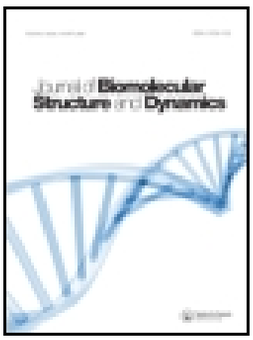

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Spectroscopic investigations on DNA binding profile of two new naphthyridine carboxamides and their application as turn-on fluorescent DNA staining probes

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Abstract

Two new 10-methoxydibenzo[b,h][1,6]naphthyridine-2-carboxamide derivatives (**R1** and **R2**) have been synthesized and characterized using different spectral techniques. The binding of these probes with DNA was investigated using spectral (Electronic, fluorescence, ^{1}H NMR and circular dichroism) and molecular docking studies. These probes exhibited a strong fluorescence around 440 nm upon excitation around 380 nm. Electronic and competitive fluorescence titration studies, in HEPES [(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)] buffer/dimethyl sulfoxide (pH 7.4) medium, suggest that these probes bind strongly to DNA, which is substantiated by ^{1}H NMR study. The binding constants are calculated to be 5.3×10^7 and 6.8×10^6 M^{-1} for **R1** and **R2**, respectively. From the results of spectral studies, it is proposed that the mechanism of binding of these probes with DNA is through minor groove binding mode, which is further confirmed by circular dichroism and molecular docking studies. Initial cell viability screening using MTT (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay shows that normal Vero cells are viable towards these probes at nano molar concentration, which

is the concentration range employed in the present study for DNA staining (IC_{50} in the order of 0.023 mM). The enhancement in fluorescence intensity of these probes upon binding with DNA enables the staining of DNA in agarose gel in gel electrophoresis experiment. The sensitivity of these probes is comparable with that of ethidium bromide and DNA amounts as low as 4 nano gram are detectable.

Keywords: DNA; Minor groove; Fluorescent; Naphthyridine; Staining

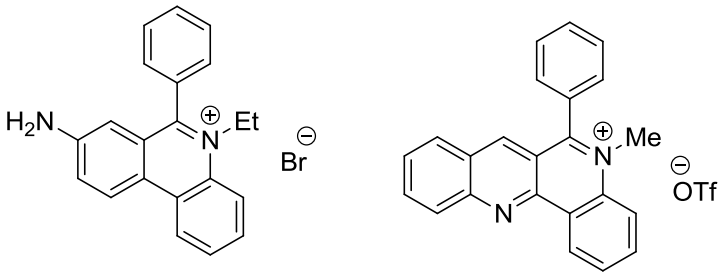
Abbreviations: CD - Circular dichroism; CT-DNA - calf thymus DNA; DMEM - Dulbecco's Modified Eagle *Medium*; DMSO - Dimethyl sulfoxide; EB - ethidium bromide; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IR - Infra red; mM - Milli molar; MTT - 3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NMR - Nuclear magnetic resonance; TLC – Thin layer chromatography.

1. Introduction

In order to design newer and more efficient deoxyribonucleic acid (DNA) targeted drugs, the mechanism of binding of small molecules with DNA is important. Review of literature revealed that variety of compounds has been reported so far for their DNA binding affinity [Ahmad et al., 2016; Bhaduri *et al.*, 2018; Ester et al., 2009; Fu et al., 2014; Hranjec *et al.*, 2007; Husain *et al.*, 2017; Khalaf *et al*., 2016 & Khan *et al*., 2012; Konovalov et al., 2018; Liang et al., 2012]. Although the binding modes of various classes of these molecules have been investigated in detail, only less attention was paid to the binding of those molecules which are used as DNA staining agents. In molecular biology, to achieve complete understanding of biological processes, it is necessary to visualize DNA, which is described as one of the nature's most elementary conduits. Electrophoresis is the widely used method to detect DNA wherein DNA staining is carried out with substances like fluorescent probes, silver stain and visible organic dyes. Among

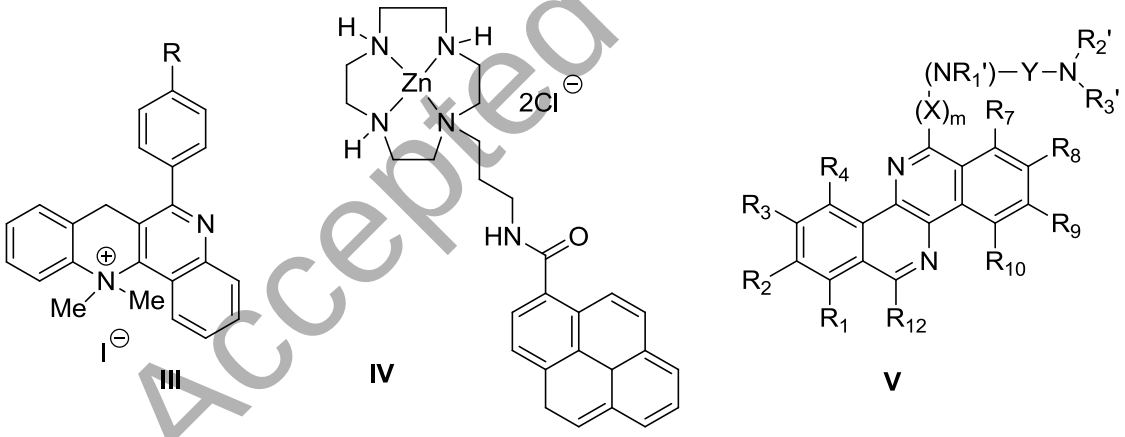
these, silver stain is reported to have advantages like high sensitivity, less toxic and use of simple chemicals and equipment [Bassam *et al.,* 2007]. Over the years, visible dyes such as methylene blue, crystal violet, methyl orange, Nile blue, brilliant cresyl blue, ethyl violet, basic fuchsin and N-719 have been reported as DNA staining agents. However, these visible dye based DNA staining methods are not able to fulfill the requirements of molecular biology. Further, Nile blue and methylene blue are suggested to be mutagenic [Daru et al.,1989; Yang et al., 2001;Yang et al., 2000; Torris et al., 1993;Cong et al, 2010; Chen et al., 2013 & Sonmezoglu et al., 2015].

Among the fluorescent probes reported so far, ethidium bromide (EB), **I,** is widely used for the visualization of DNA on gels. Even though EB is very popular among researchers and commonly used in gel electrophoresis, being mutagenic and carcinogenic, its handling and safe disposal requires considerable attention [Lunn et al., 1987]. Hence, the search for newer fluorescent probes for DNA staining is on. In an attempt, Okuma et al. have synthesized dibenzo[b,h][1,6]naphthyridines (**II**) via one pot protocol and showed that these compounds can be applied as a fluorescent DNA-binding compounds. But, fluorescence titration studies revealed that fluorescence intensity of these compounds decreased (turn-off) on binding with DNA [Okuma et al., 2017]. Very recently, the same research group has reported N-methylated **II**, having general structure **III**, as turn-on fluorescent probes for the detection of DNA. However, these probes showed only 3-10 times enhancement in fluorescence upon intercalation with DNA in Tris buffer (pH 7.6) containing 0.1% DMSO [Okuma et al., 2017].



I II

Schmidt et al. [Schmidt et al., 2010] have reported a pyrene-labeled Zn(II)-cyclen complex (**IV**) for the staining of DNA in agarose gels wherein the metal chelate coordinates reversibly to the DNA phosphate backbone inducing the formation of pyrene excimers, which is used for the detection of DNA. However, they have reported three more similar Zn(II) chelates, which failed to identify DNA in the gel with significant emission signal. Popov et al. [Popov et al., 2010] have patented the use of novel dibenzo[c,h][1,5]naphthyridines of formula (**V**) as DNA probes as well as the method of marking DNA using these probes.



III IV V

Review of literature also revealed that, when compared to DNA-ligand binding studies, only few compounds have been reported so far as fluorescent DNA probes and hence the present work. The main objectives, therefore, of the present endeavor are synthesis, characterization and DNA binding studies of two new naphthyridines derivatives (**R1** and **R2**) using various spectral

techniques. Molecular docking study was also carried out to substantiate the results of spectral studies. The applicability of these compounds as fluorescent DNA staining probes in agarose gel has also been screened and discussed.

2. Experimental

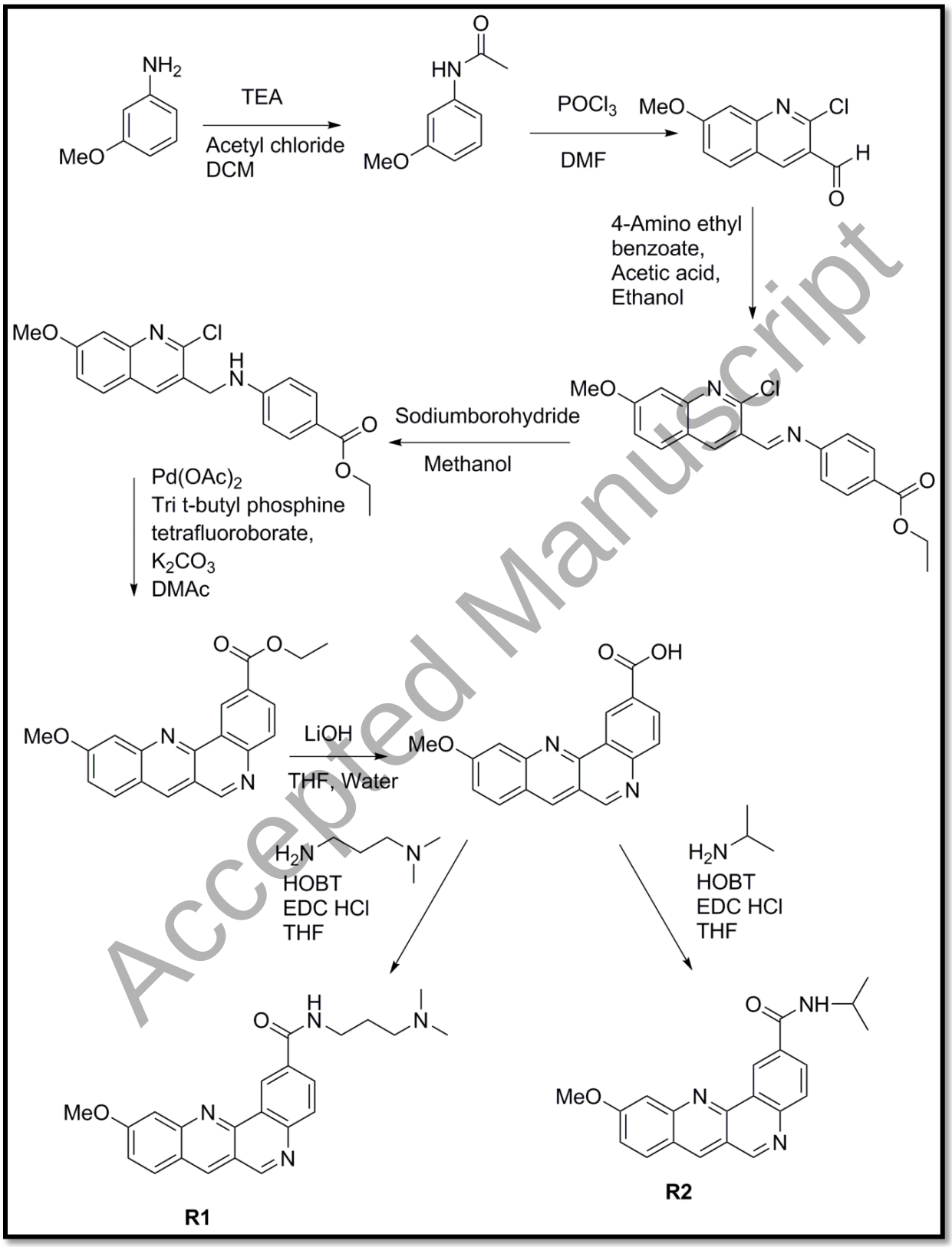
2.1. Synthesis of probes. The probes, 10-methoxydibenzo[b,h][1,6]naphthyridine-2-carboxamide derivatives **R1** and **R2**, were synthesized as depicted in Scheme 1. To the stirred solution of 10-methoxydibenzo[b,h][1,6]naphthyridines-2-carboxylic acid (200 mg) in tetrahydrofuran (4.0 mL, 20 v) hydroxybenztriazole (1.3 eq), EDC HCl (2.0 eq) and the corresponding amine (2.0 eq) was added at ambient temperature and stirred for 24 h. After completion of reaction (by TLC), the solution was concentrated to get thick syrup. Reaction mass was extracted with dichloromethane (100 mL) and washed with water (2 x 400 mL), brain solution (400 mL), and then dried over anhydrous sodium sulfate and concentration of the organic layer yielded crude product. The crude was subjected to column chromatography to obtain desired pure products as yellow solids.

The probes were characterized using ^{1}H and ^{13}C NMR, IR and mass spectral techniques and the results obtained are:

N-(3-(dimethylamino)propyl)-10-methoxydibenzo[b,h][1,6]naphthyridine-2-carboxamide (**R1**): Yield: 76%, m.p: 176-178^{o}C. ^{1}H NMR (400 MHz, DMSO-$d_{6,}$ δ ppm): 1.72-1.78 (m, 2H), 2.1 (s, 6H), 2.30-2.40 (m, 2H), 3.41 (q, 2H, *J* = 6.2 Hz), 4.0 (s, 3H), 7.44-7.47 (m, 1H), 7.71 (d, 1H, *J* = 8.4 Hz), 8.18 (dd, 1H, *J* = 8.6 Hz, 2.2 Hz), 8.26-8.32 (m, 2H), 8.98 (d, 1H, *J* = 9.2 Hz), 9.28 (s, 1H), 9.50 (s, 1H), 9.60 (s, 1H) (Fig. S1); ^{13}C NMR (100 MHz, DMSO-$d_{6,}$ δ ppm) δ: 27.0, 38.2, 45.2, 55.9, 57.1, 106.1, 118.2, 121.3, 122.9, 123.2, 124.2, 128.9, 129.2, 130.8, 133.2, 138.1,

147.2, 151.9, 155.9, 162.9, 165.8 (Fig. S2); IR (KBr, cm^{-1}): 3289, 2950, 2760, 2361, 1628, 1414, 1231, 1172, 1159 (Fig. S3); LCMS (ESI) m/z found for $C_{23}H_{24}N_4O_2$ $[M+H]^+$: 389.8 (Fig. S4).

N-isopropyl-10-methoxydibenzo[b,h][1,6]naphthyridine-2-carboxamide (**R2**): Yield: 82%, m.p 178-180^oC. ^1H NMR (400 MHz, DMSO-d_6, δ ppm): 1.25-1.27 (d, 6H, *J* = 6.8 Hz), 4.07 (s, 3H), 4.18-4.27 (m, 1H), 7.44-7.47 (m, 1H), 7.73 (d, 1H, *J* = 8.4 Hz), 8.17 (dd, 1H, *J* = 8.6 Hz, 2.2 Hz), 8.26-8.33 (m, 2H), 8.68 (d, 1H, *J* = 9.2 Hz), 9.30 (s, 1H), 9.54 (s, 1H), 9.65 (s, 1H) (Fig. S5); ^{13}C NMR (100 MHz, DMSO-d_6, δ ppm) δ: 22.4, 41.3, 56.0, 106.1, 118.2, 121.3, 122.8, 123.4, 124.1, 129.09, 129.13, 130.7, 133.5, 138.0, 147.15, 147.22, 151.8, 155.8, 162.9, 165.2 (Fig. S6); IR (KBr, cm^{-1}): 3285, 2972, 2935, 1630, 1418, 1231, 1170, 849, 695 (Fig. S7); LCMS (ESI) m/z found for $C_{21}H_{19}N_3O_2$ $[M+H]^+$: 346.8 (Fig. S8).



Scheme 1. Synthesis of probes **R1** and **R2**

3. Results and Discussion

The new naphthyridine-2-carboxamides (**R1** and **R2**) have been synthesized and characterized using ^{1}H and ^{13}C NMR, FT-IR and mass spectral techniques. DNA binding behaviour of these probes was investigated using UV-Vis, fluorescence and ^{1}H NMR spectral studies. Circular dichrosim and molecular docking were carried out to substantiate the proposed mode of binding of these probes with DNA. The cell viability of these probes against normal cell line was also screened to understand the toxicity of these probes. DNA staining studies were carried out using plasmid DNA in agarose gel.

3.1 Cell viability test

Initially the cytotoxicity effect of these probes was screened against normal Vero (African Green Monkey kidney cell lines) cells using MTT (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay as reported by us earlier [Vennila et al., 2018]. In a typical experiment, the Vero cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. Upon treatment with **R1** and **R2** the cell viability was evaluated by MTT. The IC_{50} values (the concentration that inhibited cell viability to 50% of the control) were determined by non-linear regression method and the results obtained are given in Figure 1. The IC_{50} values were found to be 0.02317 and 0.02391 mM for **R1** and **R2**, respectively. The results indicated that more than 50% of normal Vero cells would be viable at concentrations lower than these values. Herein the present study the concentrations of the probes employed for DNA staining studies are in the order of ng/ml and hence they are safe to normal cells.

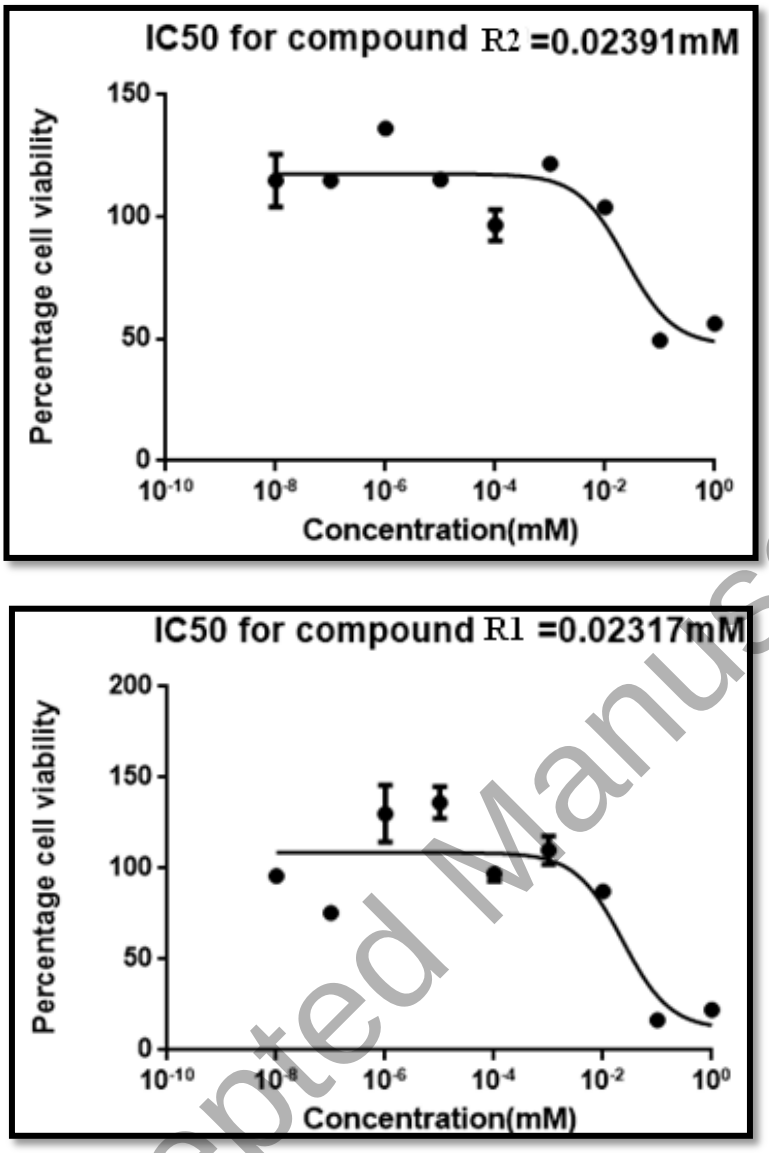
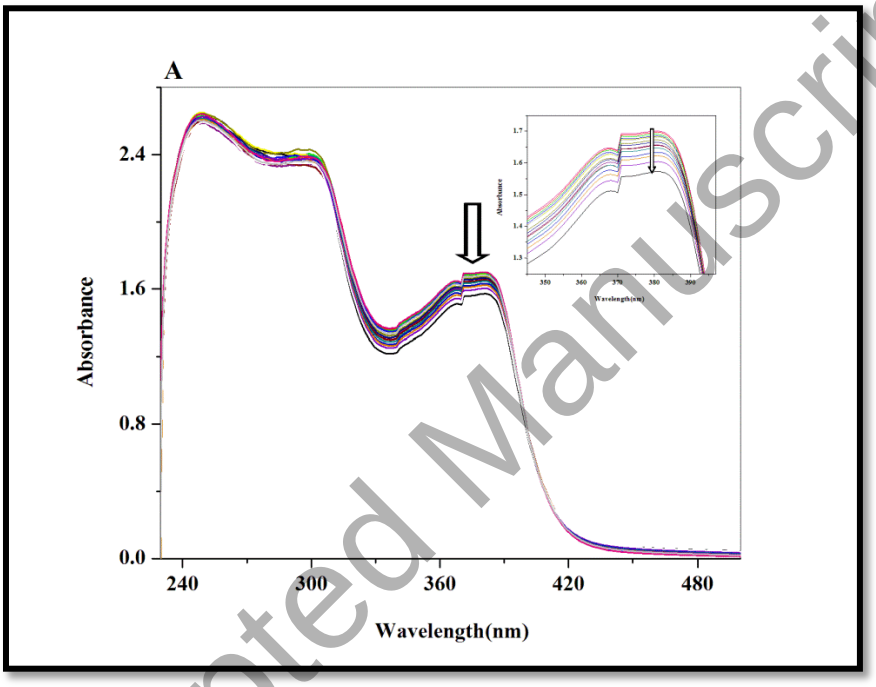


Fig. 1. Cell viability against **R1** and **R2**

3.2 UV-Vis spectral study

UV-Vis spectral titration studies were used to study the binding ability of the probes **R1** and **R2** with CT-DNA. Usually hypochromism arises due to strong interaction between probes and the base pairs of DNA [Kosiha et al., 2019]. In the present study, the interaction between the probes and CT-DNA was investigated in HEPES buffer/DMSO (pH 7.4) medium. UV-Vis

spectral titrations were carried out using a fixed concentration of the probe with addition of incremental amounts of CT-DNA (Fig. 2). It is evident from the Figure that upon addition of increasing amounts of CT-DNA to the probes, the absorption intensities decreased (hypochromic effect) suggesting strong binding between the probes and CT-DNA [Kosiha et al., 2019].



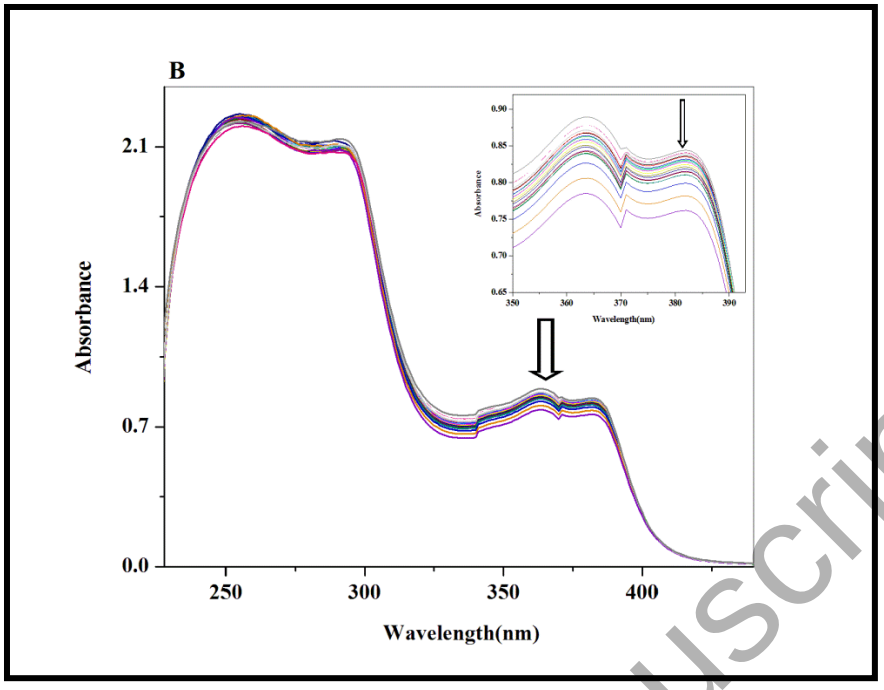


Fig. 2. UV-Vis spectra of **A**) **R1** (1 µM) with incremental addition of CT-DNA (0-50 nM) and **B**) **R2** (150 µM) with incremental addition of CT-DNA (0-200 nM) in HEPES buffer/DMSO (pH 7.4) medium

3.3 Circular dichroism study

Circular dichroism (CD) is a powerful tool to identify the conformational changes of DNA upon binding with analyte molecules. The CD spectra of CT-DNA (1 µM) obtained in the absence and presence of these probes (5 µM) are shown in Figure 3. It is evident from the figure that pure DNA exhibited a positive band at 277 nm which corresponds to base stacking and a negative band at 245 nm due to helicity. These two bands are characteristic of DNA in the right-handed B form. Analyte molecules which bind to DNA through groove and electrostatic binding modes would show no or less perturbation in stacking and helicity bands, while those bind via intercalation mode enhances the intensity of these bands significantly. In the present study, both **R1** and **R2** perturbed the intensities of both these bands only to a lesser extent upon binding with

DNA. This observation suggested that these probes may bind to DNA through groove binding mode. Usually groove binding doesn't bring large structural/conformational changes in DNA [Mi et al., 2015].

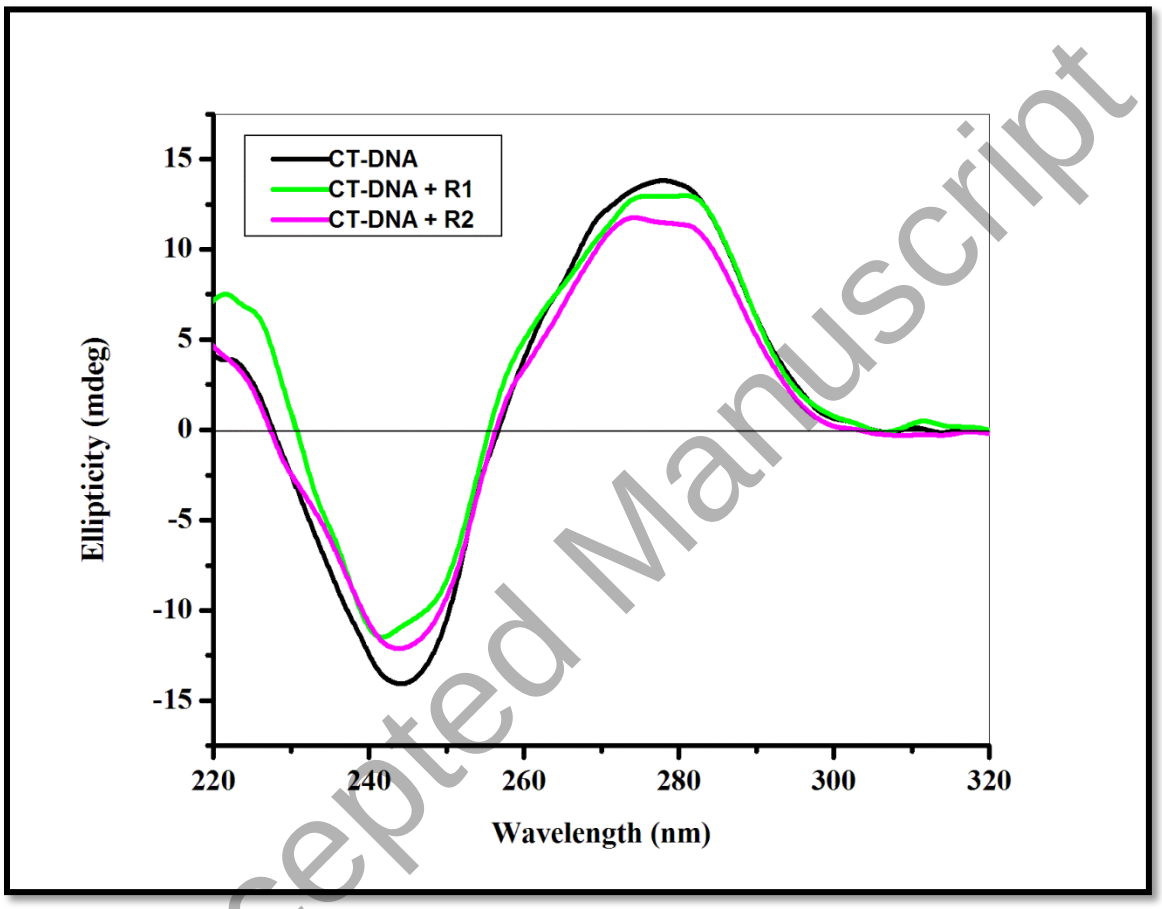


Fig. 3. Circular dichroism spectra of CT-DNA in the presence of probes **R1** and **R2** in HEPES buffer/DMSO (pH 7.4) medium

3.4 Fluorescence spectral study

Fluorescence spectral technique is one of the effective methods to investigate the interaction of molecules with DNA. The fluorescence spectra of **R1** and **R2** were recorded in the absence and presence of varying amounts of CT-DNA in HEPES buffer/DMSO (pH 7.4)

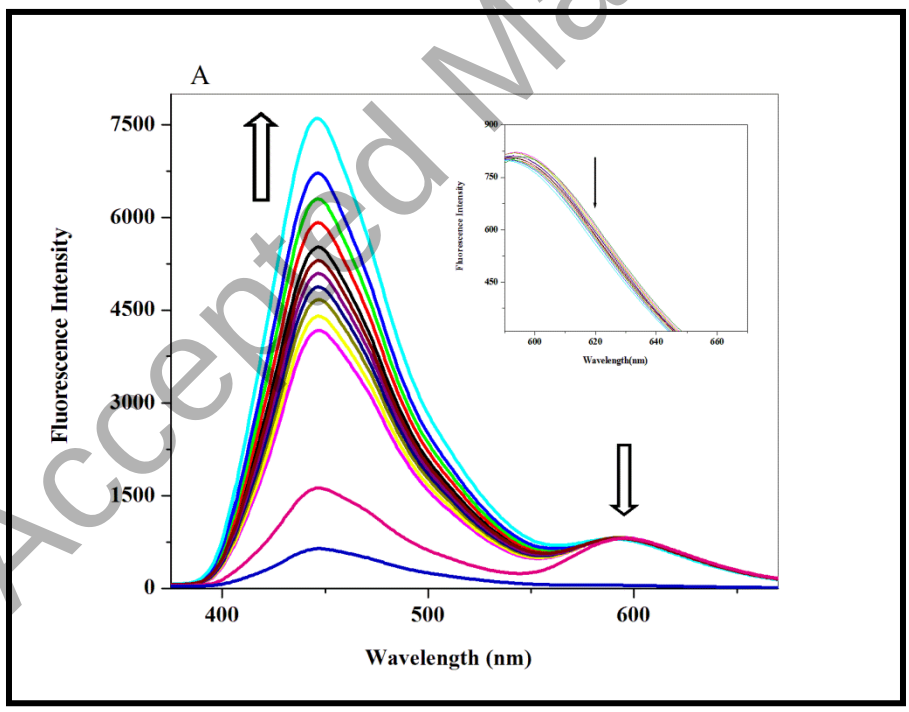
medium (Fig. S9 and S10). The probe **R1** (0.25 mM) on excitation at 380 nm emits strongly at 450 nm, whose intensity increased appreciably upon addition of 0-100 nM CT-DNA. Likewise, the probe **R2** (1 mM) also showed similar enhancement in fluorescence (λ_{ex} 383 nm; λ_{em} 438 nm) upon adding 0-200 nM CT-DNA. The binding constants for the interaction were determined from the fluorescence enhancement data according to the following Benesi–Hildebrand equation [Jayasudha et al., 2017].

$$(F_\alpha – F_o)/ (F_x – F_o) = 1/K\,[\text{CT-DNA}]$$

Where F_o, F_x and F_α are the fluorescence intensities of the receptor in the absence of DNA, at a given concentration and concentration for complete interaction, respectively. The binding constant (K) was calculated from the slope of linear plot ($r > 0.99$) of $(F_\alpha – F_o)/(F_x – F_o)$ versus 1/[CT-DNA] (Fig. S11 and S12). The binding constants for the interaction of CT-DNA with **R1** and **R2** were found to be 5.3×10^7 and 6.8×10^6 M^{-1}, respectively. The observed magnitude of the binding constants indicated a strong interaction between the probes and DNA. The binding constants (K in M^{-1}) of **R1** and **R2** are much higher than that of many cyanine-styryl dyes (10^4) reported for fluorescent DNA staining by Bohlander and Wagenknecht [Bohlander et al., 2013] and that of asymmetric cyanine dyes (10^4 - 10^5) reported by Kaloyanova and co-workers [Kaloyanova et al., 2011].

Generally, EB is used as a spectral probe to investigate the mode of binding between analyte molecules and DNA. The fluorescence of EB increases after intercalating into DNA. If the analyte molecule intercalates into DNA, it leads to decrease in fluorescence intensity of EB bound DNA system. In the present study, fluorescence spectra of EB/CT-DNA system in the absence and presence of increasing amounts of **R1** and **R2** were recorded and depicted in Figure

4. As seen from the Figure, upon addition of increasing amounts of the probes the emission intensity around 600 nm, which corresponds to EB/CT-DNA system, decreased slightly with a concomitant enhancement in fluorescence intensity around 440 nm due to binding of the probe with DNA. An isosbestic point around 580 nm is observed. Such a very marginal quenching of the fluorescence of EB/CT-DNA system by the added probe indicated that the probe may not bind to DNA through intercalative mode of binding [Okuma et al., 2017& Kosiha et al., 2019]. This observation suggested that these probes may bind to DNA via groove binding mode. Further, the enhancement in fluorescence intensity of the probes around 440 nm would facilitate the identification of DNA during its staining process.



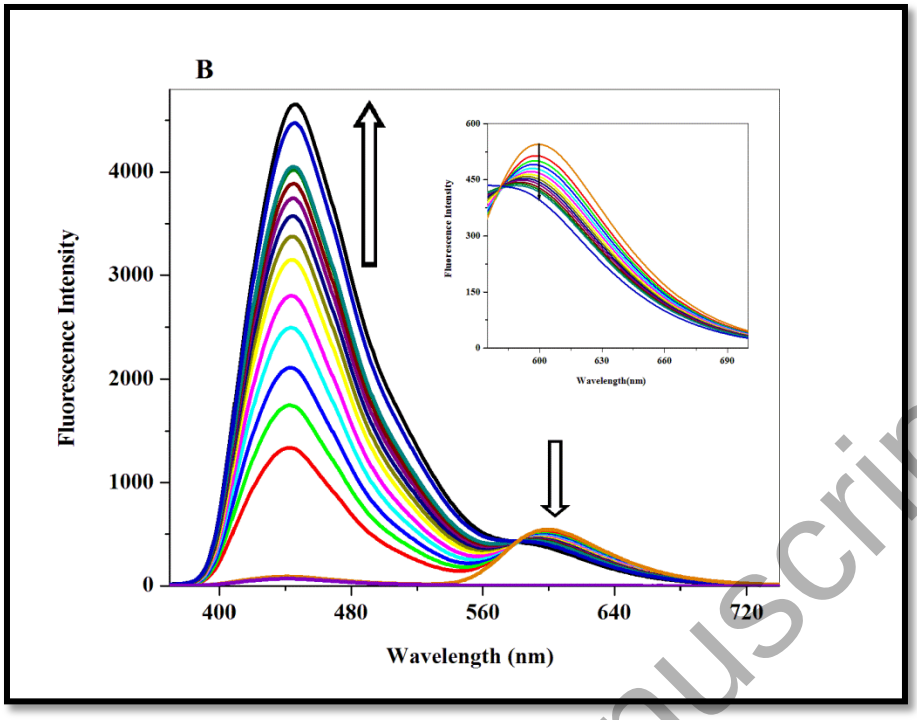


Fig. 4. Fluorescence spectra of EB/CT-DNA system upon addition of **A**) **R1** (0-10 µM) and **B**) **R2** (0-50 µM) in HEPES buffer/DMSO (pH 7.4) medium

3.5 ^{1}H NMR spectral study

In order to substantiate the results of optical spectroscopic studies on the binding of these probes with DNA, ^{1}H NMR spectral study was also carried out. ^{1}H NMR spectral study was carried out in DMSO-d_6 with guanosine nucleoside as a model for DNA [Kumar et al. 2016]. ^{1}H NMR spectrum of pure guanosine exhibited signals at 10.633, 7.939 and 6.461ppm corresponding to –NH, imidazole C-H and –NH_2 protons, respectively (Fig. S13). Upon addition of one equivalent of these probes, the signal due to –NH proton showed appreciable down field shift suggesting interaction between the probes and DNA through H-bonding (Fig. S14 and S15). The observed Δδ values are 0.049 and 0.019 ppm for **R1** and **R2**, respectively. The order of the magnitude of Δδ is in line with the binding constant values of the probe-DNA complex determined using fluorescence titration data. Further, the signal due to the –NH_2 protons after the

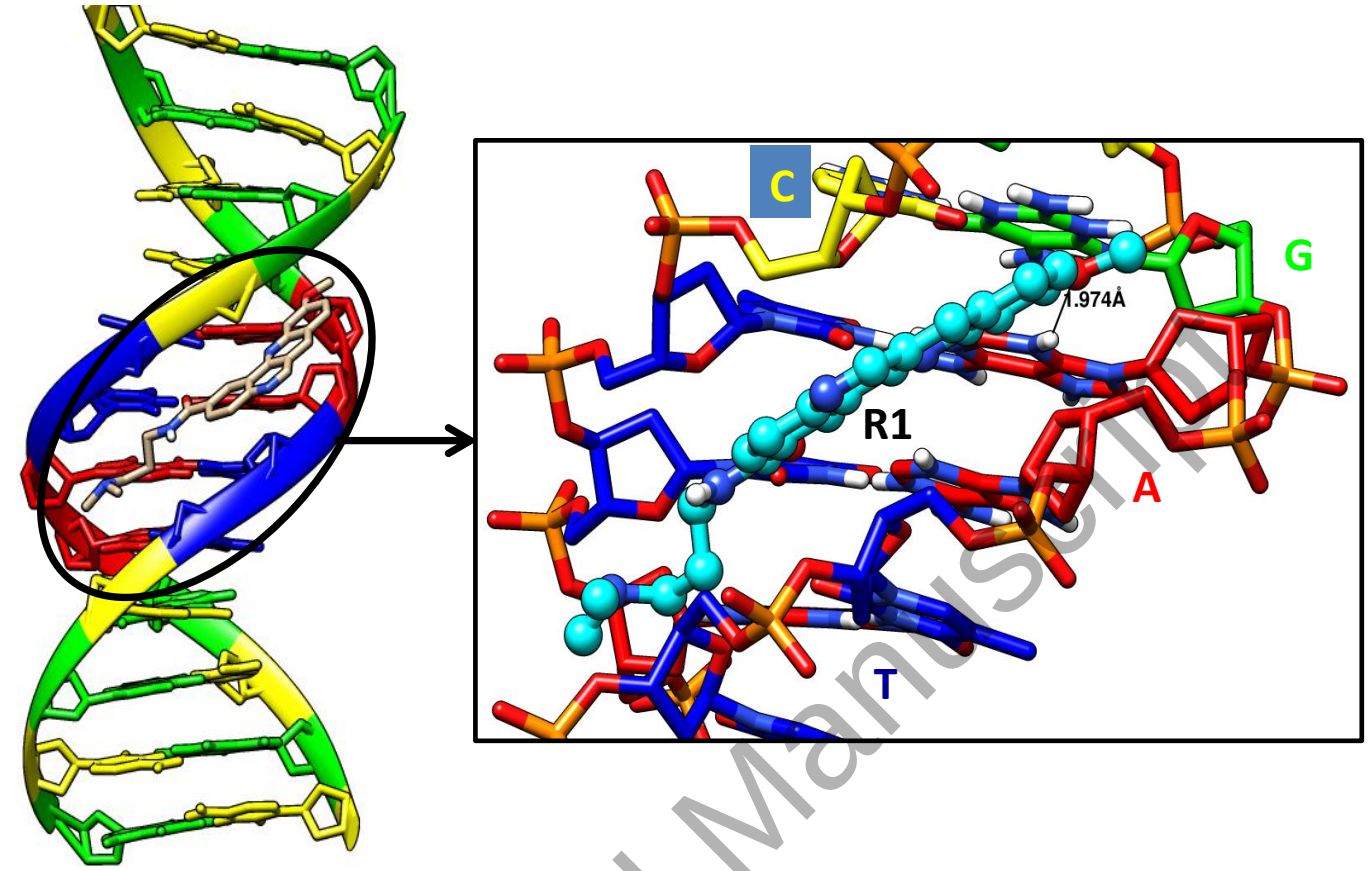
addition of these probes showed relatively lesser down field shifts (Δδ 0.033 ppm for **R1** and 0.015 ppm for **R2**). On the other hand the signal corresponds to the imidazole C-H proton experienced down field shift only to a very little extent (Δδ < 0.01 ppm). These observations suggested that these probes bind to DNA through H-bonding interaction and most probably using N-H protons.

3.6 Molecular docking

To understand and rationalize the mode of binding of the probes **R1** and **R2** with DNA, molecular docking was carried out for these probes with DNA duplex of sequence d(CGCGAATTCGCG)$_2$ dodecamer (PBD ID: 1BNA) using Gaussian software. The docking results suggested that these probes bind to AT sequences in the minor groove of the DNA, which is in accordance with the results of spectral studies (Fig. 5). Further these probes bind to the minor groove via a H-bond formation between the O-atom of methoxy substituent at position 10 and N-H moiety of DNA. This observation is in accordance with the fact that minor groove binders preferentially bind to AT sites in the minor groove of DNA via reversible non-covalent interactions [Badhuri et al., 2018 & Khan et al., 2012]. Relative binding energies of DNA-probe docked structures are calculated to be -11.59 and -10.47 kcal/mol for the probes **R1** and **R2**, respectively. The magnitudes of the binding energy indicated strong binding affinities of the probes for DNA. A competitive displacement docking has also been carried out to further establish the binding mode of these two probes with DNA. As seen from Figure 6, competitive docking with ethidium bromide showed that these probes bind to the minor groove of DNA while ethidium bromide intercalates into DNA bases, as expected [Husain et al. 2017]. Thus, the molecular docking results corroborated well with the results of spectral studies.

3.7 DNA staining studies

For electrophoresis study, 1% agarose gel was prepared in 1X TAE with different concentrations of the probes. The gel was allowed to get solidified for 30-40 min at room temperature. Upon solidification, the gel was immersed in the tank containing 1X TAE buffer. Plasmid DNA (TOPO TA clone with PCV1 construct cloned into it) samples were mixed in 6:1 ratio with 6X DNA loading dye (New England Biolabs). The agarose gel was allowed to run at 100 V, 100 mA and 10 W for 40 min and visualized under geldoc (Syngene G: Box model No. Chemi HR-1.4; Software: Genesys). The gel photographs obtained are depicted in Figures 6 and S16. As seen from Figure 7, the signal of nano gram amount of DNA can easily been obtained in the staining using these probes. Further, the detection limit of these probes is similar to that of EB and in both the cases the bands with good intensity are clearly visible to naked eye. From the results in Figure S14, it is evident that down to 4 ng of DNA can be successfully detected by the fluorescent probes under investigation, which is comparable with that of EB. The results indicated that the staining with these fluorescent probes is efficient and sensitive for DNA analysis and comparable with that of EB, the commonly used fluorescent staining agent. Furthermore, though these probes **R1** and **R2** are fluorescent in nature they don't exhibited any emission upon mixing with agarose gel and they do so only upon binding with DNA.



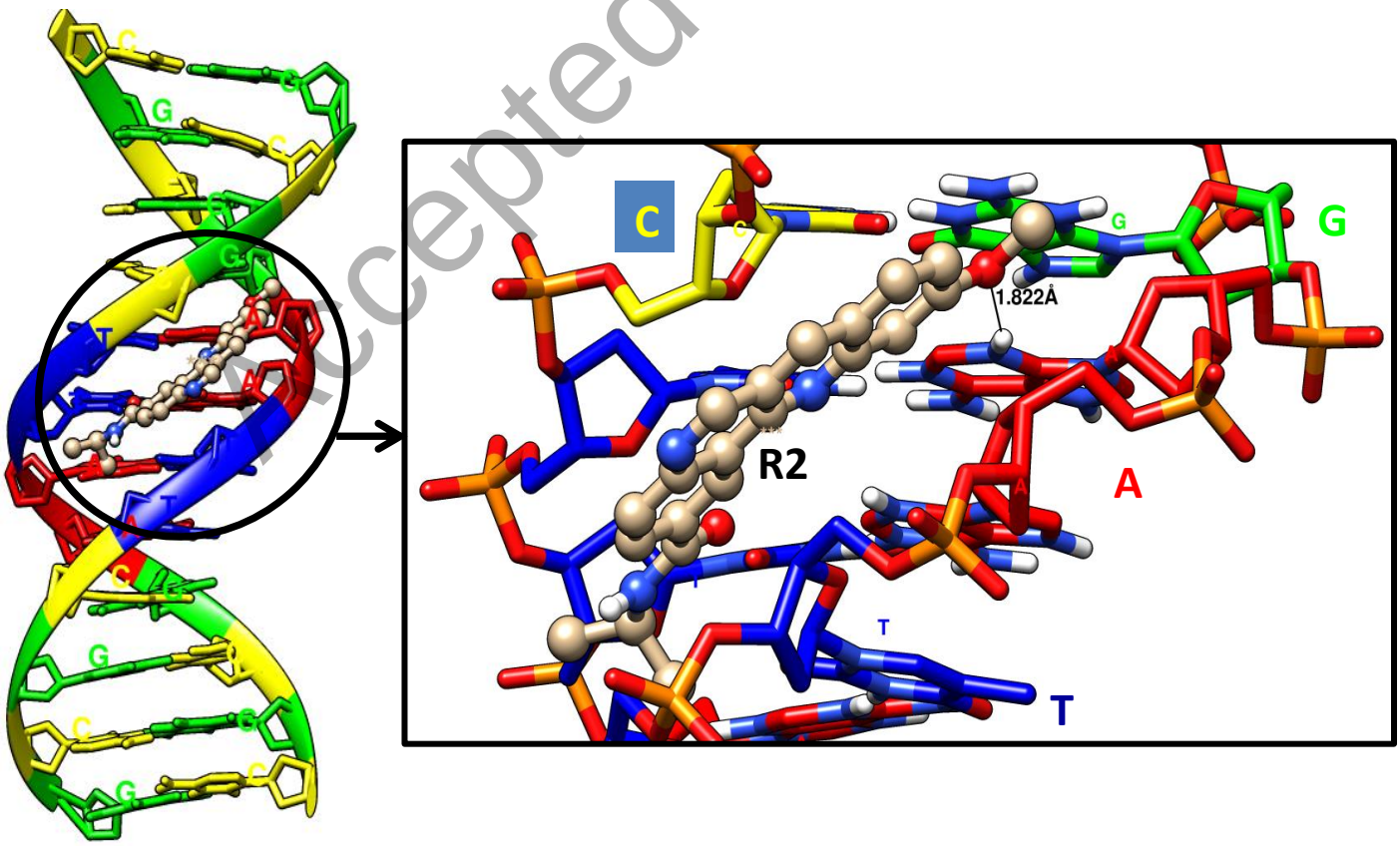


Fig. 5. Docking results depicting minor grove binding of **R1** and **R2** in AT sequence region with N-H…O hydrogen bond

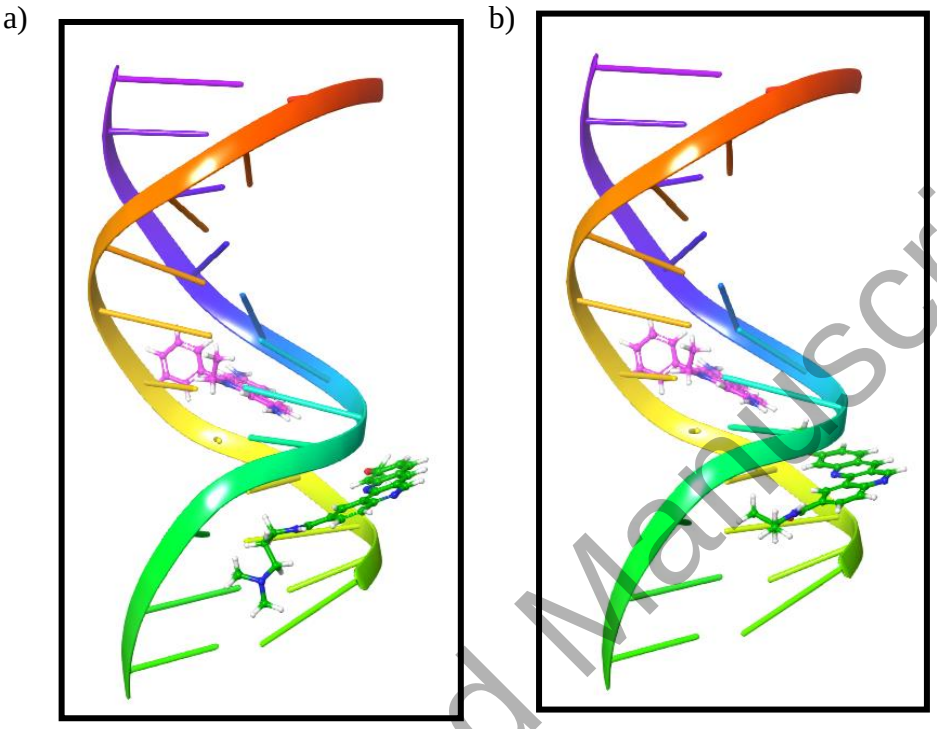


Fig. 6. Competitive molecular docked structures a) probe **R1** (green) and EB (pink); b) probe **R2** (green) and EB (pink)

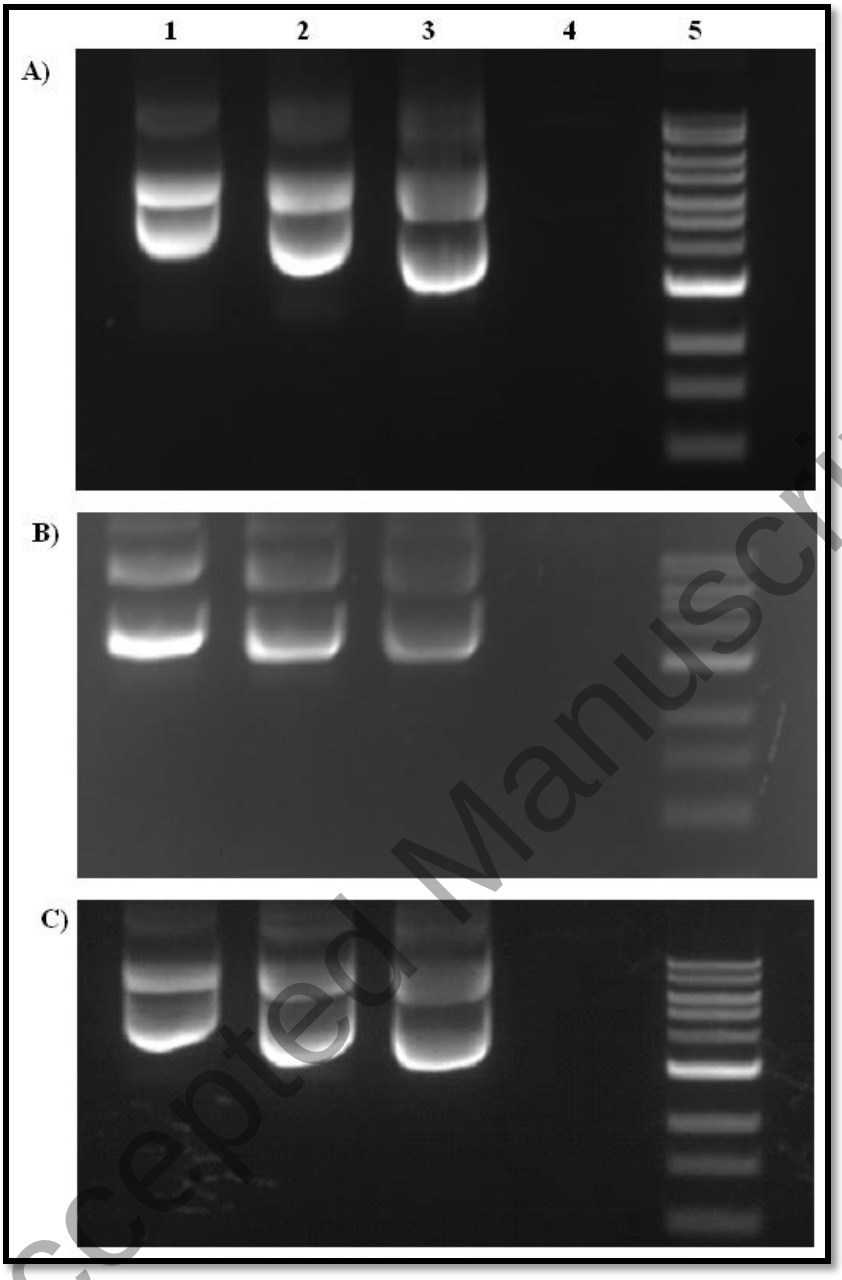


Fig. 7. Staining of DNA fragments with **R1** (**A**), **R2** (**B**) and EB (**C**) on a 1% agarose gel ([Probe] = 10 ng/mL). Lane (1): 100 ng of plasmid; (2): 200 ng of plasmid; (3) 500 ng of plasmid; (4) cDNA; (5) 1 kb ladder

4. Conclusion

New 10-methoxydibenzo[b,h][1,6]naphthyridine-2-carboxamide derivatives **R1** and **R2**, were synthesized, characterized and screened for their ability to bind with DNA. These probes were used to stain and visualize DNA during agarose gel electrophoresis. During gel electrophoresis, these probes stained DNA with same clarity as EB in the nM concentration range. As low as 4 ng of DNA can be visualized in the normal gel electrophoresis experiment in agarose gel. The mechanism of binding of these probes with DNA has been established using UV-Vis and fluorescence titration experiments along with ^{1}H NMR, CD and molecular docking studies. The results of these studies suggested binding of these probes to minor groove of DNA. The probes bind strongly with DNA accompanied with an enhancement in fluorescent intensity. Above all, these probes were found to be non-toxic towards cells in the concentration range employed. Therefore, these probes can be used to stain and visualize DNA in gel electrophoresis using agarose gel.

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