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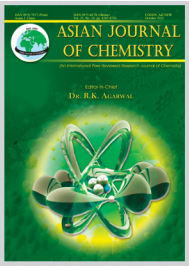

DNA Binding Studies on the Interaction of Myricetin-Bi(III) Complex with Double Stranded DNA by UV-VIS Spectrophotometry and Voltammetry

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The interaction of bismuth(III) complex of 3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone known as myricetin with calf thymus double stranded ds-DNA has been investigated using UV-visible spectrophotometry and electrochemical techniques. The observation of an isosbestic point at 312 nm follow by a hypochromicity at 326 nm showed the formation a new complex between bismuth ion and myricetin. The calibration curves for ds-DNA in the absence and presence of Bi(III)-myricetin complex differ significantly in the slopes due to the interaction between two species. The electrochemical behaviour of the new complex bound to ds-DNA has been compared with myricetin alone as a flavonoid. As a result of the interaction with ds-DNA, the reduction peak current related to Bi(III)-myricetin decreased by increasing concentration of ds-DNA. The diffusion coefficients of the complex in the absence (D_f) and presence (D_b) of ds-DNA were calculated as 5.37×10^{-7} cm^2 s^{-1} and 4.55×10^{-7} cm^2 s^{-1} respectively. The binding constant ($K = 9.0 \times 10^4$ L mol^{-1}) and binding site size ($s = 0.80$) of the complex-myricetin interacting with ds-DNA were obtained simultaneously by non-linear fit analysis. The DNA binding constant determined reveal that the Bi(III)-myricetin complex interact with ds-DNA electrostatically due to the coulombic interactions.

Key Words: Myricetin, Bi(III), ds-DNA, Isosbestic point, Flavonoids, UV-Vis spectroscopy, Cyclic voltammetry.

INTRODUCTION

DNA plays major role in the life process because it carries important informations and instructs the biological synthesis of proteins and enzyme through the process of replication and transcription of genetic information. DNA is quite often the main cellular target for studies with smaller molecules of biological importance like carcinogens, steroids and several classes of drugs. The investigation of drug-DNA interactions is of current general interest and importance[1-3], especially for the designing of new DNA-targeted drugs and the screening of these *in vitro*. Generally, the interactions of small molecules with DNA involve three binding modes: intercalation, groove binding and long-range assembly on the molecular surfaces of nucleic acids[4]. The intercalative binding is stronger than other two binding modes because the surface of intercalative molecule is sandwiched between the aromatic, heterocyclic base pairs of DNA[5,6].

The flavonoids are a large group of polyphenolic natural products that are widely distributed in higher plants. Such compounds are increasingly being recognized as possessing a broad spectrum of biological activities and important therapeutic applications[7]. It is generally considered that the flavonoids form coordination complexes with some essential trace metals and it is believed that this is an active form of the compounds, which is medicinally beneficial[8]. The interaction of rare earth metal complexes with DNA has attracted much attention in recent years, because rare earth metals have more physiological activities and lower toxicities after coordinating with a ligand[9]. Kang *et al.*[10] reported that quercetin and quercetin-Eu^{3+} can both bind to DNA, but quercetin binds to DNA mainly by electrostatic attraction and the complex bind to DNA by both intercalation and electrostatic attraction, moreover, the anti-tumor activities of lanthanides complexes of quercetin with Eu^{3+}, La^{3+} and Gd^{3+} are superior to quercetin[11]. In previous study, we found that the morin-Bi^{3+} complex can bind to base pairs of DNA by intercalation[12], while the binding mode of morin with DNA is a non-intercalating mode. The phenomena of morin and its rare earth metal complexes exhibiting different antitumor activities lead us to consider the interaction of the other flavonoids with DNA.

Myricetin (3,3´,4´,5,5´,7-hexahydroxyflavone) (Fig. 1), a naturally occurring flavonoid, is classified as a flovonoid with strong antioxidant effects. Oxidative stress plays a key role in various neurological diseases such as ischemia and Alzheimer's disease. Many techniques have been employed to study the interaction of metal complexes with DNA,

including fluorescence spectroscopy[13], UV-spectrophotometry[14], voltammetry[15], circular dichroism[16], mass spectrometry[17], *etc*., UV-VIS absorption and voltammetry are regarded as effective methods among these techniques because they are sensitive, rapid and simple.

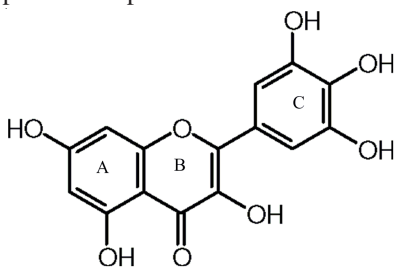

Fig. 1. Chemical structure of myricetin

In this work, the binding mechanism between myricetin-Bi^{3+} complex and calf thymus DNA has been investigated by the application of UV-VIS absorption, cyclic voltammetry and DNA melting techniques. It is presumed that this will be helpful to further understanding the mechanism of interactions between DNA and myricetin's bismuth complexes as well as further understanding of its pharmacological effects. The knowledge gained from this study should be valuable for the new therapeutic reagents for tumors and other diseases.

EXPERIMENTAL

UV-VIS absorption spectra were measured on a Shimadzu model 1650 UV-VIS spectrophotometer with the use of a 1 cm cell. Electrochemical studies were carried out using a Metrohm instrument, Model 797 VA, computrace with stand three-electrodes containing a hanging mercury drop electrode (HMDE) as a working electrode, a carbon rod as an auxiliary electrode and an Ag/AgCl (3.0 mol L^{-1} KCl) reference electrode. All experiments, unless specified otherwise, were carried out at room temperature (25 ± 1 ºC).

Deoxyribonucleic acid sodium salt from calf thymus (Sigma Chem. Co., USA) was used without further purification and its stock solution was prepared by dissolving an appropriate amount of DNA in doubly distilled water and stored at 4 ºC. The concentration of DNA in stock solution was determined by UV absorption at 260 nm using a molar absorption coefficient ε_{260} = 6600 L mol^{-1} cm^{-1}. Purity of the DNA was checked by monitoring the ratio of the absorbance at 260 nm to that at 280 nm. The solution gave a ratio of > 1.8 at A_{260}/A_{280}, which indicates that DNA was sufficiently free from protein[16]. Myricetin stock solution (1.0×10^{-3} mol L^{-1}) was prepared by dissolving its powder (Sigma Chem. Co., USA) in deionized water and stored in a cool and dark place. Phosphate buffer (20 mmol L^{-1}, pH 7.5) and was purchased from Fluka (USA). Neutral red dye stock solution (1.0×10^{-3} mol L^{-1}) was prepared by dissolving its crystals (Sigma-Aldrich) in water and diluted to the required volume.

RESULTS AND DISCUSSION

Interaction between Bi(III) and myricetin: The UV-VIS absorption spectra of myricetin in the presence of Bi(III) (with increasing concentration) were examined in the phosphate buffer solution (pH 7.5) containing 20 mmol L^{-1} KCl (Fig. 2). Myricetin has two maxima bands; at 252 nm (benzoyl of ring A) and the other at 327 nm (cinnamoyl of ring B)[18,19]. By adding gradually Bi(III) ions into the myricetin solution at pH 7.5 the absorption intensity of band at 327 nm decreased gradually and a new absorption peak appeared at 292 nm. Moreover, the isosbestic point at 312 nm indicated that myricetin could form a complex with Bi(III) and the binding is homogeneous. The stoichiometry of the complex was determined as 1:1 (Bi(III): myricetin) using mole ratio method (Fig. 3).

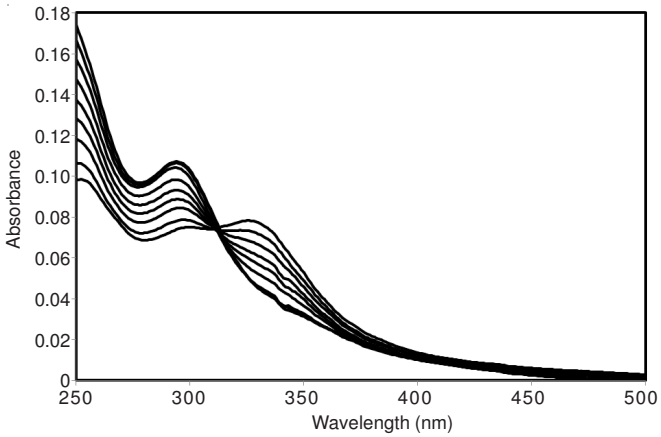

Fig. 2. Absorption spectra of myricetin in presence of Bi(III) at different concentrations. Conditions: [Bi^{3+}] = 0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 µmol L^{-1} for curves 1-9 and $C_{myricetin}$ = 10.0 µmol L^{-1}, phosphate buffer (0.02 mol L^{-1}, pH 7.5)

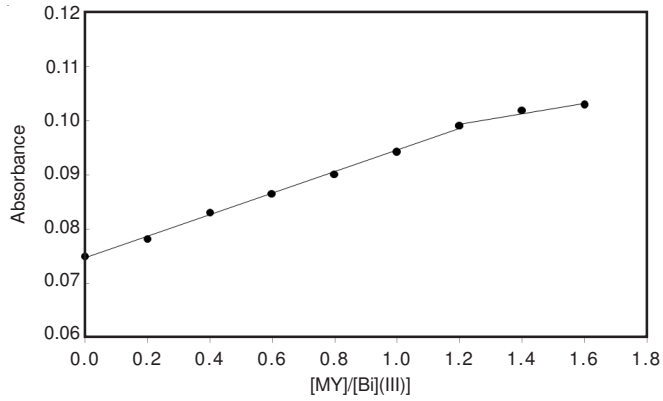

Fig. 3. Mole ratio plot of myricetin in presence of Bi(III) at different concentrations. Conditions: [Bi^{3+}] = 0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0 and 16.0 µmol L^{-1}, $C_{myricetin}$ = 10.0 µmol L^{-1}, phosphate buffer (0.02 mol L^{-1}, pH 7.5)

Electronic absorption spectra of Bi(III)-myricetin complex in the presence of DNA: UV-VIS absorption spectra of 100 µmol L^{-1} myricetin in the present of Bi(III) ion in the same concentration has shown in Fig. 4. There is one absorption band with maximum wavelength at 292 nm in the absenence of ds-DNA. The absorption band increased continuously due to the highly overlapping spectra by increasing concentration of ds-DNA. In a similar way, the spectrum of ds-DNA in the absence of Bi(III)-myricetin studied at different ds-DNA concentrations. The significant difference at the slopes of two calibration curves in the absence and presence of Bi(III)-myricetin (Fig. 5) demonstrates that a small molecule such as bismuth complex of myricetin could affect on the structure of ds-DNA.

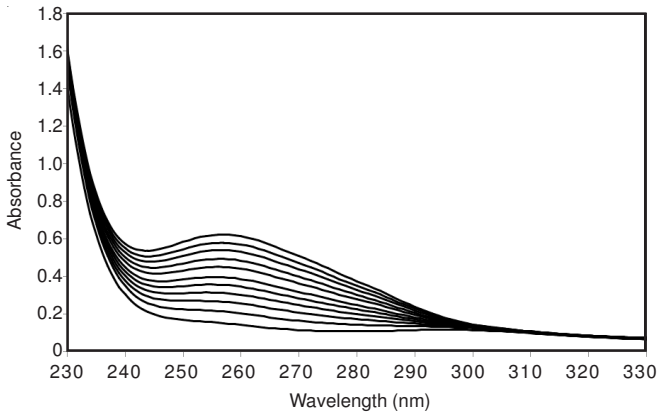

Fig. 4. Absorption spectra of Bi(III)-myricetin in different concentrations of ds-DNA. C_{DNA} = 0.0, 6.1, 12.2, 18.3, 24.4, 30.5, 36.6, 42.7, 48.8, 54.9, 60.3 and 61.0 μmol L^{-1} for curves 1-12, $C_{Bi(III)}$ = 10.0 μmol L^{-1}, $C_{myricetin}$ = 10.0 μmol L^{-1} in phosphate buffer (0.02 mol L^{-1}, pH 7.5)

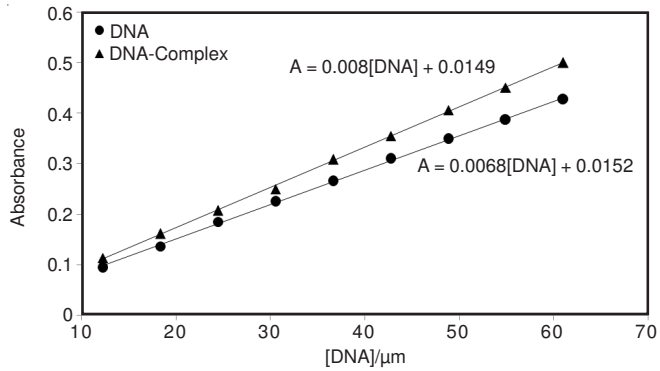

Fig. 5. Calibration curves for ds-DNA in the absence and presence of Bi(III)-myricetin at 260 nm. Conditions: phosphate buffer (0.002 mol L^{-1}, pH 7.5), $C_{myricetin}$ = 10 μmol L^{-1}, $C_{Bi(III)}$ = 10 μmol L^{-1}

Melting studies: Heat and alkali can destroy the double helix structure of DNA and change it into a single helix at the melting temperature (T_m). Interaction of small molecules with DNA can affect the T_m value. Intercalation binding can stabilize the double helix structure and T_m value increases above 5-8 ºC, but the non-intercalation binding causes no obvious increase in T_m[20,21]. The values of T_m for ds-DNA in the absence and presence of Bi(III)-myricetin were determined, respectively, by monitoring the maximum absorbance values of the systems as a function of temperature ranging from 43 to 79 ºC. The melting curves have shown in Fig. 6. It can be seen that the T_m value of ds DNA in the absence of Bi(III)-myricetin is approximately 58 ºC. The observed melting temperature of DNA in the presence of complex is 67 ºC. The interaction of the metal complex with ds-DNA can cause the T_m value to be increased, suggesting that intercalation binding is one of the major mechanisms involved in the reaction of Bi(III)-myricetin complex and ds-DNA.

Fig. 7 displays cyclic voltammo-grams of the bismuth-myricetin system between 0.15 and -0.5 V (*versus* Ag/AgCl), in the absence of myricetin and bismuth ion in phosphate buffer pH 7.5. The voltammogram of myricetin in presence of Bi(III) shows the amplification in the reduction peak current of bismuth ion at more negative potentials. Comparison of the voltammograms shows that the height of bismuth reduction peak depends on the concentration stage and also on the presence or absence of myricetin, which reveals adsorption nature of the process. The height of the Bi(III)-myricetin complex reduces in the presence of ds-DNA. The binding of the metal complex to ds-DNA should lead to a significant decrease of peak current due to the formation of Bi(III)-myricetin-DNA adduct with smaller diffusion coefficient[22-24].

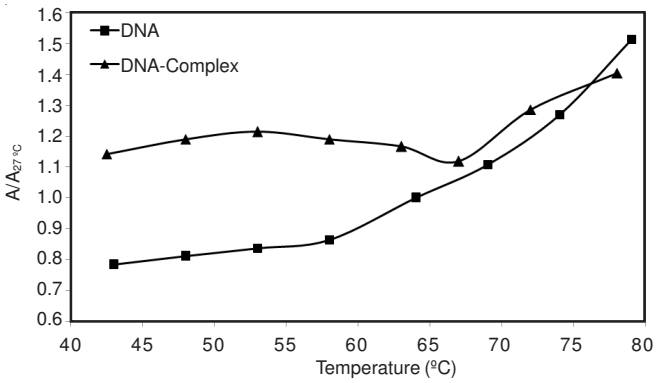

Fig. 6. Melting curves for ds-DNA in the absence and presence of Bi(III)-myricetin at 260 nm. Conditions: $C_{myricetin}$ = 10.0 μmol L^{-1}, $C_{Bi(III)}$ = 10.0 μmol L^{-1}, C_{DNA} = 9.2 μmol L^{-1} in phosphate buffer (0.02 mol L^{-1}, pH 7.5).

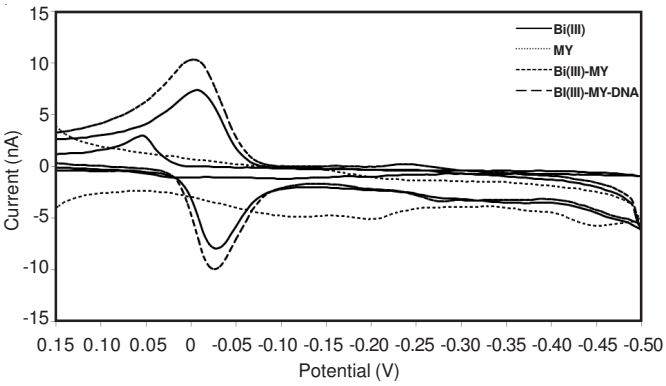

Fig. 7. Cyclic voltammetry of Bi(III), myricetin, Bi(III)-myricetin complex and Bi(III)-myricetin in the presence of ds-DNA on the surface of HMDE. Conditions: $C_{Bi(III)}$ = 20.0 μmol L^{-1}, $C_{myricetin}$ = 10.0 μmol L^{-1}, C_{DNA} = 9.2 μmol L^{-1}, ν = 50.0 mV s^{-1}, pulse amplitude 2.0 mV, phosphate buffer (0.002 mol L^{-1}, pH 7.5)

Tafel plot analysis: In order to determine the apparent transfer coefficients (α) and the number of electron transferred (n) of Bi(III)-myricetin complex in absence and presence of ds-DNA, the cathodic Tafel plots of Bi(III)-myricetin and Bi(III)-myricetin-DNA plotted on hanging mercury drop electrode using the cyclic voltammetry. By linear regression fit of the cathodic Tafel plots, cathodic Tafel slopes were determined which were 21.34 V Dec^{-1} and 15.20 V Dec^{-1} in the absence and presence of ds-DNA respectively. Thus, the values of αn_a calculated as 1.26 and 0.90 for reduction of Bi(III)-myricetin and Bi(III)-myricetin-DNA adduct, respectively.

According to the slopes of the I_p - $\nu^{1/2}$ plots (ν is the scan rate) in the absence and presence of ds-DNA with the equations of $I_p = 2.36 \times 10^{-8}\ \nu^{1/2} - 2.07 \times 10^{-9}$ and $I_p = 2.17 \times 10^{-8}\ \nu^{1/2} - 2.03 \times 10^{-9}$, the diffusion coefficients for the complex in the absence $(D_o)_f$ and presence $(D_o)_b$ of ds-DNA calculated as 5.37×10^{-7} and 4.55×10^{-7} cm^2s^{-1} respectively.

Determination of binding constant and the binding site size: When the non-specific binding of an electroactive molecule (EM), to a binding site (S), composed of s base pairs, on a DNA duplex, produces a bound species, EM-S:

$$EM + S = EM - S \quad (1)$$

The microscopic equilibrium constant for binding is:

$$K = \frac{C_b}{C_f C_s} \quad (2)$$

where, C_b, C_f and C_s represent the equilibrium concentration of EM in EM-S, free EM and free S, respectively. The total concentration of the electroactive molecule, C_t, is:

$$C_t = C_b + C_f \quad (3)$$

and the average of number of binding sites (x) along a DNA duplex molecule with an average total number of base pairs L is:

$$x = \frac{L}{s} \quad (4)$$

Thus, the total concentration of binding sites can be represented as xC_{DNA}:

$$xC_{DNA} = C_b + C_S \quad (5)$$

$$\text{where, } C_{DNA} = \frac{C_{NP}}{2L} \quad (6)$$

here, C_{NP} is the nucleotide phosphate concentration. Based on Carter *et al.*, the binding constant, K, can be expressed as the following form[25,26]:

$$K = \frac{C_b}{C_f(\frac{[NP]}{2s} - C_b)} \quad (7)$$

for an irreversible reaction at 25 ºC, the peak current (I_p) can be calculated as[27]:

$$I_p = B[(\alpha n_a)_f^{1/2} D_f^{1/2} C_f + (\alpha n_a)_b^{1/2} D_b^{1/2} C_b] \quad (8)$$

Making appropriate substitutions and eliminating C_b and C_f from eqn. (8), a new equation was obtained:

$$I_p = B\left\{(\alpha n_a)_f^{1/2} D_f^{1/2} C_t + [(\alpha n_a)_b^{1/2} D_b^{1/2} - (\alpha n_a)_f^{1/2} D_f^{1/2}]\right\} \times \left[\frac{b - \left(b^2 - \frac{2K^2 C_t [NP]}{s}\right)^{1/2}}{2K}\right] \quad (9)$$

Eqn. (9) is valid for the assumption of non-cooperative, non-specific binding to DNA with the existence of one type of discreet binding site. The binding constant (K) and the binding site size (s) of EM-DNA could be obtained by non-linear fit analysis of the experimental data (Fig. 8) according to the equation. With addition of DNA, the peak currents decreased sharply, at concentrations more than 18 mmol L^{-1}, the decrease in peak current became slight, then remained independent with the concentration of DNA, which showed that Bi(III)-myricetin interacted with ds-DNA quantitatively and the interactive balance has been established when R was 1.5. A non-linear fit analysis of the data to Eq. (11) yielded $K = 9.0 \times 10^4$ L mol^{-1} and s = 0.80. The overall results demonstrate that Bi(III)-myricetin binds to DNA with a high association constant and covers almost one base pair. Here, the actual meaning of s is the number of DNA base pairs covered (or made inaccessible to another molecule) by a binding molecule[28].

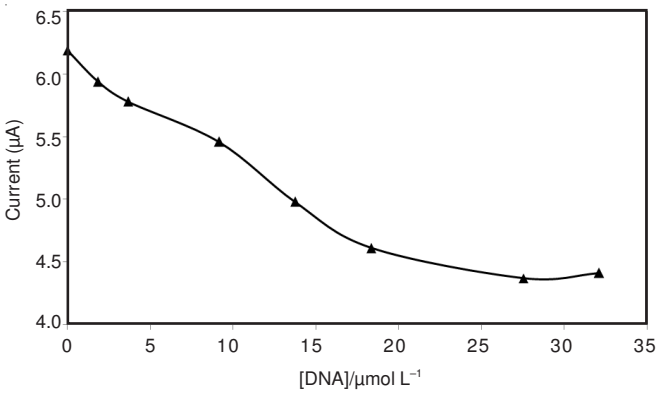

Fig. 8. Binding curve of Bi(III)-myricetin complex at different concentrations of ds-DNA. Cds-DNA = 0.0, 1.8, 3.7, 9.2, 13.7, 18.3, 27.5 and 32.1 μmol L^{-1}. $C_{Bi(III)}$ = 30.0 μmol L^{-1} and $C_{myricetin}$ = 20.0 μmol L^{-1} in phosphate buffer (0.002 mol L^{-1}, pH 7.5), ν = 50 mV s^{-1}, pulse amplitude 2 mV

Conclusion

In 0.02 mol L^{-1} phosphate buffer (pH 7.5) solution, Bi(III) makes a complex with myricetin and undergoes a process of 3-electron reaction on the surface of hanging mercury drop electrode. Also, Bi(III)-myricetin can bind with ds-DNA by electrostatically mode at pH 7.5 in which Bi(III)-myricetin has positive charges and easily interact with ds-DNA. In addition, binding constant (K) and binding site size (s) of the complex-DNA adduct were obtained simultaneously by non-linear fit analysis according to the equation, which is suitable to study interaction of ds-DNA with irreversibly electroactive molecules. According to the slopes of the I_p - $\nu^{1/2}$ plots the diffusion coefficients for the complex in the absence $(D_o)_f$ and presence $(D_o)_b$ of ds-DNA calculated as 5.37×10^{-7} and 4.55×10^{-7} cm^2 s^{-1} respectively.

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