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DNA Binding Study of Methanolic Extract of *Pisonia aculeata* Linn. Having Anticancer Activity

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Abstract: ***Anticancer drugs interact with Deoxyribonucleic acid (DNA) in many different ways. These include intercalation, non-covalent groove binding, covalent binding/cross-linking, DNA cleaving and nucleoside-analog incorporation. As a result of complex formation occurring between DNA and drug, the thermo dynamic stability and the functional properties of DNA change. Understanding how complexation affects both the structural and mechanical properties of DNA is an important step towards elucidating the functional mechanism of binding agents. The present investigation was carried out by performing Spectrophotometric methods, Viscosity measurements and Electrochemical method. In Spectrophotometric methods, Methanolic extract of Pisonia aculeata (MPA) leaves showed analogous spectral changes and Remarkable increasing in the absorbance found around 260 nm. In Viscosity measurements, a classical intercalative mode causes a significant increase in viscosity of DNA due to an increase in separation of base pairs at intercalation sites and hence an increase in overall DNA length and showed slight increase in the flow time of DNA on increasing concentration of extract. Whereas in Electrochemical method, the peak current initially increased due to the absorption of the DNA bound complex onto the electrode surface and then decreased on the addition of DNA to the complex, which is suggestive of an interaction between the complex and DNA. A decrease in the peak-to-peak separation which is consistent with non-coordinating intercalative binding of the complexes through the planar aromatic rings between the DNA base pairs. It can be conclude that MPA showed good anticancer activity.***

Keywords: ***Methanolic extract of Pisonia aculeata (MPA), DNA, Spectrophotometry, Electrochemical, Viscosity measurements***

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1. Introduction

Deoxyribonucleic acid plays an important role in the life process because it carries heritage information and instructs the biological synthesis of proteins and enzymes through the process of replication and transcription of genetic information in living cells. Studies on the binding mechanism of some small molecules with DNA have been identified as one of the key topics during the past few decades (Chen *et al.*, 1999, Nararro, 1998). Moreover it is of great help to understand the structural properties of DNA, the mutation of genes, the origin of some diseases, the action mechanism of some antitumour and antivirus drugs and, therefore, to design new and more efficient DNA targeted drugs to deal with genetic diseases. Anticancer drugs interact with DNA in many different ways. These include intercalation, non-covalent groove binding, covalent binding/cross-linking, DNA cleaving and nucleoside-analog incorporation (Bleckburn and Gait, 1990). As a result of complex formation occurring between DNA and drug, the thermo dynamic stability and the functional properties of DNA change (Graves, 2000). Understanding how complexation affects both the structural and mechanical properties of DNA is an important step towards elucidating the functional mechanism of binding agents. Numerous biological experiments have demonstrated that DNA is the primary intracellular target of anticancer drugs due to the interaction between small molecules and DNA, which cause DNA damage in cancer cells, blocking the division of cancer cells and resulting in cell death (Li et al., 1996, Zuber, et al., 1998, Hecht, 2000). Of these studies, the interaction of plant extract with DNA has gained much attention. This is due to their possible application as new therapeutic agents and their phytochemical properties which make them potential probes of DNA structure and conformation (Erkkila et al., 1999, Metcalfe et al., 2003).

In order to develop new antitumor drugs which specifically target DNA, it is necessary to understand the different binding modes a complex is capable of undertaking. Basically, plant extract interact with the double helix DNA in either a non-covalent or a covalent way. The former way includes three binding modes: intercalation, groove binding and external static electronic effects. Among these interactions, intercalation is one of the most important DNA binding modes as it invariably leads to cellular degradation. It was reported that the intercalating ability increases with the planarity of ligands (Kumar, 1985, Xu *et al*., 2003). Additionally, the coordination geometry and ligand donor atom type also play key roles in determining the binding extent of complexes to DNA (Mahadevan, 1997).

An understanding of the modes of binding of selected extract to DNA is required to illustrate the principles governing the DNA recognition by such functional molecules, that is, the factors that decide the affinity and specificity of the complexes for DNA base sequence. Cationic complexes have been found to both intercalate into DNA and bind non-covalently in a surface-bound groove-bound Fashion (Barton *et al*., 1986). To assess the mode of DNA binding have been employing several spectroscopic, electrochemical and other techniques.

2. Materials and Methods

2.1 Chemicals

All reagents and chemicals were procured from Merck, Mumbai, India. Solvents used for electrochemical and spectroscopic studies were purified by standard procedures (Perrin DD, *et al*., 1980). DNA was purchased from Bangalore Genei (India). Agarose (molecular biology grade), ethidium bromide (EB) were obtained from Sigma, St.Louis (USA). Tris (hydroxymethyl) amino methane-HCl (Tris–HCl) buffer solution was prepared using deionized, sonicated triply distilled water.

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2.2 Experiments

All the experiments involving the interaction of MPA with Calf thymus (CT) DNA were carried out in Tris–HCl buffer (50 mM Tris–HCl, pH 7.2) containing 5% ethanol at room temperature. A stock solution of CT DNA was prepared by dissolving the CT DNA in the Tris-HCl buffer. Solutions of CT DNA in the above buffer gave a ratio of UV absorbance at 260 and 280 nm, A_{260}/A_{280} of 1.87, indicating that the CT DNA was sufficiently free from protein (Reichmann *et al*., 1954). The CT DNA concentration per nucleotide was determined by absorption spectroscopy at 260 nm using the molar absorption coefficient ε_{260} (6600 M^{-1} cm^{-1}) **(**Charies *et al*., 1982**)**

2.2.1 Absorption spectroscopic studies

Electronic absorption spectra were measured on a Shimadzu UV-1601 spectrophotometer in 5 mM Tris-HCl buffer (pH 7.1) containing 50 mM NaCl at room temperature. MPA was dissolved in absolute ethanol at a concentration of 5×10^{-3} M. Working solutions were prepared by dilution of the MPA in the absolute ethanol in 5mM Tris-HCl buffer to concentration of 50 µM.

Absorption titration experiments were performed by maintaining the extract concentration as constant at 50 µM while varying the concentration of the CT DNA within 0 to 400 µM. While measuring the absorption spectra, equal quantity of CT DNA was added to both the extract solution and the reference solution to eliminate the absorbance of CT DNA itself. From the absorption data, the intrinsic binding constant K_b was determined from the following equation (1):

$$[DNA]/(\varepsilon_a-\varepsilon_f) = [DNA]/(\varepsilon_b-\varepsilon_f) + [K_b(\varepsilon_b-\varepsilon_f)]^{-1} \text{ ---------- (3.1)}$$

where ε_a, ε_f, ε_b correspond to A_{obsd}/[extract], the extinction coefficient for the free extract, and the extinction coefficient for the extract in the fully bound form, respectively. A plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA], where [DNA] is the concentration of CT DNA in base pairs, gives K_b as the ratio of slope to intercept.

2.2.2 Viscosity measurements

Viscosity experiments were carried on an Ostwald viscometer, immersed in a thermostatic water-bath maintained at a constant temperature at 30.0 ± 0.1°C. DNA samples of approximately 0.5 mM were prepared by sonicating in order minimize complexities arising from DNA flexibility (Satyanarayana *et al*., 1983). Flow time was measured with a digital stopwatch three times for each sample and an average flow time was calculated. Data were presented as $(\eta/\eta^0)^{1/3}$ versus the concentration of the MPA, where η is the viscosity of DNA solution in the presence of complex, and η^0 is the viscosity of DNA solution in the absence of complex. Viscosity values were calculated after correcting the flow time of buffer alone (t_0), $\eta = (t - t_0)/ t_0$. (Chauhan *et al*., 2007)

2.2.3 Electrochemical methods

Cyclic voltammetric study was performed on a CHI 620C electrochemical analyzer with three electrode system of glassy carbon (GC) as the working electrode, a platinum wire as auxiliary electrode and Ag/AgCl as the reference electrode. All the voltammetric experiments were carried out in single-compartment cells of volume 5-15 ml.

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Solutions were deoxygenated by purging with N_2 prior to measurements. Increasing amounts of CT DNA were added directly in to the cell containing the MPA solution (5 X 10^{-3} M, 5 mM Tris-HCl/50 mM NaCl buffer, pH 7.1). The concentration ranged from 0 to 400 µM for CT DNA. The solution in the cuvette was thoroughly mixed before each scan. All the experiments were carried out at room temperature.

3. Results and Discussion

The interaction of small molecules with DNA plays an important role in many biological processes. These associative interactions with the DNA molecules can cause dramatic changes in the physiological functions of DNA that might be responsible for the cytotoxic behavior of the small molecules (Marmur, 1961). Their DNA binding capacity was evaluated based on interaction with calf thymus (CT) DNA.

3.1 DNA Binding Studies

With the aim of elucidating possible interactions of the MPA extract studied with DNA, the corresponding absorption spectra were recorded. Experiments with MPA extract showed analogous spectral changes. In all cases, a remarkable increasing in the absorbance around 260 nm occurred. This can be indicative of classical intercalation of the extract into DNA strands. Usually, upon DNA addition the DNA band at 260 nm shows a striking hyperchromism (increasing absorbance) or hypochromism (decreasing absorbance) due to distortions in the DNA helix, caused by firmly bound or intercalated extract.

3.2 Absorption titration

The electronic absorption spectra of the MPA extract in presence of increasing amounts of CT DNA in 5 mM Tris-HCl, 50 mM NaCl, pH 7.2 buffer is shown in Fig. 1. In the UV region, the intense absorption bands with maxima of 404.5 nm and 396.5 nm for MPA, was attributed to intra ligand π - π* transition. On increasing the concentration of CT-DNA resulted in the hypochromism and blue-shift in the UV-spectra of the MPA, These spectral characteristics suggested that MPA might bind to DNA by an intercalative mode due to strong stacking interaction between aromatic chromophore of the extract and base pairs of the DNA. After intercalating the base pairs of DNA, the π* orbit of the intercalated extracts could couple with the π orbital of base pairs, thus decreasing the π – π* transition energy and further resulting in the blue-shift or red-shift. On the other hand, the coupling of π orbit was partially filled by electrons, thus decreasing the transition probabilities and concomitantly, resulting in the hypochromism.

The intrinsic binding constants K_b were obtained by monitoring the changes in the absorbance for the extracts with increasing concentration of DNA. K_b was obtained from the ratio of slope to the intercept from the plots of [DNA]/ε_a– ε_f) *versus* [DNA] (Table 1).

MPA exhibited peculiar hypochromic and bathochromic shifts (Red shift) in the absorption spectra on binding to DNA, a typical characteristic of DNA intercalation (Takenaka *et al*., 1990, Fukuda *et al*., 1990). The other absorption peak showed at 665 nm in the absence of DNA and the incremental addition of DNA to MPA, no significant change in the wavelength and very weak absorption change is shown in Fig 1. Since, only the π* orbit of the extract could couple with the partly filled π orbital by electrons of base pairs.

As a means to further clarify the mode of binding of extract to CT DNA, viscosity measurements were carried out by varying concentration of extract. A classical intercalative mode causes a significant increase in viscosity of DNA due to an increase in separation of base pairs at intercalation sites and hence an increase in overall DNA length. By contrast, extract that bind exclusively in the DNA grooves by partial and/or non-classical intercalation, under the

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same condition, typically cause less positive or negative or no change in DNA solution viscosity (Satyanarayana *et al*., 1992a & 1993b). Fig. 2 shows a slight increase in the flow time of DNA increasing concentration of extract, which is not as pronounced as those observed for the classical intercalator ethidium bromide (Wang e*t al*., 2004). This indicated that extract prefer to engage in DNA groove binding or surface binding with its overall size resulting in an increase in DNA viscosity, rather than an intercalative DNA interaction.

Electrochemical methods have contributed substantially to our understanding of anticancer agents (Bersier *et al*.,1992) and these have been used in cancer therapy in a variety of ways. Practical application of electrochemistry includes the determination of electrode reduction mechanisms. Due to the existing resemblance between electrochemical and biological reactions we can assume that the reduction mechanisms taking place at the electrode and in the body share similar principles. On the other hand, analytical determination of drugs used in therapy is necessary in order to avoid toxic effects in treated patients. The development of new methods capable of determining minimal drug concentration, both in pharmacological compounds and in biological fluids is important.

In addition, in our investigations, it was found that DNA either the natural or denatured one (including thermal denaturation and sonic denaturation), almost has the same effect on the cyclic voltammetric behaviour of MPA.

Cyclic voltammetric experiments were performed by maintaining the concentration of MPA while varying the concentration of CT DNA within 0-400 μM and the voltammetric responses were recorded.

The cyclic Voltammogram of MPA in the presence of DNA and presence of CT DNA is shown in Fig. 3 and the electrochemical data are summarized in the Table 2.

In the absence of CT DNA, the redox couple cathodic and anodic peaks appear at -0.728 V and -0.613 V respectively for MPA (Epa = -0.613 V, Epc = -0.728 V, ΔEp = 0.115 V and $E_{1/2}$ = -0.671 V). The ratio of i_{pc}/i_{pa} is approximately unity. This indicated that the reaction of the MPA on the glassy carbon electrode surface is quasi-reversible redox process. The incremental addition of CT DNA to the MPA causes a negative shift in $E_{1/2}$ of 42 mV and a decrease in ΔEp of 20 mV. The ip_c/ip_a values also decrease in the presence of DNA. The decrease of the anodic and cathodic peak currents of the MPA in the presence of DNA is due to decrease in the apparent diffusion coefficient of the MPA binds with the DNA macromolecules.

As observed in the Cyclic Voltammogram experiments, an increase or decrease of the peak current was observed for extract. The peak current increased initially and then decreased. The initial increase in the peak current was due to the absorption of the DNA bound complex onto the electrode surface (Yang *et al*., 2004). The decrease in peak current on the addition of DNA to the complex was suggestive of an interaction between the complex and DNA (Annaraj *et al*., 2005). A decrease in the peak-to-peak separation was observed, which was consistent with non-coordinating intercalative binding of the complexes through the planar aromatic rings between the DNA base pairs (Lu X *et al*., 2004). The formal potential shifts slightly towards the positive side and is attributed to characteristic behavior of intercalation of the complexes into the DNA double-helix (Srinivasan *et al*., 2005), and suggests that MPA extract bind to DNA at different rates (Vaidyanathan and Nair, 2005, Dryhurst and Niki, 1988).

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Figure 1: Electronic Absorption Spectra of MPA in the Absence (dash line) and Presence (dark line) of Increasing Amounts of DNA

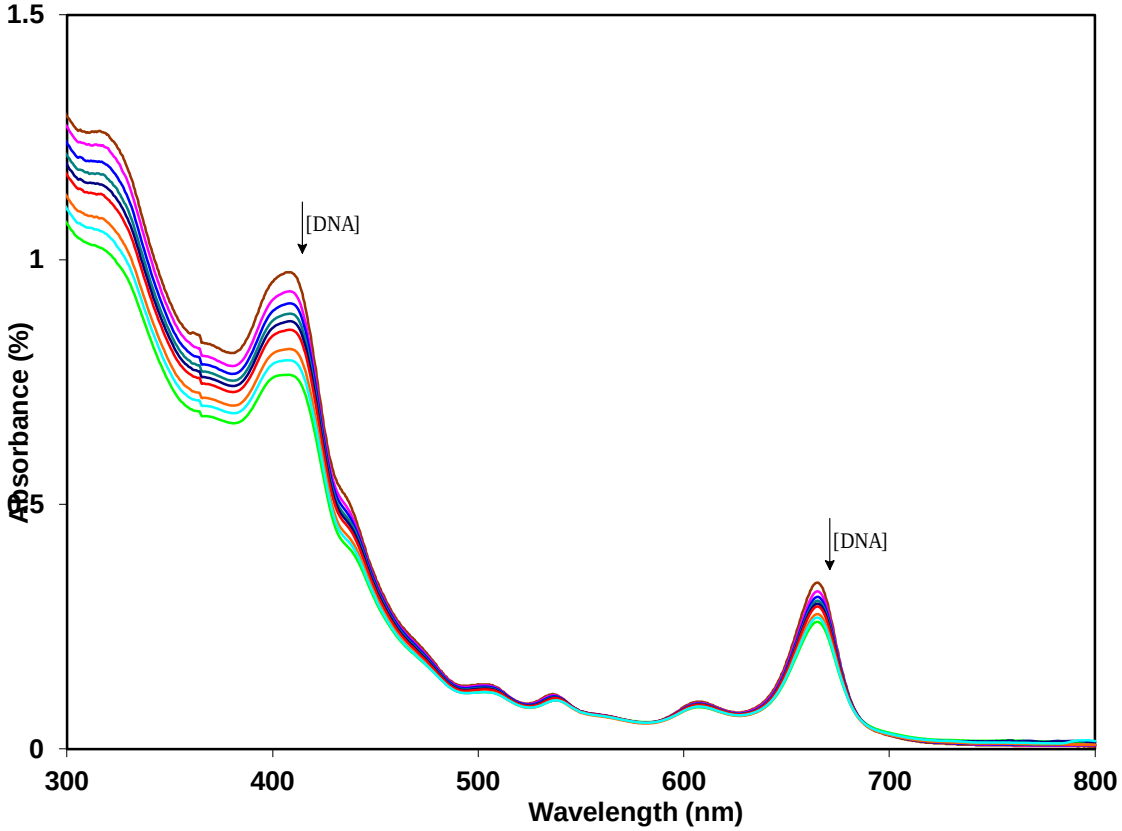

Table 1: Absorption Spectral Properties of MPA

Complexes	λmax		Δλ (nm)	H % = [(ε_f– ε_b)/ ε_f X 100].	K_b (M^{-1})
	Free	Bound			
MPA	404.5	396.5	8.0	23.6	2.56 x 10^6

The intrinsic binding constants K_b are obtained by monitoring the changes in the absorbance for the extracts with increasing concentration of DNA. K_b is obtained from the ratio of slope to the intercept from the plots of [DNA]/ε_a– ε_f) versus [DNA]

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Figure 2: The Effect of MPA on the Relative Viscosity of Calf Thymus DNA in 5 mM Tris–HCl/50 mM NaCl buffer (pH 7.1).

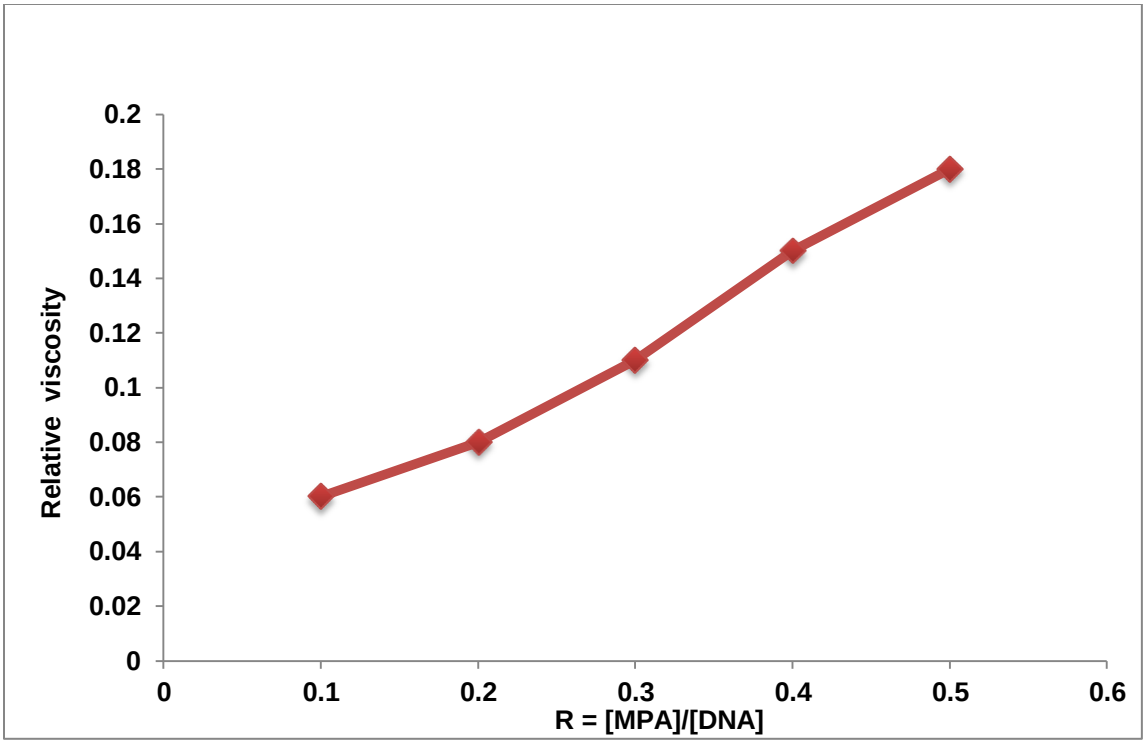

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Figure 3: Cyclic Voltammogram of MPA Both in the Absence (blue line) and Presence (other color line) of Different Concentration of DNA in 50 mM NaCl, 5 mM Tris-HCl, pH 7.2. Scan Rate 100 mV s^{-1}

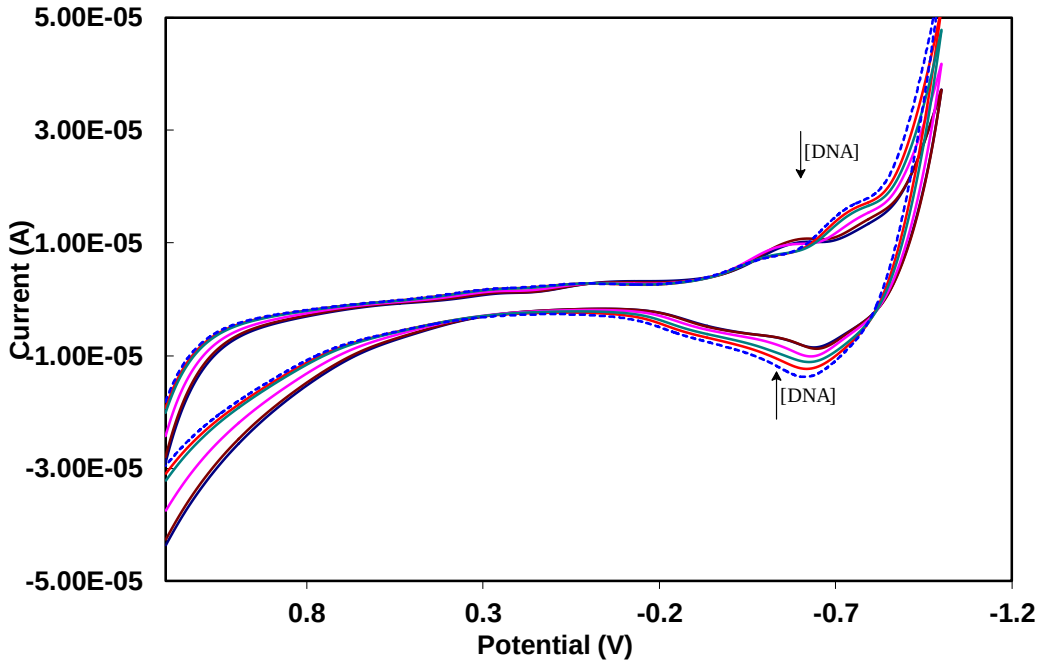

Table 2: Electrochemical Parameters of the Compound MPA

R = [DNA]/[IA]	Ipc x 10^{-5}	Ipa x 10^{-5} (A)	Epc (V)	Epa (V)	ΔEp (mV)	E1/2 (V)
0	1.65	-1.38	-0.728	-0.613	0.115	-0.671
1	1.62	-1.24	-0.735	-0.618	0.117	-0.677
2	1.53	-1.11	-0.746	-0.625	0.121	-0.686
3	1.42	-1.01	-0.759	-0.632	0.127	-0.696
4	1.38	-0.878	-0.78	-0.645	0.135	-0.713

R^a is the ratio of the concentration of the extract to that of DNA, $(\Delta Ep)^b = |Epa - Epc|$ and $(E_{1/2})^c = (Epa + Epc)/2$

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4. Conclusion

In this study, we reported that the variations of the cyclic voltammetric behavior of MPA extract in the medium on addition of DNA can be used to probe the interaction between these species and to electrochemically determine DNA. The results reported demonstrate that, rather straight forward electrochemical methods can be used to characterize the intercalative interaction between an anticancer drug or other electro active species and DNA and to calculate the binding constant.

In electrochemistry considerable progress has recently been made in the development of new and sophisticated techniques to study anticancer drugs–DNA interaction. The field of anticancer drug design will naturally take advantage of this progress. Electrochemical approach has been successively used to determine the anticancer drug–DNA interaction and can contribute to drug discovery and effective treatment for cancer through providing knowledge regarding the efficacy of candidate drug binding with DNA and through providing information of the mechanism of the DNA–drug interaction. The value of electrochemical approaches in studying the anticancer drugs–DNA interaction is that it is a relatively clean chemical system, it is relatively easy to control and can be studied in aprotic and aqueous solutions thus allowing one to evaluate the behavior of free radicals generated in biological systems. The versatility of the electrochemical methodology allows the mimicking of the multitude of biological environments in which the conditions can be widely varied in the attempt to resemble them. Different ranges of pH, oxygen content in the electrochemical cell and solvents of diverse properties can be used.

At the same time, MPA has been thought to be crucial in enhancing covalent bond formation with DNA. This paradoxical situation kindled us to extract and to study their DNA-binding ability, followed by their anticancer activity, to arrive at a conclusion regarding their mechanism of action. To achieve this, we chosen MPA, since they have demonstrated potent activity against various tumor cell lines studies. The MPA have been shown to possess DNA-binding abilities.

The interaction of small molecules with DNA plays an important role in many biological processes. These associative interactions with the DNA molecules can cause `dramatic changes in the physiological functions of DNA that might be responsible for the cytotoxic behavior of the small molecules (Marmur J. 1961). Their DNA binding capacity was evaluated based on interaction with calf thymus (CT) DNA.

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A Brief Author Biography

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Working as Associate Professor in Pharmacognosy, at Rasiklal M. Dhariwal Institute of pharmaceutical Education and Research, Chinchwad, Pune-19. Total working experience is 12 yrs. in academics and research. Interested in research of Isolation, characterization of active constituents present in plants and Evaluation of crude drugs, plant extracts and marketed preparation herbals by Chromatographic techniques like TLC, HPTLC, HPLC, GC and spectrometric Techniques like U.V., I.R., N.M.R. Published 5 research papers in the journals of national and international repute.

