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Effect of *Phyllanthus polyphyllus* extract on DNA binding studies

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Abstract

Phyllanthus polyphyllus has demonstrated potent in vitro anticancer activity against various tumor cell lines. For a better understanding of the mechanism of action, binding studies of methanolic extract of *Phyllanthus polyphyllus* (PP) with Calf Thymus (CT) DNA were studied using absorption spectroscopy, viscometry and cyclic voltammetry. PP displayed binding properties to the CT-DNA and was found to interact with CT-DNA through intercalation, as demonstrated by a hypochromic effect and blue-shift in the UV spectra. An increase in the viscosity of CT-DNA was observed. The changes in the current and potential in cyclic voltammetric experiments demonstrate intercalative interaction between teh PP and the CT-DNA.

Keywords: *Phyllanthus polyphyllus* ; Absorption Spectroscopy;Viscometry; Cyclic Voltammetry; Calf Thymus DNA; Intercalation.

Introduction

Deoxyribonucleic acid plays an important role in biological systems 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 [1]. Numerous biological experiments have demonstrated that DNA is the primary intracellular target of anticancer drugs; interaction between small molecules and DNA can cause DNA damage in cancer cells, blocking the division of cancer cells and resulting in cell death [2, 3]. 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 [4].

The investigation of drug-DNA interaction is vital for understanding the molecular mechanisms of the drug action and designing specific DNA-targeted drug[5]. Basically, plant extract interacts with the double helix DNA in either a non-covalent or a covalent way. The non-covalent way of binding includes three binding modes: (i) intercalation between the base pairs; (ii) groove binding, interactions with the DNA groove; and (iii) electrostatic interaction, electrostatic attractions with the anionic sugar-phosphate backbone of DNA. Intercalative binding and groove binding are related to the grooves in the DNA double helix, while the electrostatic binding takes place out of the groove [6]. Among these interactions, intercalation is one of the most important DNA binding modes as it invariably leads to cellular degradation.

An understanding of the modes of binding of plant 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 [7].

Various techniques have been extensively employed to study the interaction of small molecules with DNA, such as spectroscopic methods [8], nuclear magnetic resonance [9], x-ray diffraction, viscosity measurement [10] and electrochemical measurements [11] etc.

These investigations form a theoretic guide for the design of new anticancer drugs and chemical treatments of tumor and virus. They are also very valuable for probing the mechanism of the interaction between anticancer drugs and DNA and establishing convenient methods to effectively choose specific anticancer drug. Our earlier studies have demonstrated that *Phyllanthus polyphyllus* has potent *in vitro* anticancer activity against various tumor cell lines. The aim of this study was to investigate the binding mechanism of methanolic extract of *Phyllanthus polyphyllus* (PP) with Calf Thymus DNA using absorption spectroscopy, viscometry and cyclic voltammetry.

Material and Methods

Chemicals

All reagents and chemicals were procured from Merck, Mumbai, India. Solvents used for electrochemical and spectroscopic studies were purified by standard procedures [12]. 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 triple distilled water.

Experiments

All the experiments involving the interaction of PP with CTDNA were carried out in Tris–HCl buffer (50 mM Tris–HCl, pH 7.2) containing 5% alcohol 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. The CT DNA concentration per nucleotide was determined by absorption spectroscopy at 260 nm using the molar absorption coefficient $_{260}$ (6600 M^{-1} cm^{-1}).

DNA binding experiments

Absorption spectroscopic studies

UV 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. PP was dissolved in methanol at a concentration of 5 10^{-3} M. Working solutions were prepared by dilution of the PP in methanol 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):

$[DNA]/(_a - _f)=[DNA]/(_b - _f)+[K_b(_b - _f)]-1$ --(1)

where $_a$, $_f$, $_b$ 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 $[DNA]/(_a - _f)$ versus [DNA], where [DNA] is the concentration of CT DNA in base pairs, gives K_b as the ratio of slope to intercept.

Viscosity measurements

Viscosity experiments were carried on an Ostwald viscometer, immersed in a thermostated water-bath maintained at a constant temperature of 30.0 ± 0.1 C. DNA samples of approximately 0.5 mM were prepared by sonication in order to minimize complexities arising from DNA flexibility [13]. 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 PP, 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), $\eta = (t - t_0)/ t_0$ [14].

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. 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 PP solution (5 X 10^{-3} M, 5 mM Tris-HCl/50 mMNaCl 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.

In a typical cyclic voltammetric titration, a fixed concentration of the PP extract was taken and DNA solution in buffer was added in different ratios as done in the absorption titration, and the voltammetric response was recorded.

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 [15]. The DNA binding capacity was evaluated based on interaction with CTDNA.

Electronic Absorption spectral studies

Absorption spectroscopy is one of the most important and useful methods to investigate the binding of any drug with DNA [16, 17]. When a small molecule interacts with DNA, it may form a complex that exhibits absorption spectrum which is different from that of the small molecule. The addition of DNA to a solution of an intercalator results in a characteristic shift of the absorption maximum to longer wavelengths (bathochromic shift or red shift) and a decrease (hypochromocity) or increase (hyperchromicity) of the absorbance [18]. The intercalation mode of micromolecule could locally elongate the DNA helix by separating the stacked base pairs and partially unwinds the DNA helix at the intercalation site [19]. Consequently, it induces the structural perturbation of the DNA duplex, which might hinder the replication of DNA and inhibit protein synthesis. These changes could cause small increase in absorption peak of double-strand DNA (dsDNA) at 260 nm, and this phenomenon proved that the opening of the dsDNA helix occurred [20].

The electronic absorption spectra of the PP extract in presence of increasing amounts of CT DNA in 5 mM Tris-HCl, 50 mMNaCl, pH 7.2 buffer is shown in Fig 1. In the UV region, the intense absorption bands with maxima of 390 nm for PP, were attributed to intra ligand - * transition. On increasing the concentration of CT-

DNA resulted in the hypochromism and blue-shift in the UV-spectra of the PP. The hypochromism is associated with the intercalative binding of small molecules to the base pairs of DNA, because of strong stacking interactions between the aromatic chromophore of the small molecules and the base pairs of DNA [21]. After intercalating the base pairs of DNA, the * orbital of the intercalated extracts could couple with the orbital of base pairs, thus decreasing the – * transition energy and further resulting in the blue-shift or red-shift. On the other hand, the coupling of orbital was partially filled by electrons, thus decreasing the transition probabilities and concomitantly resulting in the hypochromism.

The other absorption peak was showed at 661.5 nm in the absence of DNA, and upon the incremental addition of DNA to extract, no significant change in the wavelength and very weak absorption change was seen shown in Fig 1. Since, only the * orbit of the extract could couple with the partly filled orbital by electrons of base pairs.

From the ultraviolet and visible absorption spectra of the extract in the absence, and presence, of DNA (Fig. 1), it was observed that the PP Extract solution exhibited peculiar hypochromic and bathochromic shifts in the absorption spectra on binding to DNA, a typical characteristic of DNA intercalation [22].

The intrinsic binding constant K_b is obtained by monitoring the changes in the absorbance for the extracts with increasing concentration of DNA in order to compare the binding strength of the complexes with CT DNA. K_b is obtained from the ratio of slope to the intercept from the plots of [DNA]/ $_a$ – $_f$) versus [DNA]. The K_b value is shown in Table 1. The high K_b value of 7.6 10^5 suggests a stronger binding towards DNA.

Viscosity measurements

As a means to further clarify the mode of binding of the PP extract to CT DNA, viscosity measurements were carried out by varying concentration of extract. Viscosity experiment is regarded as the most critical test for the binding mode of small molecules and DNA. Generally, a classical intercalation binding causes a significant increase in the viscosity of DNA solution due to an increase in lengthening in the DNA helix to accommodate the bound ligand between the adjacent base pairs [23]. In contrast, a partial and/or nonclassical intercalation binding causes reduction in viscosity as the ligand bends (or kinks) the DNA helix and reduces its effective length, while ligands that bind exclusively in the DNA grooves typically cause less pronounced changes (positive or negative) or no changes in DNA solution viscosity under the same conditions [24,25]. Fig.2 Shows a slight increase in the flow time of the DNA with increasing concentration of extract, which is not as pronounced as those observed for the classical intercalatorethidium bromide [26]. This indicates 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

Electrochemical methods have contributed substantially to our understanding of anticancer agents.These enable to evaluate and predict DNA interactions by DNA-binding compounds. Amongst the various electroanalytical techniques in general, cyclic voltammetry (CV) is by far the most versatile electrochemical method. The obtained results of CV from the redox properties of drugs and biomolecules might have fervent effects on interpretation of their in vivo redox behaviour or pharmaceutical activity [27].

Cyclic voltammetric experiments were performed by maintaining the concentration of PP while varying the concentration of CT DNA within 0-400 M and the voltammetric responses were recorded. The cyclic voltammogram of PP in the presence of DNA and absence of CT DNA are shown in Fig 3 and the electrochemical data are summarised in the Table 2. In the absence of CT DNA, the methanolic extract show only the oxidation peak -0.719 V (Epa) and no reduction peak in the absence of DNA. Incremental addition of DNA to PP, shows a decrease in the current intensity and negative shift of the oxidation peak potential. The resulting changes in the current and potential demonstrate interaction between the extract and DNA. This indicates that the reaction of the PP on the glassy carbon electrode surface is quasi-reversible redox process. The incremental addition of CT DNA to the PP causes only a negative potential shift and decreasing current intensity in anodic peak and no significance change of potential shift and current intensity in cathodic peak. The ip_c/ip_a values also decrease in the presence of DNA.

The peak current increased initially and then decreased. The initial increase in the peak current is due to the absorption of the DNA bound complex onto the electrode surface [28]. The decrease in peak current on the addition of DNA to the complex is suggestive of an interaction between the complex and DNA [29]. A decrease in the peak-to-peak separation was observed, which is consistent with non-coordinating intercalative binding of the complexes through the planar aromatic rings between the DNA base pairs [30]. The formal potential Ef shifts slightly towards the positive side and is attributed to characteristic behavior of intercalation of the complexes into the DNA double-helix [31, 32], and suggests that PP extract bind to DNA at different rates.

We report that the variations of the cyclic voltammetric behavior of PP Extract in methanol 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.

The binding of the PP Extract to DNA likely induces a stiffening effect explaining the higher reduced dichroism value measured in the absorption band of the drug than in the DNA absorption band. This behaviour is typical of intercalating agents.

Table 1:Absorption spectral properties of PP Extract

Extract	λmax		Δλ (nm)	H%=[(□r-□b)/ □fx 100]	K_b (M^{-1})
	Free	Bound			
PP	390.0	373.0	17	32.7	7.6 X 10^5

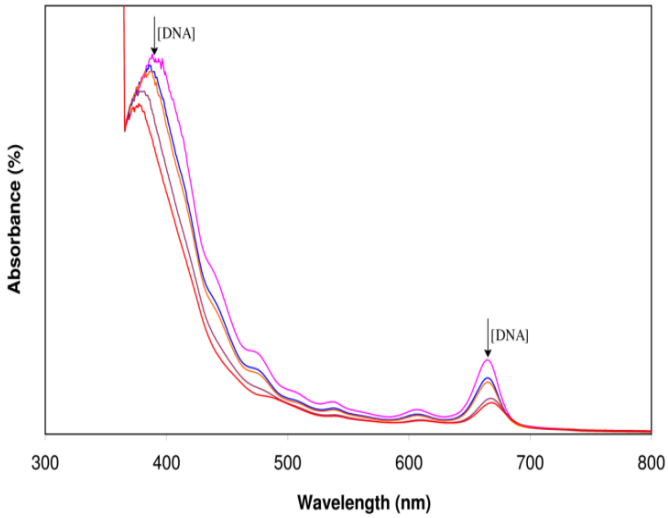

Figure 1: Electronic absorption spectra of PP in the absence (dash line) and presence (dark line) of increasing amounts of DNA caption.

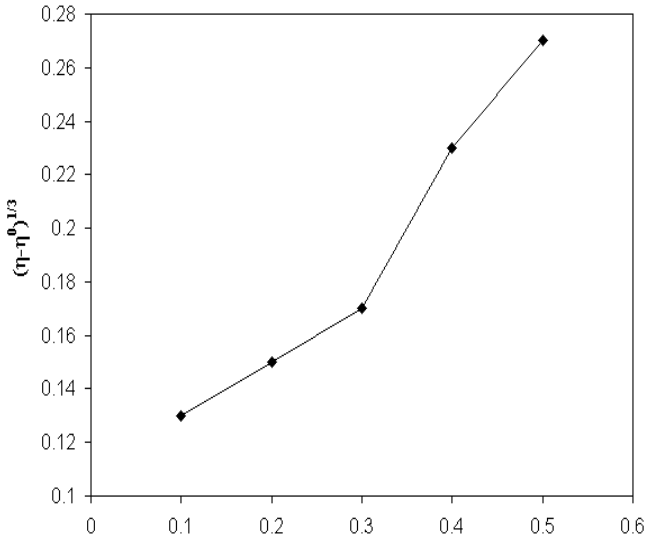

Figure 2:The effect of PP on the relative viscosity of calf thymus DNA in 5 mMTris–HCl/50 mMNaCl buffer (pH7.1).

R= [DNA]/ [PP]	Ipa x 10^{-5} (A)	Epa (V)
0	-3.19	-0.719
0.5	-2.92	-0.723
1	-2.1	-0.732
1.5	-1.83	-0.745
2	-1.52	-0.753
2.5	-1.19	-0.762
3	-0.93	-0.783

Table 2:Electrochemical parameters of the compoundPP

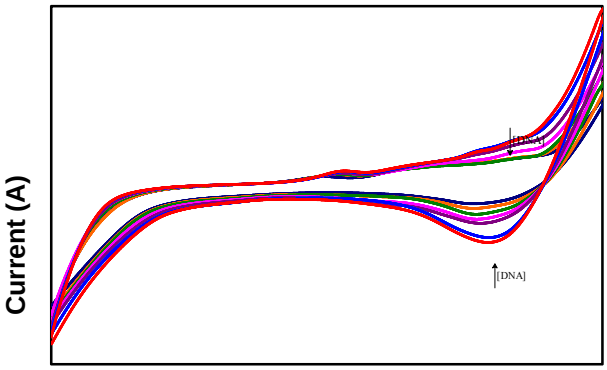

Figure 3:Cyclic voltammogram of PP both in the absence (pink line) and presence (other colour line) of different concentration of DNA in 50 mMNaCl, 5 mM Tris-HCl, pH 7.2. Scane rate 100 mV s^{-1}

Conclusion

In this work, the PP extract have been shown to possess DNA-binding abilities. The interaction of PP with calf thymus DNA was studied by absorption spectroscopy, viscometry and cyclic voltammetry. Upon binding to DNA, the absorption spectra of PP showed peculiar hypochromic effect and bathochromic shift, a slight increase in viscosity was observed in viscosity measurements, and a decrease in peak current was observed in cyclic voltammetry. The variations in the spectral characteristics, viscosity and electrochemical behavior upon binding of PP with CT-DNA indicated an intercalative mode of interaction. This study is expected to provide greater insight into the design of new anticancer drugs.

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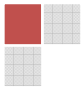

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