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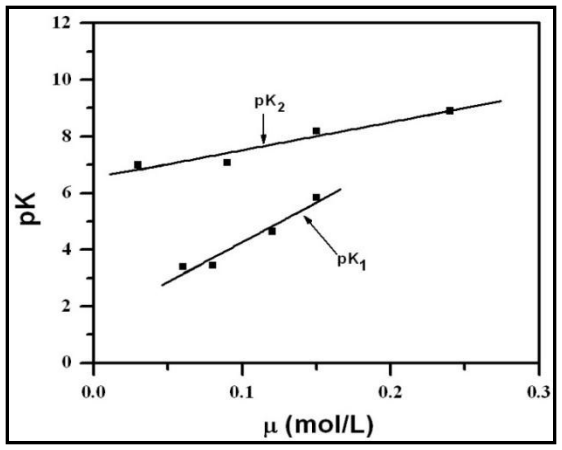
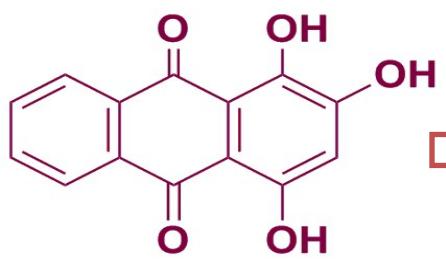
Hydroxy-9,10-anthraquinones



Dependence of pK on ionic strength



Purpurin or LH_3

Cu(II) complex of Purpurin

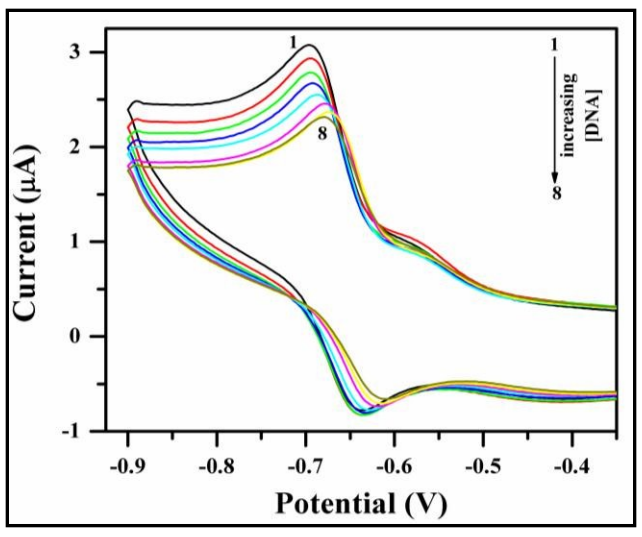
$Cu^{(II)}$-$(LH_2)_2$

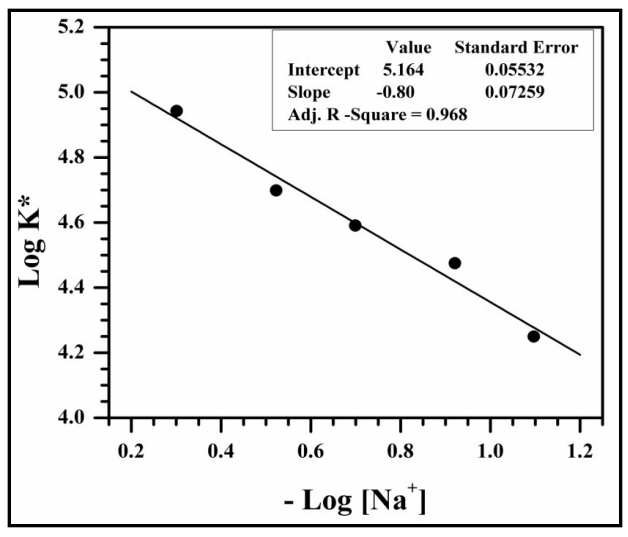
MTT assay shows Cu(II) complex of purpurin has better cytotoxic effect than purpurin on human breast cancer cells.

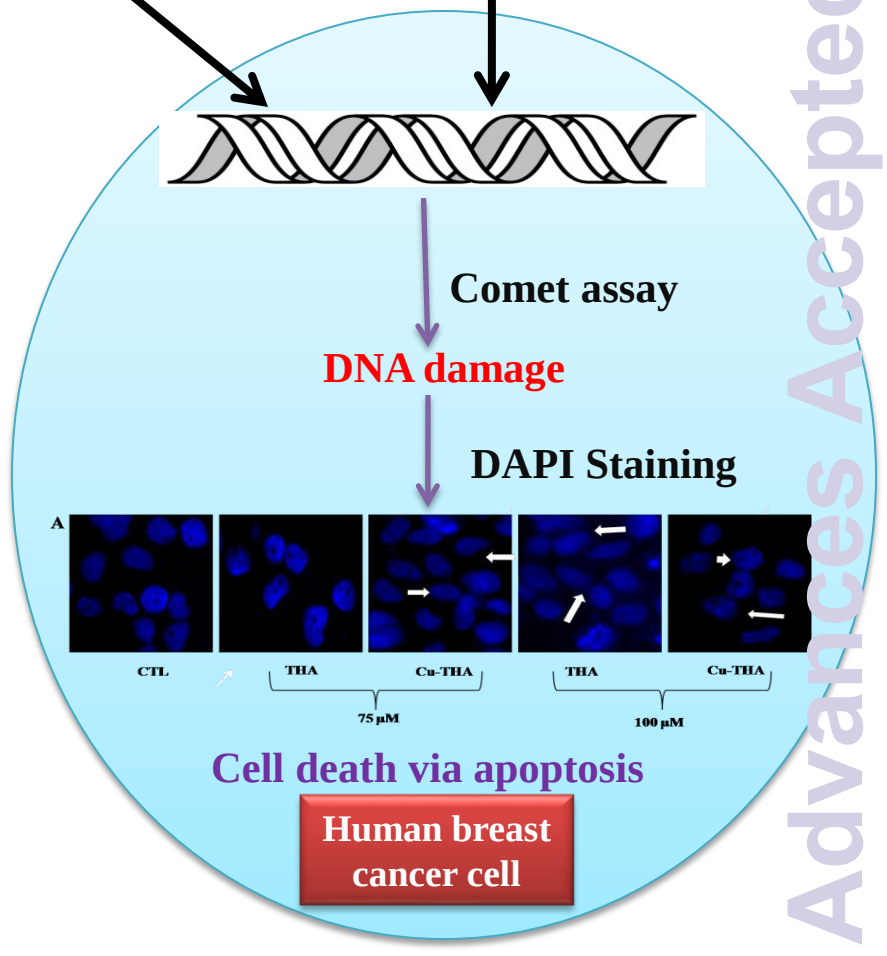
Interaction with DNA

$K' = (3.29 \pm 0.62) \times 10^4\ M^{-1}$

K* is dependent on ionic strength







Influence of ionic strength on the interaction of purpurin with DNA that helps to explain studies of it and its Cu^{II} complex on some breast cancer cells

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Abstract

Purpurin, a structural analogue to the family of anthracycline anticancer drugs was used to understand how environmental changes affect its biophysical interactions with DNA. A variation of ionic strength of the medium was attempted at constant pH. Under such varying conditions binding constant and site size of interaction were evaluated. Owing to close structural similarity with anthracyclines and the fact that the quinone moiety in these drugs control the chemical reactions, the effect of ionic strength on physicochemical and biophysical attributes of hydroxy-9,10-anthraquinones is important. With an increase in ionic strength, dissociation of the first proton of purpurin is affected to a greater extent than the second. Since pK_{a1} is connected with the generation of the mono-anion of purpurin an increase in its value implies there would be less anion in the medium leading to better DNA binding. Increased NaCl concentration neutralizes negative charges on the DNA backbone manifesting in an overall increase in binding constant for purpurin. This fact might be exploited for the use of such molecules on cancer patients. However, in case of a Cu^{II} complex of purpurin, formation of anions being almost negligible there is a marked improvement in DNA binding. Cytotoxic action of purpurin (LH_3) and its Cu^{II} complex $[Cu^{II}(LH_2)_2]$ were studied on also studied on three breast carcinoma cells and a primary human dermal fibroblast cells. The complex was seen to be better than purpurin. Results could be explained with the help of the Comet assay, γH2AX foci, DAPI staining followed by western blotting of apoptotic protein marker. Findings with purpurin and its Cu^{II} complex was compared with the anthracycline doxorubicin.

Key Words: Purpurin, Cu^{II}purpurin, pK, ionic strength, breast cancer cells, comet assay, phosphorylation of BAD.

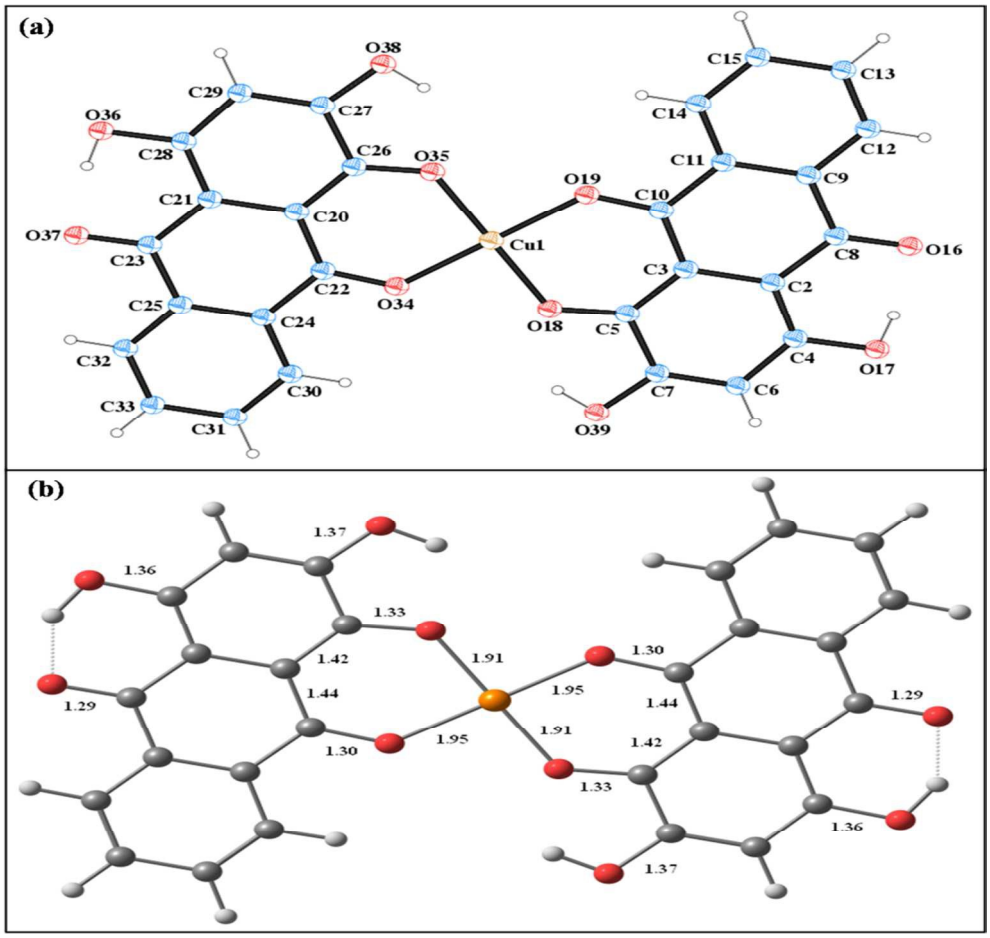
Abbreviation: THA or LH_3: 1,2,4-trihydroxy-9,10-anthraquinone or purpurin; Cu(II)-THA or Cu^{II}-$(LH_2)_2$: Cu^{II} complex of LH_3; c t DNA: calf thymus DNA; BAD: Bcl-2-associated death promoter protein

1. Introduction:

A major outcome of the research on cancer today is the ability to identify some of the reasons leading to the disease.[1-3] Recent reports suggest besides changing environmental conditions, a lot is also attributed to changing lifestyles, aspects of stress and strain that have become a part of our modern societies.[4,5] This has been further aggravated by several environmental factors contributing to initiation of cell aberration and their propagation.[5-7] Over the last few decades, a lot of effort has been made to enhance our knowledge on all possible stimuli that might be responsible.[3,4] Being aware of these, we can now take necessary precautions so that they are rendered inactive.[3-5] An important approach is to adopt proper food habits that include material capable of quenching toxic free radicals before they cause harm to cells and tissues.[4-8]

In the recent past, major breakthroughs were achieved in the area of drug development related to cancer.[9,10] These are able to check the progress of different forms of the disease in a much more convincing manner than a decade or two ago increasing the life span of cancer patients.[11,12] High quality targeted research and modern aspects of drug delivery have also been able to win over pain, trauma and side effects associated with cancer treatment, considered by many a significant achievement.[11,12] However, toxic side effects continue to be a major concern in cancer treatment, most often taking away the good aspects of a drug.[13-16] Hence, modifying established drugs in a manner that their associated toxic effects are either significantly decreased or controlled to an extent that efficacy is not compromised is a major area of research.[16-21] Anthracyclines for example, being an important class of effective chemotherapeutic agents are handicapped with cardio-toxic side effects.[22-26] Avoiding anthracyclines could be an immediate approach to remove the side effects, but that implies doing away with an effective group of chemotherapeutic agents. The medical fraternity is not prepared for this since not many alternatives are known. Hence, there is a need to broaden

the activity spectrum choosing molecules that are simple, addressing several related issues.[10,12,27-29] Hydroxy-9,10-anthraquinones present at the core of anthracyclines could be an option. They show remarkable similarity with anthracyclines on a number of fronts.[30-37] A Cu^{II} complex of purpurin for example, whose crystal structure we reported recently is an inhibitor of human DNA topoisomerase enzymes resembling doxorubicin (DOX).[36]



a) Perspective view of $Cu^{II}(LH_2)_2$ (for clarity, guest water molecules were omitted); Ellipsoids are drawn at 30% probability. b) DFT optimized structure of $Cu^{II}(LH_2)_2$. [Reproduced with permission from RSC Advances, where this was earlier published; **Vol. 3 (2014) pages 59344-59357].**

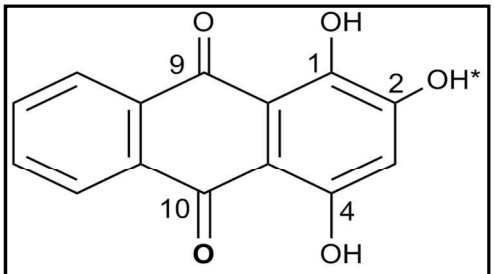
While making modifications or simplifying established drugs it is important to keep in mind several factors that either directly or indirectly influence drug action; aspects like pH, ionic strength of the medium etc. Cancer patients are known to show fluctuations of body pH and experience an

imbalance in electrolyte concentration.[27] In an earlier study, we showed binding of purpurin to DNA is affected with increase in pH and that complex formation is able to address this problem. In this study, we look at the aspect of a variation in ionic strength of the medium on physicochemical parameters like pK and biophysical parameters to see how these manifest in the molecule's interaction with DNA. The study also attempts to see the ability of purpurin and its Cu^{II} complex as effective anticancer agents on a few breast cancer cell lines. It makes an attempt to find reasons for action that are based on physicochemical, biophysical and cell biology experiments.

2. Experimental:

2.1 Materials

Purpurin (~96% purity) was purchased from Sigma-Aldrich and purified by re-crystallization from ethanol-water mixtures. The compound being photosensitive was stored in the dark. Stock solutions were prepared in ethanol (~10^{-4} M). HEPES buffer [4-(hydroxyethyl)-1-piperizine ethane sulphonic acid] was prepared using triple distilled water. Sodium nitrate (AR) and sodium chloride (AR) were used to maintain ionic strength of the medium. Calf thymus (c t) DNA was purchased from Sigma-Aldrich and dissolved in triple distilled water using 120 mM NaCl. Its absorbance was recorded at 260 and 280 nm respectively; A_{260}/A_{280} calculated. The ratio being in the range 1.8 <A_{260}/ A_{280}> 1.9, the DNA was considered sufficiently free of protein. It was also characterized using its CD spectra at 260 nm using a CD spectropolarimeter J815, JASCO. Concentration was determined in terms of nucleotide considering molar extinction coefficient at 260 nm to be 6,600 M^{-1} cm^{-1}.



Purpurin or LH_3 or LH_2H^*

2.2 Methods

2.2.1 Determination of pK for purpurin at different ionic strength

Proton dissociation constants were determined by pH-metric titration of an aqueous solution of purpurin (25 μM), varying the ionic strength of the medium. pH was recorded with the help of a pH meter [Elico LI 613, India]. Absorbance of each solution at 513 nm was plotted against pH. Purpurin exists in three distinctly different forms depending on the pH of the solution.[32] The forms are LH_2H^*, LH_2^-, L^{3-} respectively (Eqs. 1 and 2).[32]

$$LH_2H^* \rightleftharpoons LH_2^- + H^{*+} \qquad K_1 = \frac{[H^{*+}][LH_2^-]}{[LH_2H^*]} \tag{1}$$

$$LH_2^- \rightleftharpoons L^{3-} + 2H^+ \qquad K_2 = \frac{[L^{3-}][H^+]^2}{[LH_2^-]} \tag{2}$$

Absorbance (A_{obs}) at 513 nm was fitted to Eq. 3 yielding values for pK_{a1} and pK_{a2} respectively.[30-32]

$$A_{obs} = \frac{A_1}{(1 + 10^{pH - pKa1} + 10^{pH - pKa2})} + \frac{A_2}{(1 + 10^{pKa1 - pH} + 10^{pH - pKa2})} + \frac{A_3}{(1 + 10^{pKa1 - pH} + 10^{pKa2 - pH})} \tag{3}$$

A_1, A_2 and A_3 refer to absorbance due to LH_2H^*, LH_2^-, L^{3-} respectively while pK_{a1}, pK_{a2} are the pK_a values for the dissociation of LH_2H^* (Eqs 1 and 2).[32]

2.2.2 Electrochemistry on purpurin

Cyclic voltammetry was performed on an EG and G Potentiostat Model 263A, Princeton Applied Research, with power suite software for electrochemistry. Voltammograms were recorded using the three-electrode system; a glassy-carbon electrode of surface area 0.1256 cm^2 served as the working electrode, Ag/AgCl, KCl (saturated) was the reference while a platinum wire served as counter

electrode. Electrochemical measurements were done in a 20 ml electrochemical cell in aqueous solution using 0.1 M KCl as the supporting electrolyte. Before the start of each experiment the solution was purged with high purity argon for 25 minutes and in between scans for 5 minutes.

2.2.3 Interaction of purpurin with c t DNA at different ionic strengths of the medium using UV-Vis spectroscopy and cyclic voltammetry

DNA interaction was studied using separate aliquots containing a constant concentration of purpurin (75 µM) that was titrated with different concentrations of c t DNA. In case of UV-Vis spectroscopy, the total volume was kept constant at 2.0 ml while for cyclic voltammetry the volume was 3.0 ml. 15 mM HEPES buffer was used in either case. At the point of saturation, c t DNA was ~ 30 folds greater than the concentration of purpurin. Binding constant and site size of interaction were determined using standard equations.[31-37] All experiments were repeated three times.

$$L + DNA \rightleftharpoons L - DNA \qquad K_d = \frac{[L][DNA]}{[L-DNA]} \qquad (4)$$

"L" represents purpurin and "K_d" the dissociation constant for the equilibrium in Eq. 4.

Eq. 4 yields a double reciprocal equation in the form of Eq. 5.

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_{max}} + \frac{K_d}{\Delta A_{max}(C_D - C_0)} \qquad (5)$$

ΔA refers to the change in absorbance during titration, C_D the concentration of c t DNA in solution and C_0 that of purpurin. From Eq. 5 one obtains ΔA_{max} and K_{app} (= $1/K_d$) as the intercept and slope respectively. For each titration, ΔA_{max} indicates the maximum change in absorbance at the chosen wavelength for a particular concentration of NaCl at physiological pH. This was used to calculate $\Delta A/\Delta A_{max}$.

$$K_d = \frac{\left[C_0 - \left(\frac{\Delta A}{\Delta A_{max}}\right) C_0\right]\left[C_D - \left(\frac{\Delta A}{\Delta A_{max}}\right) C_0\right]}{\left(\frac{\Delta A}{\Delta A_{max}}\right) C_0} \quad (6)$$

$$C_0 \left(\frac{\Delta A}{\Delta A_{max}}\right)^2 - (C_0 + C_D + K_d)\left(\frac{\Delta A}{\Delta A_{max}}\right) + C_D = 0 \quad (7)$$

Using Eqs. 6 and 7, non-linear square fit analysis provided another value for K_{app} (= $1/K_d$).[31-36] Binding site size in terms of the number of nucleotides bound to each molecule of purpurin (n_b) was obtained from the plot of $\Delta A/\Delta A_{max}$ *vs.* $\frac{[DNA]}{[THA]}$. The data was also analyzed according to Scatchard.[38] Intrinsic binding constant (K') and site size of interaction (n) was determined where n is site size in terms of the number of purpurin molecules bound to each nucleotide.

$$r/C_f = K'(n - r) \quad (8)$$

r = C_b/C_D; C_b and C_f refer to the concentration of bound and free purpurin respectively.

Since electrochemistry experiments performed on purpurin and reported earlier showed that the compound in aqueous buffer undergoes electron exchanges that are purely diffusion controlled with no adsorption on the electrode surface, cathodic peak current (I_{pc}) was considered to be linearly proportional to its concentration.[32] Moreover, since there is no interference from DNA in the region of cathodic peak potential, a linear relationship between peak current and concentration quite logically holds good.[39-42] Applying the same analogy as that in absorption or fluorescence methods, binding parameters for interaction of purpurin with c t DNA were determined using cyclic voltammetry as well.[31-36]

ΔI is the change in current i.e. ($I_{pc}^0 - I_{pc}$) = change in cathodic peak current where I_{pc}^0 and I_{pc} are cathodic peak currents for purpurin at – 515 mV against Ag/AgCl, satd. KCl in the absence and

presence of different amounts of c t DNA. ΔI_{max} is the change at infinite time considering the compound to be totally bound to c t DNA. The double reciprocal equation (Eq. 9) yields values for ΔI_{max} and K_{app} from the intercept and slope respectively.

$$\frac{1}{\Delta I} = \frac{1}{\Delta I_{max}} + \frac{K_d}{\Delta I_{max}(C_D - C_0)} \quad (9)$$

$\Delta I/\Delta I_{max}$ denotes the fraction of purpurin bound to DNA.

$$K_d = \frac{\left[C_0 - \left(\frac{\Delta I}{\Delta I_{max}}\right)C_0\right]\left[C_D - \left(\frac{\Delta I}{\Delta I_{max}}\right)C_0\right]}{\left(\frac{\Delta A}{\Delta I_{max}}\right)C_0} \quad (10)$$

$$C_0\left(\frac{\Delta I}{\Delta I_{max}}\right)^2 - (C_0 + C_D + K_d)\left(\frac{\Delta I}{\Delta I_{max}}\right) + C_D = 0 \quad (11)$$

Eqs. 10 and 11 provide values for K_d (= $1/K_{app}$) using non-linear curve fit analysis.[39-42] The titration data from cyclic voltammetry was fitted to Eq. 8 and the corresponding Scatchard plot was drawn yielding the overall binding constant.

2.2.4 Cell culture

Three human breast cancer cell lines MCF-7, MDA-MB 231, MDA-MB 468 and one human primary dermal fibroblast (HDF) were cultured in RPMI or DMEM with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in a humid atmosphere containing 5 % CO_2.

2.2.5 MTT assay for cell viability

Anti-proliferative effect of purpurin and its Cu^{II} complex on three human breast carcinoma cell lines were determined by the MTT assay. Cells were seeded at a density of 1×10^3 cells/well in a 96 well plate. For normal HDF cells, these were seeded at a density of 1×10^5 cells/well in a 24 well plate. After 24 hour cells were exposed to purpurin (THA) or Cu^{II}-purpurin (Cu-THA) at different

concentrations for 48 hours. After incubation, cells were washed with 1 X PBS twice. Thereafter, they were treated with 0.5 mg/ml MTT solution (SRL) and incubated for 3-4 hrs at 37°C until a purple colored formazan product developed. The resulting product was dissolved in DMSO and OD was measured at 570 nm using a microplate reader (Biorad). Percentage survival was calculated considering untreated cells as 100%.

2.2.6. Single cell gel electrophoresis/Comet assay

Comet assay was performed after treating the cells with purpurin and Cu^{II} purpurin for 48 hours at the concentration of the IC_{50} dose of Cu^{II} purpurin (75 μM) and above (100 μM). Briefly, 1×10^5 cells/ml was mixed with 0.7 % LMPA and distributed onto frosted slides. The slides were then immersed in a lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl (pH 10)], containing freshly added 1% Triton-X 100 and 10% DMSO for 1 h at 4°C and placed into a horizontal electrophoresis tank filled with freshly prepared buffer (1 mM EDTA, 300 mM NaOH). After 20 min of pre-incubation (unwinding of DNA), electrophoresis was run for 20 min at a fixed voltage of 25 V and 300 mA. At the end of electrophoresis, slides were washed with a neutralization buffer (0.4 M Tris–HCl, pH 7.5) followed by staining with 20 μg/ml of ethidium bromide (SRL, India) for 15 min. The slides were then washed three times with 1× PBS and observed under a fluorescence microscope (model: Leica, Germany). Around 50 comets per slide were counted. Extension of each comet was analyzed by a computerized image analysis system (Komet software 5.5) that gave several parameters including % of tail DNA. The experiments were repeated at least three times.[43]

2.2.7. Analysis of nuclear morphology by DAPI staining

After exposure of purpurin and Cu^{II}purpurin at concentrations determined by IC_{50} dose of Cu^{II}purpurin (75 μM) and above (100 μM) for 48 hours, cells were washed several times with 1 × PBS and stained with 0.2 mg/ml of 4′, 6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories Inc.). The percentage of cells with rupture and decondensed nuclei were counted under a fluorescence microscope (Leica). At least 100 nuclei per slide were counted and photographs were taken at 40 X magnification.

2.2.8. γH2AX Foci assay

After exposure of the compounds purpurin and Cu^{II}purpurin for 48 h at their respective IC_{50} values, MCF7 cells were washed twice with 1 × PBS twice and fixed with freshly prepared 4% paraformaldehyde (Himedia, India) for 15 min at room temperature. After the fixation, cells were washed with 1 × ice cold PBS several times and treated with 0.2 % Triton X-100. Subsequently, the cells were blocked by 5% FBS and incubated with anti-γH2AX antibody diluted in wash buffer (1 : 100) containing 0.1% BSA and 0.05% Tween 20 in PBS for overnight at 4 °C in a moist chamber. After incubation, the cells were washed and labeled with appropriate secondary antibody conjugated with FITC. Finally cells were counterstained by DAPI and observed under a fluorescence microscope (Leica). At least 100 cells were counted per slides.

2.2.9. Western Blot Analysis

For western blot analysis, cells were cultured in 60 mm plates and after 24 h were treated with their respective IC_{50} of either purpurin or Cu^{II}purpurin for 48 hours. The harvested cell pellets were lysed in a lysis buffer containing 1% Triton-X 100, 50 mM sodium chloride, 50 mM sodium fluoride, 20 mM Tris (pH7.4), 1 mM EDTA (ethylene diamine tetraacetic acid), 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM sodium vanadate, 0.2 mM PMSF (Phenylmethylsulfonyl fluoride, Sigma),

0.5% NP-40 and protease inhibitor cocktail (Sigma) for 30 min on ice and centrifuged at 12,000 g for 2 min. The supernatant was collected and the protein concentration was measured by the Bradford method. Cell lysate containing equal quantities of protein (75 μg) were solubilized in Laemmli buffer, boiled for 5 min and electrophoresed on an 8-10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane. Non-specific binding was blocked with 5% non-fat dry milk in 1 × Tris–Cl, pH 7.6 with 0.1% Tween-20 (1 × TBS-T) followed by incubation with the appropriate primary antibodies Phospho-Bad (Ser 136) from Cell signaling technology, β-actin and PARP from Santa Cruz) for overnight. Membranes were washed with 1 × TBS-T and blots were incubated with anti-rabbit or anti-mouse secondary antibodies conjugated to HRP (Santa Cruz) at room temperature. Bound antibodies were detected by the ECL immunoblotting detection reagent (Santa Cruz).

3. Results and Discussion

3.1 Acid dissociation of purpurin: Dependence of pK on the ionic strength of the medium

Titration of purpurin under conditions of different ionic strength of the medium was done using NaOH. Different concentrations of NaCl and $NaNO_3$ were used for changing the ionic strength of the medium. Figure 1, A and B are typical plots for the variation of absorbance with pH at ionic strengths 0.06 (M) and 0.15 (M) respectively. Fitting the experimental data according to Eq. 3, pK_{a1} and pK_{a2} for purpurin were determined for different ionic strengths of the medium.

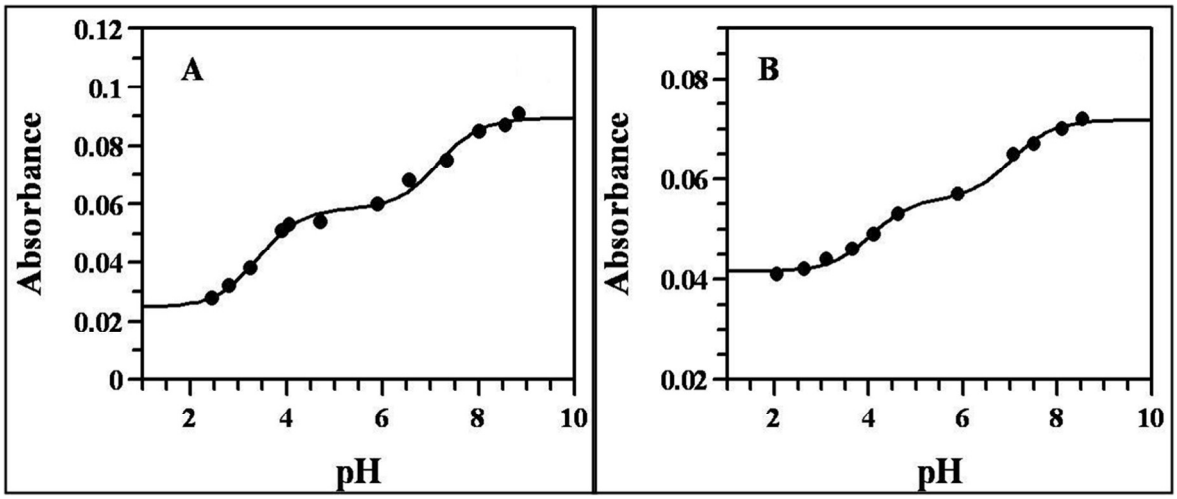


Fig. 1: pH-metric titration of purpurin, shown by a variation in absorbance at 513 nm at (A) ionic strength = 0.06 M and (B) ionic strength = 0.15 M. [purpurin] = 25 μM; T = 298 K.

It was observed as ionic strength increased both $pK_{a1}$and pK_{a2} increased indicating increase in ionic strength affects dissociation of OH groups adversely. Both pK_{a1} and pK_{a2} were individually plotted against the ionic strength of the medium (Fig. 2). An interesting fact realized from Fig. 2 was that for the same change in ionic strength, slope of the lines showing increase in pK_{a1} and pK_{a2} were different; the plot for pK_{a1} being comparatively steeper indicating that an increase in ionic strength of the medium affects the first dissociation to a much greater extent than the second.

From the determination of pK_a values due to an increase in the ionic strength, it was realized that dissociation of phenolic–OH decreased that is significant for hydroxy-9,10-anthraquinones since the dissociation of the first proton is important for studies on the interaction of purpurin with DNA at physiological pH (~ 7.4).[34-36] Even a small variation in pK_a is able to cause a huge difference to the presence of the neutral and anionic forms of the molecule that affects interactions with DNA.[34-36] An increased presence of the anionic form, being seen as a hindrance to interaction with DNA at physiological pH.[34, 36] Therefore, by the same analogy we can now say, in a medium with high ionic

strength, molecules like purpurin, should show greater interaction with c t DNA as anionic species would be less.

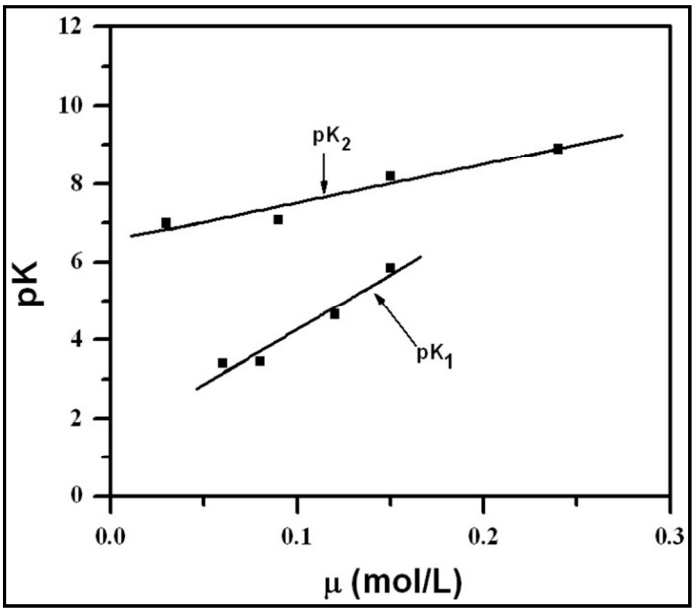


Fig. 2: Linear dependence of pK values of purpurin with variation in ionic strength of the medium at 298 K; [purpurin] = 25 μM.

3.2 Effect of ionic strength on the interaction of purpurin with c t DNA followed by UV-Vis spectroscopy

Interaction of purpurin with c t DNA was studied at physiological pH with an emphasis on the variation of the concentration of Na^+, analyzed according to the equilibrium shown in Eq. 4. Fig. 3 is a representative plot of Eq. 5 showing interaction at pH ~7.4 having NaCl concentration 0.5 M. Fig. 1S (A) is a similar plot obtained when NaCl concentration was 0.08 M. The apparent binding constant (K_{app}) obtained from these plots and similar plots having different NaCl concentrations were compared with K_{app} values obtained from non-linear square fit analysis (Table 1).

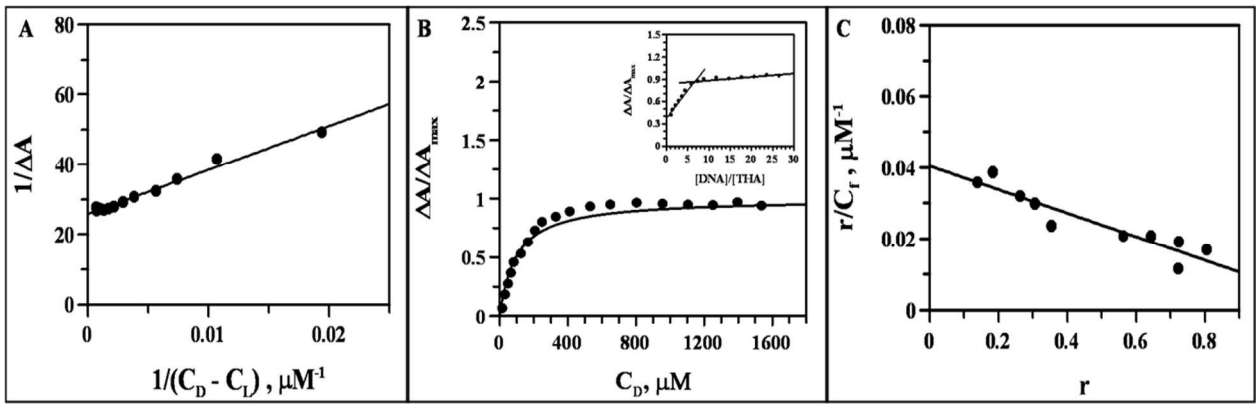


Fig. 3: (A**)** Double reciprocal plot for the interaction of purpurin with c t DNA followed by UV-Vis spectroscopy; (B) Binding isotherm of spectrophotometric study of purpurin –ct DNA and non-linear fit analysis; **Inset,** mole-ratio plot of the same; (C) Scatchard plot of UV-Vis study of purpurin – c t DNA interaction; [purpurin] = 75 μM, [NaCl] = 500 mM; [TRIS] = 15 mM of pH = 7.42; T = 298 K.

Fig. 3 (B) ([NaCl] = 0.5 M) and Fig 1S (B) ([NaCl] = 0.08 M) show plots for such interaction fitted by non-linear analysis (Eq. 7). Site size (n_b) for the interaction of purpurin with c t DNA at different NaCl concentration were also determined [Inset of Fig. 3 (B) and Fig. 1S (C), Table 1]. K_{app} values obtained either from the double-reciprocal plots or non-linear analysis when multiplied by the site size of interaction (n_b) provide overall binding constant values for the interaction between purpurin and c t DNA at different ionic strengths of the medium (Table 1). These were compared with values obtained directly from Scatchard plots (Eq. 8, Table 1). Fig. 3 (C) is a typical Scatchard plot obtained at [NaCl] = 0.5 M while Fig. 1S (D) shows the same at [NaCl] = 0.08 M. Results reveal as the concentration of Na^+ increased binding constant values for purpurin with c t DNA also increased (Table 1).

Table 1: Results of the interaction of purpurin with c t DNA for a variation of the ionic strength of the medium at pH ~ 7.40. [THA] = 75 μM, [TRIS] = 15 mM; T = 298 K.

[Na^+] in M	$K_{app} \times 10^{-4}$ (M^{-1}) from double-reciprocal plot	$K_{app} \times 10^{-4}$ (M^{-1}) from non-linear curve fitting	n_b	$K' \times 10^{-4} = K_{app} \times n_b$	$K' \times 10^{-4}$ (M^{-1}) Scatchard plot	$K' \times 10^{-4}$ (M^{-1}) B-H double-reciprocal plot
0.080	0.527 ± 0.026	0.364 ± 0.016	4.80	2.14	(1.91 – 2.53)	(0.76 – 0.89)
0.120	0.656 ± 0.032	0.683 ± 0.021	5.21	3.49	(2.47 – 3.74)	(2.25 – 3.49)
0.200	0.815 ± 0.018	0.729 ± 0.012	5.45	4.21	(3.39 – 4.23)	(3.58 – 4.39)
0.300	1.058 ± 0.022	0.789 ± 0.024	5.70	5.26	(3.63 – 4.72)	(5.04 – 6.59)
0.500	2.074 ± 0.013	1.496 ± 0.015	5.80	10.35	(8.77 – 11.28)	(5.44 – 9.60)

As expected from studies on the dissociation of purpurin at increased ionic strength at physiological pH and on the knowledge of the interaction of NaCl with a negative polymer like DNA we found through this study there was a substantial increase in binding constant values for purpurin interacting with c t DNA (Table 1).[44, 45] As stated earlier, at physiological pH purpurin exists in two different forms neutral and mono-anionic.[36] Hence, both these forms participate during interaction with DNA at physiological pH.[34, 36] The mono-anionic form owing to a negative charge on it faces repulsion from DNA while the neutral form is in a slightly better position to interact.[36] Through earlier studies on hydroxy-9,10-anthraquinones we showed that the tendency of the neutral form to bind DNA was much greater than the anionic form and we predicted if the presence of the anionic form in the medium could be decreased, then binding of such hydroxy-9,10-anthraquinones to DNA would increase substantially.[34-36] In this study, with the help of physicochemical experiments we could show there is an increase in pK_a values of purpurin under conditions of high ionic strength implying that the anionic form would decrease substantially at physiological pH.[32, 36.] Hence, for conditions applied for the DNA binding experiments it is only expected that binding of purpurin to

DNA would be much better than obtained earlier.[32, 35, 36] This was in fact found in reality (Table 1) and the reasons for this increase could be attributed to a) purpurin's reluctance to generate anionic species as ionic strength increased and b) a high ionic strength of the medium enabled the formation of a strong positively charged sheath around DNA that allowed better interaction with purpurin.

Dependence of binding constants on [Na^+] is an important aspect pertaining to interaction of molecules with DNA.[44, 45] Reports suggest binding of cationic substances with DNA decrease with increase in [Na^+] while anionic substances bind better. On a simple qualitative basis, ionic strength effects are a consequence of the mutual relationship between the number and charge of counter cations surrounding DNA and the number and charge of species that bind to it. According to Manning,[44] cations in order to partially neutralize the negative charge of the DNA phosphate backbone are condensed around nucleic acids i.e. confined close to the polyanion backbone without being bound to any particular site. When a drug intercalates causing a lengthening of DNA, the phosphates by moving further apart require lesser screening counter ions for a stable system. Hence, this also initiates the release of a certain number of condensed cations. Dependence of the binding constant on [Na^+] is interpreted by Eq. 12 based on the theory of Friedman and Manning.[44]

$$(\partial \log K / \partial \log[Na^+]) = -2n_b(\varphi - \varphi^*) - z\varphi^* \qquad (12)$$

As the concentration of NaCl is increased, repulsion between the anionic form of purpurin and DNA gradually decreased due to effective shielding of negative charges on DNA by Na^+. Now since with increase in concentration of NaCl, the ionic strength of the medium also increased, dissociation of the first proton of purpurin decreased (discussed earlier). For this reason, there was a marked decrease in the concentration of the anionic form of purpurin at physiological pH,

reducing significantly the repulsion between it and c t DNA. Moreover, since the negative character of the DNA backbone also decreased as more and more NaCl was added, there resulted a better interaction leading to an increase in binding constant values.

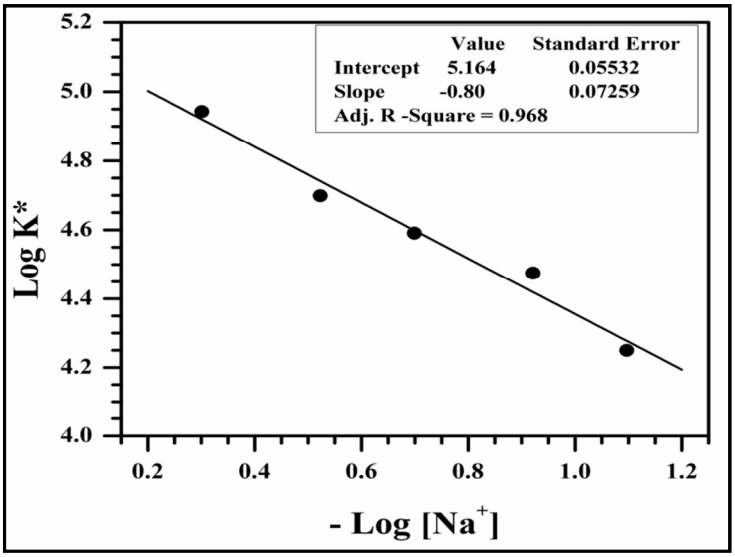


Fig. 4: Variation of intrinsic binding constants of purpurin – c t DNA interaction with the concentration of Na^+; [purpurin] =75 μM, [TRIS] =15 mM, pH = 7.42, T = 298 K.

All titrations of purpurin with c t DNA i.e. at each ionic strength of the medium, were repeated three times using different stock solutions of c t DNA. Results obtained were reproducible. An average of the overall binding constant (K′) was obtained from Table 1 for different concentrations of NaCl and this is shown in Table 2. Fig. 4 is a plot of log K′ versus –log [Na^+] that was found to be on expected lines.

Table 2: Results showing dependence of intrinsic binding constants for the interaction of purpurin with ct DNA on the ionic strength of the medium. [Purpurin] = 75 μM, pH = ~7.4 TRIS buffer = 15 mM. T = 298 K.

$[Na^+]$ in M	Intrinsic binding constant (M^{-1})	Site-size (n_b)
0.080	$(1.7767 \pm 0.8280) \times 10^4$	4.80
0.120	$(2.9875 \pm 0.7372) \times 10^4$	5.21
0.200	$(3.8975 \pm 0.4869) \times 10^4$	5.45
0.300	$(4.9950 \pm 1.2226) \times 10^4$	5.70
0.500	$(8.7725 \pm 2.4547) \times 10^4$	5.80

3.3 Interaction of purpurin with c t DNA by cyclic voltammetry

The interaction was studied in 10% ethanol containing 10 mM HEPES buffer (physiological pH ~ 7.4) having 0.12 M NaCl. Separate solutions were taken having a constant concentration of purpurin and different concentrations of c t DNA. Cyclic voltammetry of each solution was done and the change in cathodic peak current at -692 mV was used to construct binding isotherms. Cyclic voltammograms of the compound in the absence and presence of different amounts of c t DNA is shown in Fig. 5. The cathodic peak current was seen to undergo a positive shift of 25–35 mV corresponding to an increase in DNA concentration indicating intercalation of the compound between the base pairs.[46] Under identical experimental conditions, cyclic voltammetry of pure c t DNA did not show any cathodic or anodic peaks indicating it was not responsible for any electrochemical activity.[39] Decreased cathodic peak current (I_{pc}) obtained during the titration with c t DNA was understood as that obtained from the amount of free purpurin remaining.

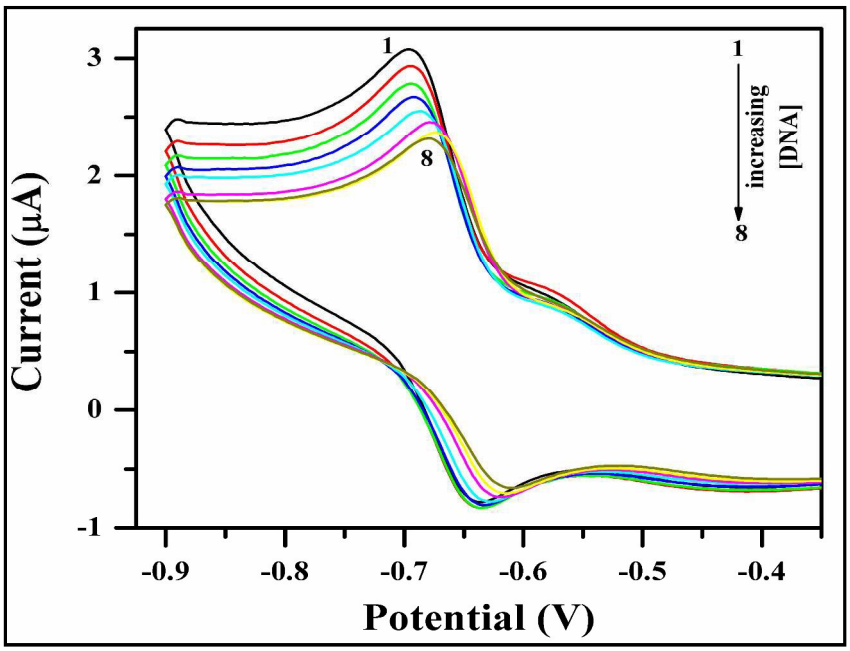


Fig. 5: Cyclic voltammogram of 100 μM purpurin in 120 mM NaCl (supporting electrolyte) and 10 mM HEPES buffer pH 7.36 in aqueous solution using glassy carbon electrode (A = 0.1256cm^2), in absence (1) and presence of different ct DNA concentrations: (2) 73.47 μM, (3) 217.55 μM, (4) 528.33 μM, (5) 853.46 μM, (6) 1159.18 μM, (7) 1585.00 μM, (8) 1849.17 μM; T = 298 K.

Fig. 6 is a typical double reciprocal plot to determine ΔI_{max} for the titration. The inverse of K_d (Eq. 9) provide values for K_{app} (Table 3). Fig. 6A shows the binding isotherm for the data on purpurin interacting with c t DNA as obtained from the titration using cyclic voltammetry fitted according to Eq. 9. Fig. 6 B is the non-linear square fit of the same data according to Eqs. 10 and 11. Values for K_{app} using cyclic voltammetry were in good agreement with those obtained using UV-Vis spectroscopy (Table 1). Multiplying K_{app} with site size of interaction (n_b) [inset of Fig. 6 B], overall binding constant (K′) was obtained. Fig. 6 C shows the Scatchard plot for the same titration using cyclic voltammetry that provides the overall binding constant and site size, n (= n_b^{-1}) tallying

appreciably with results obtained from the double reciprocal plot and non-linear square fit analysis (Table 3).

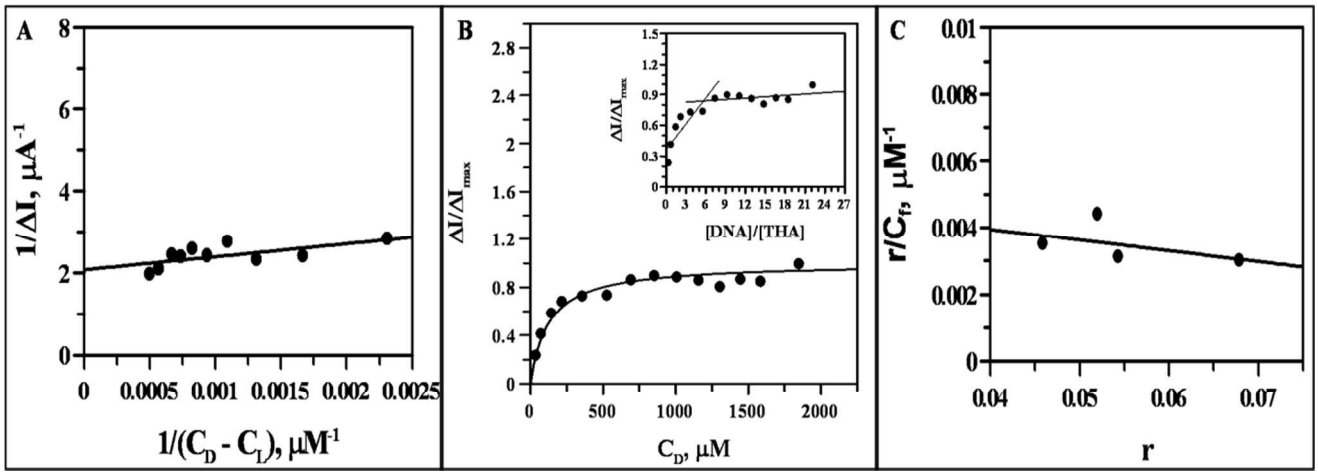


Fig. 6: (A) Double reciprocal plot showing interaction of purpurin with c t DNA followed by cyclic voltammetry; B) Binding isotherm for interaction of purpurin with c t DNA fitted by non-linear analysis; Inset of B shows a normalized increase of absorbance as a function of mole-ratio of c t DNA to purpurin; C) A Scatchard plot of the interaction of purpurin with c t DNA; [purpurin] = 100 μM, [NaCl] = 120 mM; pH = 7.36; T = 298 K.

Table 3: Results of binding parameters for the interaction of purpurin with c t DNA studied using cyclic voltammetry. [Purpurin] = 100 μM, [NaCl] = 120 mM; pH = 7.36; T = 298 K.

$K_{app} \times 10^{-3}$ (M^{-1}) from double-reciprocal plot	$K_{app} \times 10^{-3}$ (M^{-1}) from non-linear curve fitting	n_b	$K' \times 10^{-4}$ (M^{-1}) = K_{app} x n_b	$K' \times 10^{-4}$ (M^{-1}) Scatchard plot
6.59 ± 0.20	7.92 ± 0.16	5.97 ± 0.14	4.73 ± 0.15	3.29 ± 0.62

3.4 Effect of Purpurin and Cu^{II}purpurin on human breast carcinoma cell lines

Anthracyclines, like DOX, show remarkable efficacy in treating breast cancer and have been quite successfully and extensively used in the last few decades.[36, 47, 48] Owing to the close similarity between purpurin and DOX with regard to hydroxy-9,10-anthraquinone we made an attempt to determine the potency of purpurin and Cu^{II}purpurin on three human breast cancer cell lines MCF7, MDA-MB 231 and MDA-MB 468. Cells were treated with purpurin [THA] or Cu^{II}purpurin [Cu(II)-THA] for 48 hours in the concentration range 25 µM to 150 µM. Results of the assay show Cu^{II}purpurin was more effective on all three human breast cancer cells in a dose dependent manner (Fig. 7 A-C).

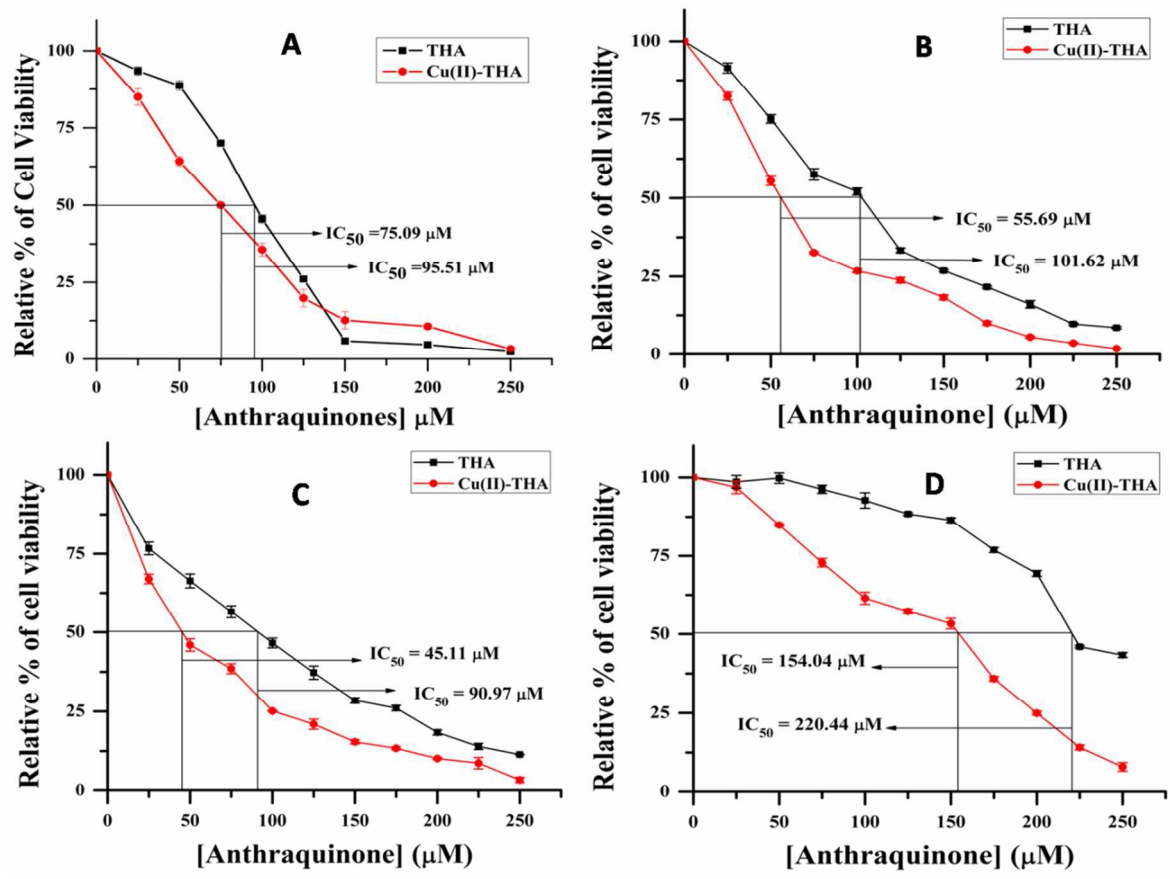


Fig. 7: MTT assay on human breast cancer cell lines (A) MCF-7, (B) MDA-MB 231, (C) MDA-MB 468 and (D) HDF in the presence of purpurin and Cu^{II}purpurin at 48 h of treatment.

Data are presented as % survival relative to the untreated control. All data are the mean ± SD of three independent experiments.

It was important that we see the performance of the compounds on normal cells as well. For this reason, Cu^{II}purpurin and purpurin were tried on human dermal fibroblast (HDF) cells. This being required for therapeutic applications, the effects had to be considered. Interestingly, it was observed that Cu^{II}purpurin was much less toxic to HDF than on the three breast cancer cells. In case of purpurin, the IC_{50} value could not be determined accurately as most survival values in the treated concentration range were higher than 50% (Fig. 7D). IC_{50} values for all types of cells are summarized in Table 4.

Table 4: Summary of IC_{50} doses of three human breast carcinoma cells and one human dermal fibroblast cell

Cell line	**IC_{50} (µM)**	
	Purpurin	Cu^{II} purpurin
MCF-7	**95.51**	**75.09**
MDA-MB 231	**101.62**	**55.69**
MDA-MB 468	**90.97**	**45.11**
HDF	**220.44**	**154.04**

Our results are in good agreement with previous reports where we showed Cu^{II} purpurin [Cu^{II}-$(LH_2)_2$] was more effective on ALL MOLT-4 cells than purpurin as it inhibits both human DNA topoisomerase I and human DNA topoisomerase II enzymes.[36] The fact that the IC_{50} values for

Cu^{II}purpurin are lower than purpurin on all breast cancer cells suggest a role for Cu^{II} in the complex in inducing cell death.[36, 49] The study on breast cancer cells and normal cells provide certain interesting observations in the back drop of the performance of the established drug doxorubicin. Being simpler with regard to synthesis and less costly than most anthracycline drugs that are in use, our chosen molecule and its Cu^{II} complex could be a viable alternative.

3.4.1 Comet assay

Cytotoxic effects of purpurin and Cu^{II}purpurin are most likely linked to the DNA-damaging effects of the compounds. The comet assay was performed since it is a more direct assay to study genotoxic effects of any substance. As shown in Fig 8A, Cu^{II}purpurin produced more DNA damage compared to purpurin at 75 μM and 100 μM respectively. At the IC_{50} dose of the complex, DNA damage was absent for purpurin. In consistence with this data, the percentage of tail DNA increased significantly ($p<0.01$) for Cu^{II}purpurin treated cells after 48 hours of treatment (Fig. 8 B). The availability of copper for interaction with DNA could be one reason that might have shown such enhanced genotoxic effect of the complex in comparison to purpurin.[23,49] Therefore, considering a probable enhanced cellular uptake of the complex followed by an effective binding of it with the DNA of the cells Cu^{II}purpurin was able to induce greater DNA damage as observed in the comet assay.[36,49] Besides interaction of Cu^{II}purpurin with DNA also revealed compared to purpurin, binding of the former was much greater and that binding constant values for the complex did not decrease with an increase in the pH of the medium as was observed for purpurin itself.[36] All these attributes add up to indicate why the complex is better than purpurin on all the three breast cancer cells.

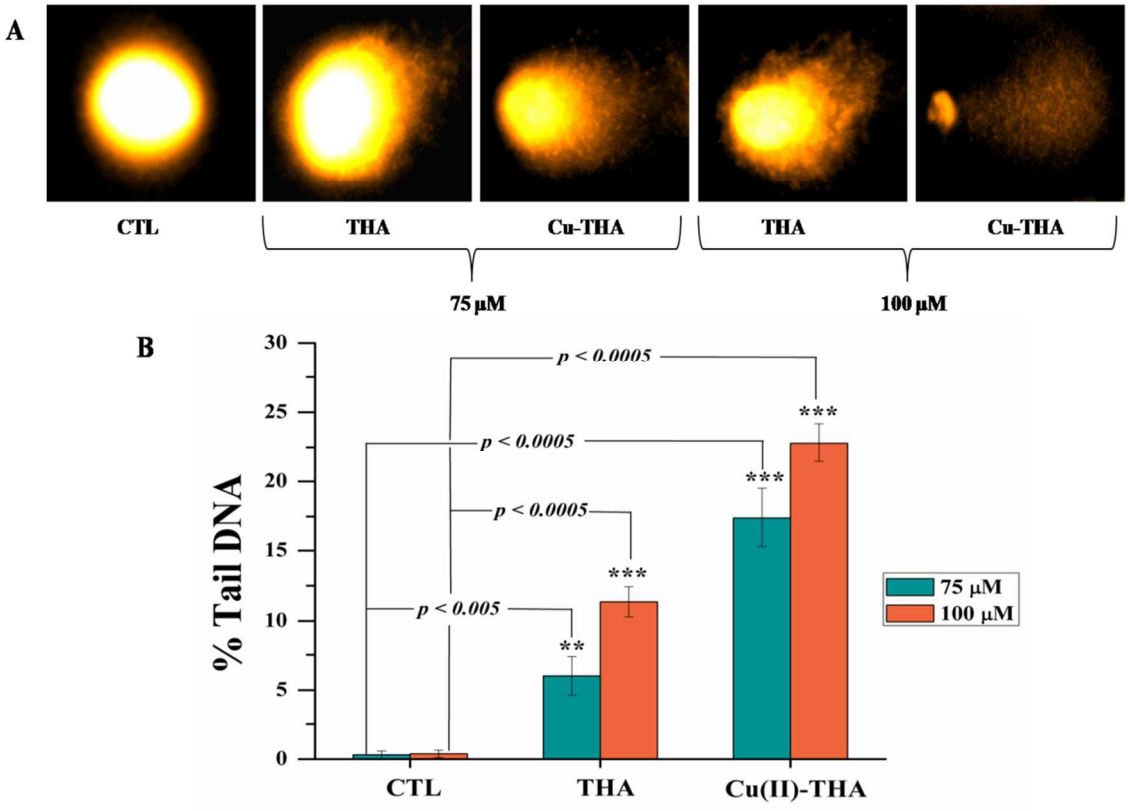


Fig. 8: Induction of DNA damage on MCF-7 cells by purpurin [THA] and Cu^{II}purpurin [Cu(II)-THA] at a concentration of 75 µM and 100 µM respectively for 48 h after which the genotoxicity was quantified by comet assay. (A) Representative images of comet assay with respect to untreated control. (B) Histogram is % of Tail DNA of comets for THA and Cu-THA with respect to their untreated control of both 75 µM and 100 µM after 48 h of treatment. Values are the mean ± standard deviation (SD; vertical bars) of three independent experiments. **(p <0.005) and ***(P <0.0005) denoted the statistically significant difference compared to the untreated control.

3.4.2 γH2AX Foci assay

One step further to validate the previous experiment, we determined γH2AX foci formation in MCF7 cells in the presence of purpurin or Cu^{II}purpurin for 24 h at their respective IC_{50} values. As shown in Fig 9 A, Cu^{II}purpurin produced more γH2AX foci compared to purpurin at 75 µM and 100 µM respectively. At the IC_{50} dose of the complex, γH2AX foci was almost absent for purpurin.

In consistence with this data, the percentage of γH2AX foci containing cells increased for Cu^{II}purpurin treated cells for both the concentrations [75 μM and 100μM] (Fig. 9B). Thus, Cu^{II}purpurin is more effective in inducing DNA damage compared to purpurin alone.

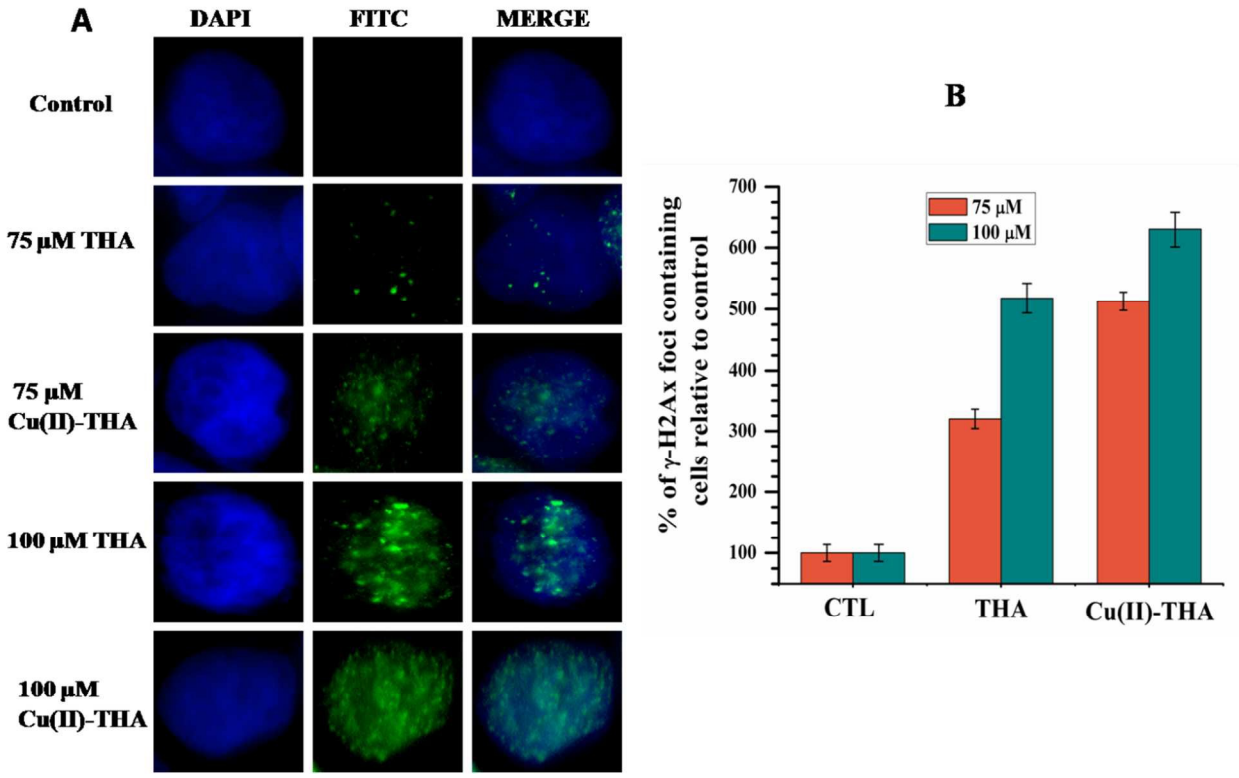


Fig. 9 Induction of γH2AX foci in MCF7 cells in the presence of purpurin and Cu^{II}purpurin at a concentration of 75 μM and 100 μM respectively for 48 hours. (A) Representative images of γH2AX foci assay with respect to untreated control DNA counter stained with DAPI were shown in blue and γH2AX foci appeared in green. (B) Histogram shows the % of γH2AX foci for purpurin and Cu^{II}purpurin with respect to their untreated control of 75 μM and 100 μM after 48 hours of treatment. Values are the mean ± standard deviation (SD; vertical bars) of three independent experiments.

3.4.3 DAPI staining

Nuclear morphology and nature of cell death was studied by DAPI staining (Fig. 10A). The percentage of apoptotic cells are represented graphically (Fig. 10B). When cells were treated with

100 µM of either compound for 48 hours we found 15 to 20 % cells were apoptotic in nature following treatment with Cu^{II}purpurin, whereas for purpurin, the value was only 5 to 10%. DNA damage and subsequent induction of apoptosis in case of the complex treated breast cancer cell MCF-7 implicates its potential therapeutic application.

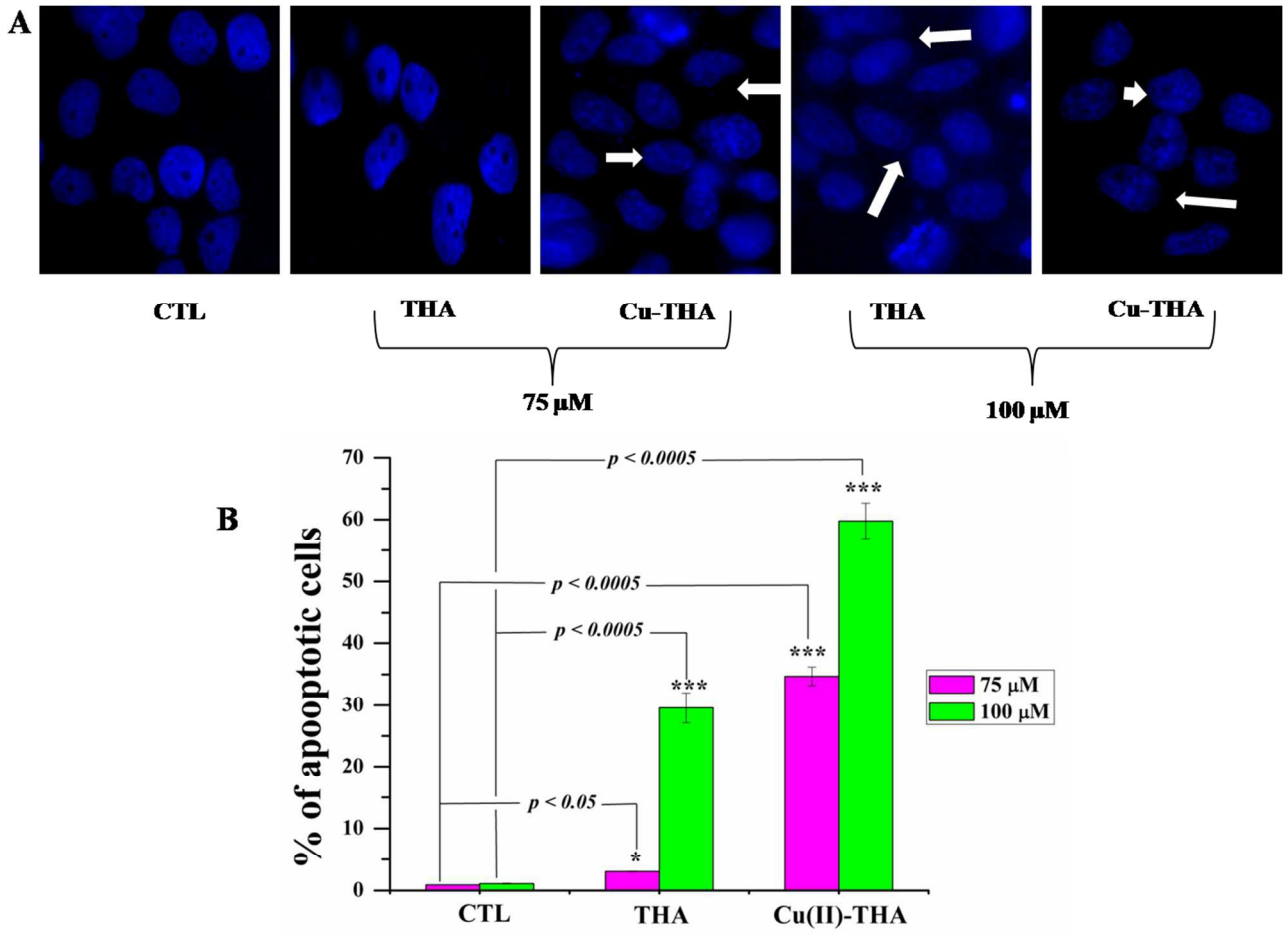


Fig. 10: (A) Fluorescence micrographs of DAPI stained MCF-7 cells in 40X magnification. Arrow represents the nucleus of apoptotic cells. (B) % of apoptotic cells as determined by DAPI staining followed by fluorescence microscopic observations. Each value represents the mean ± S.D. of three independent experiments. * ($p < 0.05$) and *** ($P < 0.0005$) denote the statistically significant difference compared to untreated control.

3.4.4 Western Blotting

To establish induction of apoptosis, we further immunoblotted the MCF-7 cell extract with antibodies specific for apoptosis marker proteins. As seen in Fig. 11 the amount of BAD phosphorylation (ser136) was reduced. For induction of apoptosis dephosphorylation of BAD is necessary and our results indicate, Cu^{II}purpurin treatment resulted in induction of apoptosis. Further, cleavage of PARP by caspase 3 is also an indication of the apoptosis process. In Fig.11 we also demonstrated that after treatment of Cu^{II}purpurin (75 μM and 100 μM), PARP was cleaved to 90 kD and 55 kD. In case of the purpurin treated cells a slight cleavage of PARP was observed at 100 μM concentration which is the IC_{50} value of purpurin (Table 4). Thus at higher concentration purpurin is able to induce DNA damage (Fig. 8 and Fig 9) that may be responsible for the induction of apoptosis.

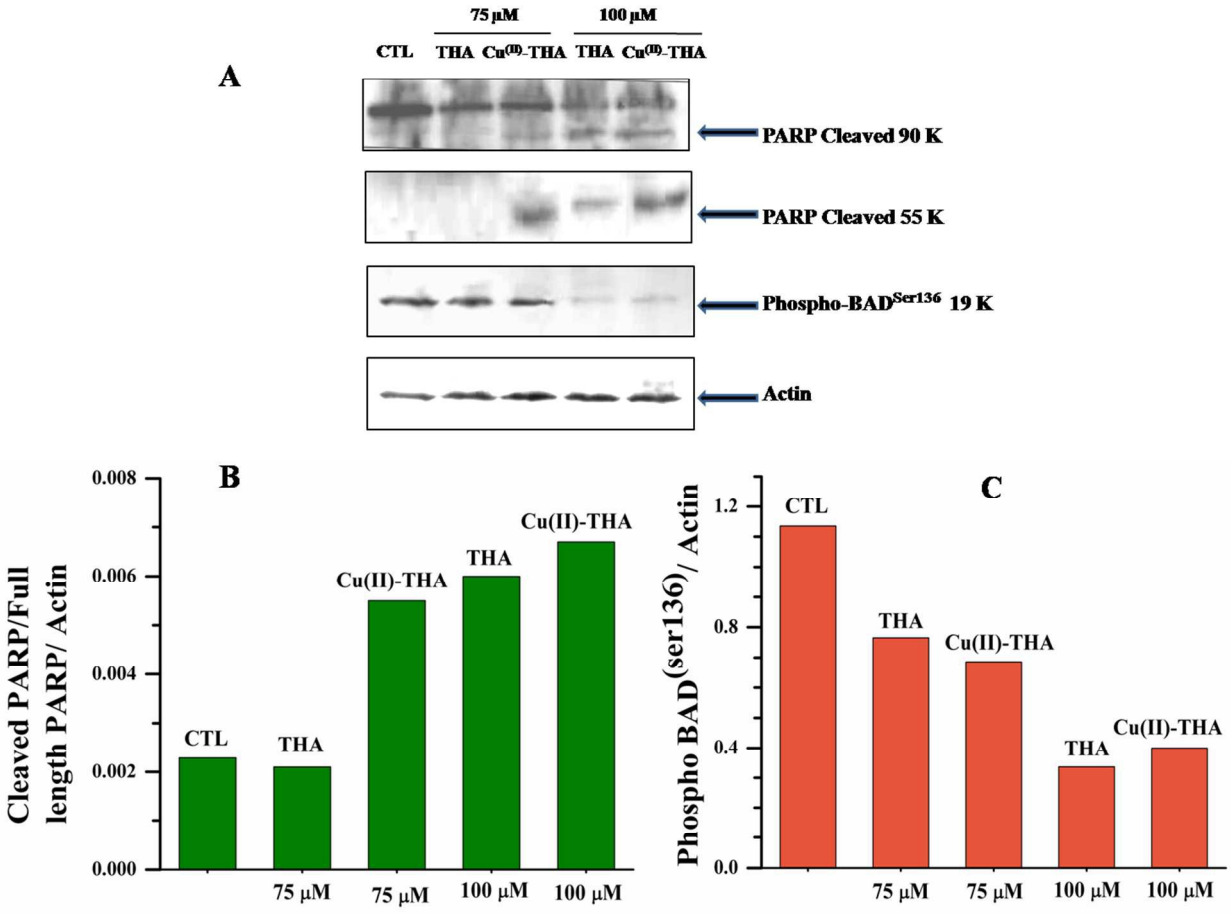


Fig. 11: (A) Western blot analysis of apoptosis marker protein in the MCF-7 cell extract after the cell was treated with purpurin and Cu^{II}purpurin at 75 μM and 100 μM respectively following a 48 hours treatment. The bottom panel (B) and (C) corresponds to quantification data analysis of these proteins with respect to loading control β-actin.

Conclusion:

An attempt was made to see the efficacy of purpurin and its mononuclear complex with Cu(II) $[Cu^{II}\text{-}(LH_2)_2]$ on three different breast cancer cell lines in the backdrop of an established anthracycline doxorubicin which they closely resemble. A greater ability shown by the complex to stop cell proliferation and damage to DNA in these cells was attributed to the presence of Cu^{II} that probably caused an efficient cellular uptake in the first place. Cu^{II} being important to cancer cells, enabled a better interaction with DNA established through an earlier study with the complex using UV-Vis spectroscopy and cyclic voltammetry.[39] Physicochemical studies performed on purpurin at increased ionic strength of the medium help to discuss the reasons behind purpurin showing a greater tendency to bind DNA at high ionic strengths of the medium that explains its activity on the different breast cancer cells used in this study. Although the complex was found to be more potent in killing breast cancer cells compared to purpurin but on human primary dermal fibroblast cells it was non-toxic. Therefore, considering earlier findings of this complex on DNA topoisomerase enzymes[36] we suggest it holds a promise for anticancer activity. Doxorubicin, an established anticancer drug, found to be effective on breast cancer and our molecules resembling it both structurally and with their demonstrated activity on several breast cancer cells could be an important alternative.

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References:

1) G. Danaei, S. Vander Hoorn, A. D. Lopez, C. J. Murray, M. Ezzati, *Lancet* 2005, **366(9499)**, 1784-1793.
2) K. Morishita, *Hidden Truth of Cancer*, George Ohsawa Macrobiotic Foundation, East West Center for Macrobiotics, Chico, California, **2010**.
3) World Cancer Report 2014, International Agency for Research on Cancer, World Health Organization, **2014**, ISBN 9283204298.
4) P. Anand, A. B. Kunnumakkara, C. Sundaram, K. B. Harikumar, S. T. Tharakan, O. S. Lai, B. Sung, B. B. Aggarwal, *Pharm Res.,* 2008, **25** 2097-2116, doi: 10.1007/s11095-008-9661-9.

5) P. Irigaray, J. A. Newby, R. Clapp, R, L. Hardell, V. Howard, L. Montagnier, S. Epstein, D. Belpomme, *Biomed. Pharmacother.* 2007, **61**, 640–658 doi:10.1016/j.biopha. 2007.10.006. PMID 18055160.

6) J. Kravchenko, I. Akushevich, K. G. Manton, *Cancer mortality and morbidity patterns in the U. S. population: an interdisciplinary approach*, Springer, Berlin, 2009. ISBN 0-387-78192-7

7) B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, P. *The preventable causes of cancer* in Chapter 23: Cancer in *Molecular biology of the cell* (4th ed.), New York: Garland, Science, 2002. ISBN 0-8153-4072-9.

8) T. Byers, M. Nestle, A. McTiernan, C. Doyle, A. Currie-Williams, T. Gansler, M. Thun, and the American Cancer Society 2001, *CA Cancer J Clin*. 2002, **52**, 92-119.

9) G. J. Kelloff, S. M. Lippman, A. J. Dannenberg, C. C. Sigman, H. L. Pearce, B. J. Reid, E. Szabo, V. C. Jordan, M. R. Spitz, G. B. Mills, V. A. Papadimitrakopoulou, R. Lotan, B. B. Aggarwal, R. S. Bresalier, J. Kim, B. Arun, K. H. Lu, M. E. Thomas, H. E. Rhodes, M. A. Brewer, M. Follen, D. M. Shin, H. L. Parnes, J. M. Siegfried, A. A. Evans, W. J. Blot, W. H. Chow, P. L. Blount, C. C. Maley, K. K. Wang, S. Lam, J. J. Lee, S. M. Dubinett, P. F. Engstrom, P. F.; F. L. Meyskens Jr, J. O'Shaughnessy, E. T. Hawk, B. Levin, W. G. Nelson, W. K. Hong, AACR Task Force on Cancer Prevention. Progress in chemoprevention drug development: the promise of molecular biomarkers for prevention of intraepithelial neoplasia and cancer--a plan to move forward, *Clin Cancer Res*., 2006, **12**, 3661-3697.

10) S. Hoelder, P. A. Clarke, P. Workman, *Molecular Oncology*, 2012, **6**, 155–176.

11) J. D. Benson, Y.-N. P. Chen, S. A. Cornell-Kennon, M. Dorsch, S. Kim, M. Leszczyniecka,, W. R. Sellers, C. Lengauer, *Nature*, 2006, **441**, 451-456.

12) M. De Palma, D. Hanahan, *Mol. Oncol*. 2012, **6,** 111-127.

13) L. Gianni, B. J. Corden, C. E. Myers, The biochemical basis of anthracycline toxicity and antitumor activity. In Reviews in Biochemical Toxicology, Hodgson E, Bend J, Philpot, R. M., Eds.; Elsevier Biomedical, New York, 1983; pp 1-82.

14) S. V. Geiger, N. Lange, P. Suhl, V. Heinemann, H. J. Stemmler, H. J. *Anticancer Drugs,* 2010, **21**, 578-90; doi: 10.1097/CAD.0b013e3283394624.

15) C. L. Loprinzi, W. I. Bensinger, D. E. Peterson, C. Messner, Eds. Understanding and managing chemotherapy side effects, Cancer Care, National Office 275 Seventh Avenue New York, NY10001, 2014.

16) C. Vergely, S. Delemasure, Y. Cottin, L. Rochette, *Heart Metab*., 2007, **35**, 1–7.

17) A. Nagy, A. V. Schally, P. Armatis, K. Szepeshazi, G. Halmos, M. Kovacs, M. Zarandi, K. M. Groot, A. Miyazaki, Jungwirth, J. Horvath, Medical Sciences, Proc. Natl. Acad. Sci. USA, 1996, ***93***, 7269-7273.

18) M. M. L Fiallo and A. Garnier-Suillerot, *Biochim. Biophys. Acta*, 1985, **840**, 91-98.

19) H. Beraldo, A. Garnier-Suillerot, L. Tosi, F. Lavelle, Biochemistry, 1985, **24**, 284-289.

20) C. Santini, M. Pellei, V. Gandin, M. Porchia, F. Tisato, C. Marzano, *Chemical Reviews*, 2014, **114**, 815-862.

21) A. Kheirolomoom, L. M. Mahakian, C. Y. Lai, H. A. Lindfors, J. W. Seo,; E. E.Paoli, K. D. Watson, E. M. Haynam, E. S. Ingham, L. Xing, R. H. Cheng, A. D. Borowsky, R. D. Cardiff, K. W. Ferrara, *Mol Pharm*., 2010, **7**, 1948-1958.

22) A. J. M. Ferreri, E. Campo, A. Ambrosetti, F. Ilariucci, J. F. Seymour, R. Willemze, G. Arrigoni, G. Rossi, A. Lopez-Guillermo, E. Berti, M. Eriksson, M. Federico, S. Cortelazzo, S. Govi, N. Frungillo, S. Dell'Oro, M. Lestani, S. Asioli, E. Pedrinis, M. Ungari, T. Motta, R.

Rossi, T. Artusi, P. Iuzzolino, E. Zucca, F. Cavalli, M. Ponzoni, *Ann. Oncol*. 2004, **15**, 1215-1221.

23) E. V. Barry, S. E. Lipshultz, S. E. Sallan, Anthracycline-induced cardiotoxicity: natural history, risk factors, and prevention, in American Society of Clinical Oncology 2008 Educational Book (Govindan R., Ed.), *American Society of Clinical Oncology*, Alexandria, 2008, pp. 448–453.

24) M. I. Gharib, A. K. Burnett, Eur. J. Heart. Fail. 2014, **4**, 235-242.

25) D. Iarussi, P. Indolfi, F. Casale, V. Martino, M. T. Di Tullio, R. Calabrò, *Paediatr. Drugs*, 2005, **7**, 67-76.

26) D. Cardinale, A. Colombo, G. Lamantia, N. Colombo, M. Civelli, G. D. Giacomi, M. Rubino, F. Veglia, C. Fiorentini, C. M. Cipolla, *J. Am. Col. Cardiol*., 2010, 55, 213-220.

27) V. Vukovic, I. F. Tannock, *Br. J. Cancer,* 1997, **75**, 1167-1172.

28) I. F. Tannock, D. Osoba, M. R. Stockler, D. S. Ernst, A. J. Neville, M. J. Moore, G. R. Armitage, J. J. Wilson, P. M. Venner, C. M. Coppin, K. C. Murphy, *J. Clin. Oncol*., 1996, **14**, 1756-1764.

29) Y. Zhao, E. B. Butler, M. Tan, *Cell Death and Disease*, 2013, Targeting cellular metabolism to improve cancer therapeutics, *Cell Death and Disease*, e532; doi:10.1038/cddis.2013.60.

30) S. Das, A. Saha, P. C. Mandal, *Talanta*, 1996, **43**, 95-102.

31) P. S. Guin, S. Das, P. C. Mandal, *J. Inorg. Biochem*., 2009, **103**, 1702-1710.

32) P. Das, P. S. Guin, P. C. Mandal, M. Paul, S. Paul, **S.** Das, *J. Phy.Org. Chem*., 2011, **24**, 774-785.

33) P. S. Guin, S. Das, P. C. Mandal, *J. Sol. Chem*. 2011, **40**, 492-501.

34) S. Mukherjee, P. Das, S. Das, *J. Phy.Org. Chem.*, 2012, **25**, 385-393.

35) S. Mukherjee, P. K. Gopal, S. Paul, S.; Das, *J. Anal. Oncol*., 2014, **3**, 122-129.

36) P. Das, C. K. Jain, S. K. Dey, R. Saha, A. D. Chowdhury, S. Roychoudhury, S. Kumar, H. K. Majumder, S. Das, *RSC* Advances, 2014, **3**, 59344-59357**.**

37) S. Roy, P. Mondal, P. S. Sengupta, D. Dhak, R. C. Santra, S. Das, P. S. Guin, Dalton Transactions, 2015, **44,** 5428-5440. DOI: 10.1039/C4DT03635B

38) G. Scatchard, *Ann. N. Y. Acad. Sci*., 1949, **51**, 660-672.

39) P. S. Guin, P. Das, S. Das, P. C. Mandal, *Int. J. Electrochem.* 2012, **2012**, Article ID 183745, 10 pages, doi:10.1155/2012/183745.

40) P. S. Guin, P. C. Mandal, S. Das, *ChemPlusChem* 2012, **77**, 361 – 369.

41) R. C. Santra, K. Sengupta, R. Dey, T. Shireen, P. Das, P. S. Guin, K. Mukhopadhyay, S. Das, *J. Coord. Chem*. 2014, **67**, 265-285.

42) R. C. Santra, D. Ganguly, J. Singh, K. Mukhopadhyay, S. Das, *Dalton Trans*. 2015, **44**, 1992-2000. doi: 10.1039/c4dt03362k.

43) N. P. Singh, M. T. McCoy, R. R. Tice, E. L. Schneider, *Exp. Cell Res*. 1988, *175*, 184-191.

44) R. A. G. Friedman, G. S. Manning *Biopolymers* 1984, **23**, 2671-2714.

45) M. Cusumano, A. Giannetto, *J. Inorg. Biochem*.1997, **65,** 137-144.

46) A. J. Bard, L. R. Faulkner, *Electrochemical Methods Fundamental and Applications*, 2nd ed., John Wiley & Sons. New York, 2001.

47) J. L. Speyer, M. D. Green, A. Zeleniuch-Jacquotte, J. C. Wernz, M. Rey, J. Sanger, E. Kramer, V. Ferrans, H. Hochster, M. Meyers, et al. *J Clin Oncol*. 1992, **10**, 117–127.

48) A. Moreno-Aspitia, E. A. Perez, *Clin*. *Ther*. 2009, **31**, 1619–1640.

49) T. Deb, P. K. Gopal, D. Ganguly, P. Das, M. Paul, M. B. Saha, S. Paul, S. Das, *RSC Adv*.**,** 2014, **4**, 18419-18430.