] 5α-cholestan-6-one[50] and compounds **1(a, b)**, **2(a, b)** and **3(a, b)** were prepared according to the literature procedure.[15f]

*General method for the synthesis of steroidal hydrazones (**1d-3d**)*

The steroidal ketones (**1c-3c**) reacted with hydrazine hydrate in ethanol solution in the presence of few drops of acetic acid in cold conditions. After completion of the reaction (monitored by TLC), the precipitate thus obtained was filtered, washed with water, air dried and recrystallized from methanol to afford respective products (**1d-3d**).[51]

*General method for the synthesis of steroidal hydrazone derivatives (**1e-3e**)*

To a solution of steroidal hydrazone (**1d-3d**) (1.5 mmol) in absolute ethanol (20 ml), phthalaldehyde (1.5 mmol) was added. Then the reaction mixture was stirred at 70 ^{o}C. After completion of reaction (monitored by TLC), solvent was evaporated under reduced pressure. The residue was purified by recrystallization to afford the corresponding target products (**1e-3e**).

*3β-Acetoxy-5α-cholestan-6-(iminohydrazono)-2-methylbenzaldehyde (**1e**)*

Yield(82%), Mp: 166-168 ˚C; IR (KBr, ν cm^{-1}): 3080, 1567, 1394 (C-H aromatic), 1735 ($OCOCH_3$), 1630 (C=N), 1057, 1059 (C-O), 1696 (-CHO); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.9-8.3 (m, 5H, aromatic), 10.4 (s, 1H, aldehyde), 4.77 (m, 1H, C_3-αH, $W_{1/2}$ = 17 Hz, axial), 2.01 (s, 3H, $OCOCH_3$), 1.15 (C_{10}-CH_3), 0.73 (C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 194.6, 172.3, 162.7, 144.3, 137.6, 133.3, 131.9, 129.2, 128.7, 127.4, 71.1, 60.6, 58.3, 53.8, 43.6, 40.5, 39.2, 38.8, 36.3, 35.7, 34.6, 32.3, 31.9, 30.6, 29.2, 26.7, 25.4, 24.8, 23.5, 22.6, 21.4, 20.1, 19.5, 17.4, 16.3, 15.4, 13.7; Anal. Calcd for $C_{37}H_{54}N_2O_3$; C, 77.31; H, 9.47; N, 4.87 found; C, 77.33; H, 9.49; N, 4.85; MS (EI): *m/z* 574 [$M^{+\cdot}$].

*3β-Chloro-5α-cholestan-6-(iminohydrazono)-2-methylbenzaldehyde (**2e**)*

Yield (79%), Mp: 160-162 ˚C; IR (KBr, ν cm^{-1}): 3081, 1560, 1388 (C-H, aromatic), 1627 (C=N), 740 (C-Cl), 1700 (-CHO); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.9-8.3 (m, 5H, aromatic), 10.5 (s, 1H, aldehyde), 3.71 (m, 1H, C_3-αH, $W_{1/2}$ = 15 Hz, axial), 1.15 (C_{10}-CH_3), 0.73 (C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 196.7, 159.8, 142.3, 139.4, 137.3, 135.8, 132.2, 127.6, 125.3, 58.7, 57.4, 55.8, 52.2, 47.4, 45.2, 41.5, 40.2, 37.1, 36.3, 35.7, 34.6, 32.4, 32.9, 31.1, 29.9, 26.7, 25.1, 23.3, 21.7, 20.6, 19.2, 17.8, 16.5, 14.9, 11.5; Anal. Calcd for $C_{35}H_{51}ClN_2O$; C, 76.26; H, 9.33; N, 5.08 found; C, 76.24; H, 9.35; N, 5.09; MS (EI): *m/z* 550/552 [$M^{+\cdot}$].

*5α-cholestan-6-(iminohydrazono)-2-methylbenzaldehyde (**3e**)*

Yield (77%), Mp: 155-157 ˚C; IR (KBr, ν cm^{-1}): 3085, 1564, 1391 (C-H, aromatic), 1631 (C=N), 1702 (-CHO); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.9-8.3 (m, 5H, aromatic), 10.4 (s, 1H, aldehyde), 1.15 (s, 3H, C_{10}-CH_3), 0.73 (s, 3H, C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 195.2, 160.5, 141.7, 135.2, 134.7, 132.2, 129.8, 127.4, 126.1, 57.9, 53.3 43.0, 42.9, 39.2, 38.7, 37.9, 36.4, 34.8, 33.2, 32.4, 32.0, 30.7, 29.8, 27.6, 26.1, 25.8, 22.9, 21.5, 20.4, 19.6, 18.5, 17.7, 15.3, 12.1, 11.2; Anal. Calcd for $C_{35}H_{52}N_2O$; C, 81.34; H, 10.14; N, 5.42 found; C, 81.37; H, 10.12; N, 5.41; MS (EI): *m/z* 516 [$M^{+\cdot}$].

*General method for the synthesis of steroidal heterocyclic compounds (**4-12**)*

A mixture of steroidal hydrazone derivatives (**1e-3e**) (1mmol) and o-aminothiophenol/ o-aminophenol/ o-phenylenediamine (1.25 mmol, 1.25 equiv) in DMSO (30 mL) was refluxed for 2-6 h.[16] The progress as well as completion of reaction was monitored by TLC. After completion of the reaction, the reaction mixture was poured into water. The precipitate thus obtained was filtered, washed with water and air dried. The crude product was purified by column chromatography on silica gel column using light petroleum ether/ ether (10:1). For further purification, the compounds were recrystallized from methanol to give desirable product (**4-12**).

*3β-Acetoxy-5α-cholestan-6-(iminohydrazonomethylphenyl-2-yl)-benzo-[d]-thiazole (**4**)*

Yield(75%), Mp: 156-158 ˚C; IR (KBr, ν cm^{-1}): 3085, 1569, 1396 (C-H aromatic), 1739 ($OCOCH_3$), 1635 (C=N), 1050, 1054 (C-O), 687 (C-S-C); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.7-8.0 (m, 5H, aromatic), 4.75 (m, 1H, C_3-αH, $W_{1/2}$ = 16 Hz, axial), 2.03 (s, 3H, $OCOCH_3$), 1.15 (C_{10}-CH_3), 0.73 (C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 171.4, 161.2, 159.2, 151.1, 142.6, 135.7, 132.1, 130.2, 127.4, 126.8, 126.1, 125.9, 123.7, 122.9, 120.3, 119.9, 70.2, 58.3, 57.1, 53.8, 44.1, 42.5, 40.1, 38.1, 37.5, 35.9, 35.6, 33.2, 32.9,

28.8, 27.9, 26.1, 25.2, 24.9, 24.0, 22.9, 21.3, 21.9, 20.3, 19.7.0, 18.9, 15.1, 11.9; Anal. Calcd for $C_{43}H_{57}N_3O_2S$; C, 75.95; H, 8.45; N, 6.18 found; C, 75.91; H, 8.49; N, 6.15; MS (EI): *m/z* 679 [$M^{+\cdot}$].

*3β-Chloro-5α-cholestan-6-(iminohydrazonomethylphenyl-2-yl)-benzo-[d]-thiazole (**5**)*

Yield (72%), Mp: 162-164 ˚C; IR (KBr, ν cm^{-1}): 3086, 1562, 1393 (C-H, aromatic), 1632 (C=N), 743 (C-Cl), 685 (C-S-C); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.90-8.2 (m, 5H, aromatic), 3.91 (m, 1H, C_3-αH, $W_{1/2}$ = 14 Hz, axial), 1.15 (C_{10}-CH_3), 0.73 (C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 160.3, 157.7, 152.6, 140.8, 135.7, 134.0, 130.3, 129.1, 127.0, 127.9, 126.7, 124.2, 122.9, 120.0, 118.2, 59.6, 57.6, 55.0, 52.4, 41.4, 40.7, 39.2, 38.5, 37.9, 36.5, 35.9, 34.6, 34.0, 33.9, 31.2, 29.5, 27.2, 26.8, 23.0, 22.9, 21.6, 20.1, 20.9, 17.3, 15.1, 13.7; Anal. Calcd for $C_{41}H_{54}ClN_3S$; C, 75.02; H, 8.29; N, 6.40 found; C, 75.05; H, 8.25; N, 6.44; MS (EI): *m/z* 655/657 [$M^{+\cdot}$].

*5α-cholestan-6-(iminohydrazonomethylphenyl-2-yl)-benzo-[d]-thiazole (**6**)*

Yield (71%), Mp: 154-156 ˚C; IR (KBr, ν cm^{-1}): 3082, 1565, 1389 (C-H, aromatic), 1629 (C=N), 682 (C-S-C); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.90-8.2 (m, 5H, aromatic), 1.15 (s, 3H, C_{10}-CH_3), 0.73 (s, 3H, C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 158.7, 156.4, 151.7, 139.7, 135.9, 132.4, 130.9, 129.1, 127.4, 126.5, 125.9, 123.9, 122.6, 120.7, 118.9, 54.2, 52.9, 50.1, 45.3, 41.6, 40.2, 39.0, 37.7, 36.4, 35.3, 34.8, 33.9, 30.3, 28.1, 27.3, 26.7, 24.1, 22.9, 21.6, 21.1, 20.6, 19.5, 17.2, 16.9, 15.9, 11.5; Anal. Calcd for $C_{41}H_{55}N_3S$; C, 79.18; H, 8.91; N, 6.76 found; C, 79.21; H, 8.87; N, 6.77; MS (EI): *m/z* 621 [$M^{+\cdot}$].

*3β-Acetoxy-5α-cholestan-6-(iminohydrazonomethylphenyl-2-yl)-benzo-[d]-oxazole (**7**)*

Yield (78%), Mp: 175-177 ˚C; IR (KBr, ν cm^{-1}): 3083, 1564, 1390 (C-H, aromatic), 1736 ($OCOCH_3$), 1631 (C=N), 1053, 1059 (C-O); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 8.0-8.3 (m,

5H, aromatic), 4.79 (m, 1H, C_3-αH, $W_{1/2}$ = 17 Hz, axial), 2.02 (s, 3H, $OCOCH_3$), 1.15 (C_{10}-CH_3), 0.73 (C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 173.4, 163.4, 153.5, 145.2, 141.2, 139.5, 135.6, 130.1, 129.3, 127.9, 126.4, 123.4, 121.5, 119.4, 115.5, 108.4, 69.6, 53.2, 52.7, 50.1, 42.8, 40.2, 38.4, 37.2, 37.9, 36.1, 35.7, 34.2, 32.9, 30.9, 29.0, 28.6, 26.3, 25.1, 23.9, 22.1, 21.7, 20.9, 19.7, 17.9, 16.6, 15.4, 10.9; Anal. Calcd for $C_{43}H_{57}N_3O_3$; C, 77.79; H, 8.65; N, 6.33; found: C, 77.81; H, 8.62; N, 6.35; MS (EI): *m/z* 663 [$M^{+\cdot}$].

*3β-Chloro-5α-cholestan-6-(iminohydrazonomethylphenyl-2-yl)-benzo-[d]-oxazole (**8**)*

Yield (76%), Mp: 181-183 ˚C; IR (KBr, ν cm^{-1}): 3081, 1561, 1395 (C-H, aromatic), 1637 (C=N), 1056 (C-O), 741 (C-Cl); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.9-8.3 (m, 5H, aromatic), 3.81 (m, 1H, C_3-αH, $W_{1/2}$ = 16 Hz, axial), 1.15 (C_{10}-CH_3), 0.73 (C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 156.1, 151.3, 148.6, 143.5, 140.2, 135.9, 130.3, 129.4, 128.1, 126.9, 124.3, 122.5, 121.2, 117.9, 109.9, 59.6, 55.3, 54.6, 53.9, 41.5, 40.9, 38.7, 37.5, 36.9, 36.7, 35.4, 34.9, 33.8, 32.7, 31.4, 29.9, 27.1, 26.3, 24.9, 22.9, 21.7, 20.2, 19.1, 18.8, 15.7, 13.1; Anal. Calcd for $C_{41}H_{54}ClN_3O$; C, 76.90; H, 8.50; N, 6.56; found: C, 76.87; H, 8.52; N, 6.59; MS (EI): *m/z* 639/641 [$M^{+\cdot}$].

*5α-cholestan-6-(iminohydrazonomethylphenyl-2-yl)-benzo-[d]-oxazole (**9**)*

Yield (80%), Mp: 176-178 ˚C; IR (KBr, ν cm^{-1}): 3088, 1560, 1397 (C-H, aromatic),1634 (C=N), 1058 (C-O); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.9-8.3 (m, 5H, aromatic), 1.15 (C_{10}-CH_3), 0.73 (C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 157.3, 150.2, 149.4, 140.7, 137.6, 136.7, 132.2, 128.5, 127.2, 126.5, 124.6, 122.1, 121.5, 118.7, 111.5, 58.2, 56.7, 55.4, 48.7, 41.5, 40.1, 39.2, 38.5, 37.9, 36.5, 34.3, 33.2, 29.0, 27.9, 26.4, 25.2, 24.9, 23.6, 22.7, 21.4, 20.1, 19.8, 18.3, 17.7, 15.9, 13.2; Anal. Calcd for $C_{41}H_{55}N_3O$; C, 81.27; H, 9.15; N, 6.94; found: C, 81.31; H, 9.20; N, 6.95; MS (EI): *m/z* 605 [$M^{+\cdot}$].

*3β-Acetoxy-5α-cholestan-6-(iminohydrazonomethylphenyl-2-yl)-benzo-[d]-imidazole (**10**)*

Yield (79%), Mp: 167-169 ˚C; IR (KBr, ν cm^{-1}): 3331 (NH), 3087, 1563, 1391 (C-H aromatic), 1738 ($OCOCH_3$), 1636 (C=N), 1061, 1065 (C-O), 1310 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 5.3 (s, 1H, NH, exchangeable with D_2O), 7.9-8.2 (m, 5H, aromatic), 4.71 (m, 1H, C_3-αH, $W_{1/2}$ = 18 Hz, axial), 2.01 (s, 3H, $OCOCH_3$), 1.15 (C_{10}-CH_3), 0.73 (C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 169.9, 159.9, 147.3, 144.6, 143.8, 139.2, 138.4, 132.7, 129.3, 128.1, 126.5, 124.6, 123.1, 122.8, 113.7, 111.5, 71.5, 54.2, 52.6, 50.3, 42.8, 40.3, 39.2, 38.5, 37.5, 36.2, 35.9, 34.2, 33.7, 30.5, 29.7, 28.2, 26.4, 25.2, 24.9, 23.6, 22.5, 21.8, 20.4, 19.7, 18.2, 16.8, 14.2; Anal. Calcd for $C_{43}H_{58}N_4O_2$; C, 77.90; H, 8.82; N, 8.45 found; C, 77.86; H, 8.85; N, 8.48; MS (EI): *m/z* 662 [$M^{+\cdot}$].

*3β-Chloro-5α-cholestan-6-(iminohydrazonomethylphenyl-2-yl)-benzo-[d]-imidazole (**11**)*

Yield (77%), Mp: 152-154 ˚C; IR (KBr, ν cm^{-1}): 3335 (NH), 3084, 1566, 1387 (C-H, aromatic), 1633 (C=N), 1317 (C-N), 744 (C-Cl); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 4.7 (s, 1H, NH, exchangeable with D_2O), 7.9-8.2 (m, 5H, aromatic), 3.72 (m, 1H, C_3-αH, $W_{1/2}$ = 14 Hz, axial), 1.15 (C_{10}-CH_3), 0.73 (C_{13}-CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 160.2, 149.2, 141.5, 140.8, 139.4, 138.0, 133.7, 130.9, 129.7, 128.5, 125.4, 124.8, 120.3, 112.9, 110.5, 59.5, 57.1, 55.9, 52.7, 45.2, 43.7, 40.2, 39.5, 38.8, 37.4, 36.1, 35.4, 34.7, 33.8, 32.5, 29.3, 27.5, 26.7, 25.9, 24.1, 23.6, 22.1, 20.6, 17.3, 16.7,14.2; Anal. Calcd for $C_{41}H_{55}ClN_4$; C, 77.02; H, 8.67; N, 8.76 found; C, 77.05; H, 8.70; N, 8.75; MS (EI): *m/z* 638/640 [$M^{+\cdot}$].

*5α-cholestan-6-(iminohydrazonomethylphenyl-2-yl)-benzo-[d]-imidazole (**12**)*

Yield (81%), Mp: 164-166 ˚C; IR (KBr, ν cm^{-1}): 3342 (NH), 3089, 1568, 1388 (C-H, aromatic), 1628 (C=N), 1329 (C-N); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 4.4 (s, 1H, NH, exchangeable

with D_2O), 7.9-8.3 (m, 5H, aromatic), 1.15 (s, 3H, C_{10}-CH_3), 0.73 (s, 3H, C_{13}- CH_3), 0.91 & 0.84 (other methyl protons); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 162.5, 147.6, 145.6, 143.2, 140.3, 137.6, 134.2, 129.6, 128.1, 125.9, 124.7, 123.2, 122.8, 117.3, 112.6, 59.2, 57.5, 55.2, 49.5, 44.7, 39.1, 37.9, 36.9, 35.4, 34.7, 32.5, 30.8, 29.7, 27.9, 26.6, 25.3, 24.7, 23.4, 22.8, 21.2, 20.5, 19.8, 18.2, 17.6 15.9, 13.6; Anal. Calcd for $C_{41}H_{56}N_4$; C, 81.41; H, 9.33; N, 9.26 found; C, 81.44; H, 9.29; N, 9.21; MS (EI): *m/z* 604 [$M^{+\cdot}$].

Pharmacology

Rule of Five and bioactivity score

The physicochemical parameters including octanol partition coefficients (CLogP), Mw, HBD, HBA and TPSA were calculated using ChemBioOffice 2008. The Bioactivity score calculated using molinspiration server (http://www.molinspiration.com/cgi-bin/properties).

Anticancer activity

MTT assay

The cancerous cell lines (**HeLa**/ **Hep 3B**/ **MCF 7**) and non-cancerous cell (**PBMCs**) were maintained in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotic antimycotic solution. The cells were plated at a density of 5×10^3 cells per well in a 96-well plate, and cultured for 24 h at 37 ^oC. The cells were subsequently exposed to drugs. The plates were incubated for 48 h, and cell proliferation was measured by adding 20 µL of MTT dye (5 mg/mL in phosphate buffered saline) per well. The plates were incubated for a further 4 h at 37 ^oC in a humidified chamber containing 5% CO_2. Formazan crystals formed due to the reduction of dye by viable cells in each well were dissolved in 150 µL dimethyl sulfoxide (DMSO), and absorbance was read at 570 nm. The absorption values were expressed as the cell viability (%), according to the control group as 100%. The concentration

required for 50% inhibition of cell viability (IC_{50}) was calculated using the software "Prism 3.0".[52]

Blood peripheral mononuclear cell isolation

The blood sample (20-15 mL) was diluted with the same volume of phosphate buffer saline (PBS). After that, the diluted blood sample was layered on Ficoll-Histopaque. The mixture was centrifuged at 400 g for 30 min at 20-22 ^{o}C. The undisturbed lymphocyte layer was transferred out. The lymphocyte was washed and pelleted down with three volumes of PBS twice and resuspended RPMI-1640 media with antibiotic and antimycotic solution 10%, v/v FCS. Cell counting was performed to determine the **PBMCs** cell number with equal volume of trypan blue.[53,54]

Antioxidant activity

By DPPH assay

Steroidal heterocyclic compounds (**4-12**) were tested for their antioxidant property by 1,1-diphenylpicrylhydrazyl (DPPH) method.[55] Drug stock solution (1 mg/mL) was diluted to final concentration of **2**, **4**, **6**, **8**, **10** and **12** mg/mL in methanol. Methanolic DPPH solution (1 mL, 0.3 mmol) was added to 3.0 mL of drug solution of different concentrations. The mixture was shaken vigorously and incubated in dark at an ambient temperature for 30 min and the absorbance was measured as the decrease in the absorbance of DPPH at 517 nm resulting from the color change from purple to yellow. The decrease in absorbance is because of formation of stable molecule of DPPH on reaction with an antioxidant through donation of hydrogen or electron by an antioxidant. The free electron on DPPH radical is responsible for giving absorbance peak at 517 nm and appears purple in color. The antioxidant agent pair up through donation of electron or release of hydrogen with the free electron on DPPH radical and form

stable molecule of DPPH-H. The change of color from purple to yellow is attributed to decrease of molar absorptivity of DPPH radical when odd electron of DPPH pair up with the antioxidant agent. The resulting decrease in color is also stiochiometric with number of electrons captured. The DPPH radical-scavenging activity (%) was calculated by the following formula:

[% inhibition = $[(A_{\text{Control}}{}^{-}A_{\text{Sample}}/A_{\text{Control}}) \times 100]$

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 a negative control whereas L-ascorbic acid was used as a positive control.

By FRAP assay

FRAP assay measures the antioxidant capacity by the reduction of ferric 2,4,6-tripyridyl-s-triazine complex $[Fe^{III}(TPTZ)_2]^{3+}$ to the intensely blue colored ferrous complex $[Fe^{II}(TPTZ)_2]^{2+}$.[56] The reagent composed of 10 mM TPTZ (2,4,6-Tripyridyl-s-Triazine) (10 mL), 20 mM $FeCl_3.6H_2O$ (10 mL), and 300 mM acetate buffer (100 mL) in ratio of 1:1:10 were freshly prepared. 300 mM acetate buffer (pH 3.6) was prepared by dissolving sodium acetate trihydrate (3.1 g) in distilled water (500 mL) then glacial acetic acid (16 mL) was added and made up to the mark of 1 L with distilled water and checked for its pH. 10 mM TPTZ solution was prepared in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ was prepared in distilled water. Different concentrations of compounds (**25**, **50**, **75** and **100** µg/mL) were taken and an increase in the absorbance by $[Fe^{III}(TPTZ)_2]^{3+}$ complex was measured at 593 nm after 5 min of incubation at 37 ^{o}C. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard solution and the concentration of FRAP content in the compounds were expressed in terms of mg trolox-equivalent (TE)/g deduced from the standard curve of trolox.[57,58]

By ABTS assay

The total antioxidant activity of compounds **4-12** were measured by ABTS (2′,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay with some modification.[59] A stock solution of ABTS (2 mM) was prepared by dissolving the ABTS in phosphate-buffered saline (PBS, 50 ml) to produce a solution with a pH of 7.4. The $ABTS^{\bullet+}$ was generated by reacting 50 mL of the stock solution with 200 mL of a 70 mM aqueous $K_2S_2O_8$ solution. The resulting $ABTS^{\bullet+}$ solution was diluted with PBS to obtain a reasonable absorbance at 734 nm. A 100 µL of drug solution at different concentration (**25**, **50**, **75** and **100** mg/mL) was mixed with $ABTS^{\bullet+}$ solution (1.9 ml), and the absorbance of the mixed solution was read at ambient temperature after 3 min. The PBS solution was used as a blank sample.[60] The experiment was done in triplicates and the radical scavenging activity of the sample was calculated with the following formula:

$$SC\% = \frac{B_O - B_S}{B_O} \times 100$$

Where, B_O and B_S are the absorbance values of the blank and of the tested samples, respectively. The assay was carried out on ascorbic acid, which served as a standard.

Fluorescence Microscopy

The **HeLa** cell line was maintained in RPMI-1640 culture medium supplemented with 10% heat-inactivated FCS. The cells were plated at a density of 5×10^3 cells on glass cover slips, and cultured for 24 h at 37 ^oC. These were subsequently exposed with compounds for 48 h. Cells were fixed by 2% paraformaldehyde for 2 h followed by washing with Hank's Balanced Salt Solution (HBSS). The HeLa cells treated with compounds **4-12** were observed under Fluorescence Microscope.

Preparation for TEM and AFM

The sample for TEM analysis was prepared by placing a drop of colloidal solution on a copper grid coated with a carbon film and allowed to equilibrate for 3-5 min. The solution was wiped off with filter paper and allowed to air dry. Images were taken using TEM, JEOL JEM-2100 Electron Microscope set at an accelerating voltage of 200 kV to determine the size and morphology of the condensate material. The samples for AFM analysis of the compound **6** and its condensate with CT-DNA were prepared from the solution of the compound **6** (prepared in DMSO) and equimolar mixture of compound **6** and CT-DNA (dissolved in 0.01 M Tris-HCl buffer pH 7.2). The samples were placed drop wise on a mica wafer, then air dried at room temperature for 12 h and the images were recorded with Agilent Model 5500 using tapping mode AFM, where cantilever and AFM tip oscillate and are scanned over the surface of the dry sample.

Nonenzymatic DNA damage

In order to identify and determine the possible role of ROS, the reaction mixture (0.5 mL) containing Tris-HCl (10 mM, pH 7.5), CT-DNA (200 μg), Cu (II) (100 μM) and increasing concentrations of compound **6** (12.5 μM, 25 μM, 50 μM, 75 μM, 100 μM, 200 μM, 400 μM, 600 μM). The volume was made up to 1 mL by buffer solutions and incubated at 37 ˚C for 60 min. Reaction was stopped using 0.5 mL of trichloroacetic acid solution (TCA) (28%) and 0.5 mL of 1% thiobarbituric acid (TBA) was added, boiled for 15 min and cooled to room temperature. The intensity was read at 532 nm.[61]

In vitro acetylcholinesterase inhibition activity

The ability of synthesized steroidal compounds (**4-12**) to inhibit AChE activity was assessed by Ellman's method.[62] AChE stock solution was prepared by dissolving human recombinant AChE

(EC:3.1.1.7) lyophilized powder in 0.1 M phosphate buffer (pH 8.0) containing Triton X-100 (0.1 %). Five increasing concentrations of test compounds were assayed to obtain percent inhibition of the enzymatic activity in the range of 20-80. The assay solution consisted of a 0.1 M phosphate buffer pH 8.0, with the addition of 340 μM 5,50-dithio-bis(2-nitrobenzoic acid), 0.02 unit/mL of human recombinant AChE from human serum and 550 μM of substrate (acetylthiocholine iodide, ATCh). Increasing concentration of tested inhibitor were added to the assay solution and pre-incubated for 5 min at 37 ˚C with the enzyme followed by the addition of substrate. Initial rate assays were performed at 37 ˚C with a Jasco V-530 double beam spectrophotometer. Absorbance value at 412 nm was recorded for 5 min and enzyme activity was calculated from the slope of the obtained linear trend. Assays were carried out with a blank containing all components except AChE to account for the non-enzymatic reaction. The reaction rates were compared and the percent inhibition due to the presence of tested inhibitors was calculated. Each concentration was analyzed in triplicate and IC_{50} values were determined graphically from log concentration-inhibition curves (GraphPad Prism 4.03 software, GraphPad Software Inc.). Tacrine was used as a standard inhibitor.

Molecular docking

The rigid molecular docking studies were performed using HEX 8.0.0 software.[63] HEX is an interactive molecular graphics program for calculating and displaying feasible docking modes of DNA.[64] The initial structures of the steroidal compounds **4-12** were generated by ChemBioDraw Ultra 12.0 software. The structures of compounds were optimized for use in the following docking study. The parameters that were used for docking include: correlation type-shape only, FFT mode-3D, grid dimension-0.6, receptor range-180, ligand range-180, twist range-360, distance range-40. The crystal structure of the B-DNA dodecamer $d(CGCAAATTTCGC)_2$ (PDB

ID: 1BNA) were downloaded from the protein data bank (http://www.rcsb.org./pdb). All calculations were carried out on an Intel CORE i5, 2.6 GHz based machine running MS Windows 8 as the operating system. Visualization of the docked pose have been done using PyMol molecular graphics program.[65]

Conclusion

In conclusion, a series of new steroidal heterocyclic compounds with significant anti-tumor and antioxidant activities were successfully designed and synthesized. Their anti-tumor activity *in vitro* was evaluated against **Hep3 B** (human hepatocellular carcinoma), **MCF 7** (human breast adeno carcinoma), **HeLa** (human cervical carcinoma) cancer cell lines and on normal cells **PBMCs** (peripheral blood mononuclear cell). The results demonstrated that most of the synthesized derivatives exhibited significant anti-tumor activity; however compounds **5**, **6**, **8**, **9** and **12** exhibited excellent activity with IC_{50}<19 μM against all the cancer cell lines. In addition, compounds **10-12** were found to be good antioxidant. Nonenzymatic degradation of DNA has also been investigated. More importantly, the application of compounds **6** as DNA gene transporter was evaluated by DNA condensation and ascertained by employing TEM and AFM, which illustrated that the compound **6** induces the condensation of CT-DNA. Lipinski's 'Rule of Five' analysis predicted good oral absorption of the synthesized compounds. Moreover, the acetylcholinesterase (AChE) inhibitor activities of the steroidal derivatives were also evaluated using Ellman's method. From the results obtained we deduced that compound **4**, **7** and **10** (IC_{50} = 0.31±0.10, 0.37±0.02 and 0.39±0.03, respectively) exhibited significant inhibition on AChE among all the synthesized compounds. The molecular docking studies undertaken in the present work are in total agreement, with the primary mode of binding of the synthesized compounds with DNA, although the H-bonding and other types of interactions can also be argued. These

findings demonstrated that among steroidal heterocyclic derivatives one can find compounds with interesting therapeutic properties that could become a new group of potential anti-tumor and antioxidant agents.

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Supplementary data

Copy of ^{1}HNMR, ^{13}CNMR and HRMS spectra of all synthesized compounds.

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Synthesis, characterization of steroidal heterocyclic compounds, DNA condensation and molecular docking studies and their *in vitro* anticancer and acetylcholinesterase inhibition activities

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A facile, convenient and efficient approach for the synthesis of new series of steroidal heterocyclic compounds (**4-12**) has been performed. After characterization, the interaction of the synthesized compounds with DNA was evaluated by docking studies. MTT assay has been performed to check the *in vitro* cytotoxicity of new compounds.