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Investigation of Diuron Effect as an Environmental Pollution on the Structure and Stability of Human Hemoglobin

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ABSTRACT

Diuron is being used as an herbicide in agricultural crops and non-crops areas such as roads, garden paths, and railway lines. According to clinical studies, it is slightly toxic to mammals, birds and human health. In this study, the intermolecular interaction of Diuron and human hemoglobin was investigated using various spectroscopic methods. The UV-Vis and fluorescence results showed that the Diuron binds to Hb. According to the linear S-V plot, dynamic enhancement constant reduced with rising of temperature. Diuron formed a complex with HHb by static mechanism of enhancement and changed the conformation of Hb. The thermodynamic result suggested that the binding reaction was spontaneous and exothermic. Also, the result of synchronous fluorescence, heme degradation, thermal denaturation, aggregation and determination of surface hydrophobicity indicated that the Diuron could induce the conformational alteration, unfolding and heme degradation of Hb. bioanformatics study used for deciphering the binding location of a ligand to a biomicromolecules. According to molecular docking results the Diuron binds near the hydrophobic pocket of Hb which hydrophobic residue was located in this region.

Keywords: Diuron, Herbicide, Human hemoglobin, Stability of protein, Spectroscopy

INTRODUCTION

One of the most insoluble environmental problems is the use of the chemicals in agriculture that causes the pollution of the aquatic system and the soil [1]. Diuron (3,4-dichlorophenyl)-1,1-dimethylurea) is a Urea-derived herbicide which belongs to Phenylamide family and the subclass of Phenylurea [2]. This herbicide inhibits the photosynthesis in the photosynthetic microorganisms and plants by preventing oxygen generation and electron transport in the photosystem II (PSII) [2,3]. The high concentration of Diuron causes the photo destruction of pigment and also has an effect on the photosystem I (PSI) and photosystem II [4]. Diuron widely is used for broadleaf and grass weeds control in soil and water [5,6].

Diuron was introduced by Bayer in 1954 and was proffered with the brand names of Karmex and Direx in the market [1,6]. This compound is a colorless crystalline powder, non-ionic, odorless and with a moderate water solubility of 42 mg l^{-1} at 20 °C. The melting temperature of Diuron is at ambient temperature 158-159 °C [2,5]. It is used in the cultivation of cotton, coffee, flax, sugar cane, alfalfa and wheat in an amount of 1.8 Kg ha-1 year-1 that remains in the soil for a month to one year and remains in water for two weeks to 90 days [7]. So, it is known as a contamination source for soil, sediments and, water. Diuron is absorbed in the human body through the mouth, skin and respiration. Due to its harmful effects on the environment and human health its usage was restricted in the UK and EPA but it is still widely used in the world [6]. Some effects of Diuron on the health are ulcers, spleen, depression, anemia, reduction of the rate of erythrocytes and enhancement of the rate of methemoglobin and white blood cells [2,6]. Human hemoglobin (HHb) is a major metalloprotein and one-third of red blood cells can be infected [8]. This is a globular tetrameric protein with a

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molecular mass of 64.5 KDa and has four polypeptide chains including two identical α-chains (each having 141 amino acids) and two β-chains (having 146 amino acids) and one prosthetic group inserted in a hydrophobic pocket [9]. Hemoglobin has two subunits α1β2 that is formed from the contact of noncovalent each of α-chain with β-chain. The moiety of porphyrin consists of one atom of iron (Fe^{++}) in the center of the ring and four imidazole rings [10]. The function of hemoglobin is the transport of oxygen from lungs to tissues and carbon dioxide from the tissues to the lungs and also helps regulate the pH of the blood and this function is related to the moiety of the heme group. Hemoglobin binds to small molecules like drug, surfactant, and herbicide through the reversible binding mechanism [11]. Hemoglobin has three intrinsic fluorophores including tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe). Four polypeptide chains in HHb consist six Trp and ten Tyr residues. In each dimer there are Trp α 14, Trp β 15 and Trp β 37 residues of Trp and Tyr β 34, Tyr β 144, Tyr α 24, Tyr α 42, Tyr α 140 residues of Tyr. The intrinsic fluorescence of Hb mainly originates to Trp β 37 [12,13].

In this study,the focus is on the interaction of Diuron herbicide with hemoglobin using fluorescence spectroscopy, UV-Vis spectroscopy, and circular dichroism. The molecular dynamic was used to determine the binding site of Diuron in hemoglobin structure.

MATERIALS AND METHODS

Materials

Human hemoglobin (product number: H7379, lyophilized powder, 98%), Diuron (product number: D2425, 98%) and 1-Anilino-naphthalene-8-sulfonate (ANS) were purchased from Sigma Aldrich. Phosphate buffer solution (50 mM, PH 7.4) was prepared with the deionized water. All the experiments were carried out at ambient temperature.

Methods

UV-Vis spectroscopy. The UV-Vis studies were carried out in the absence and presence of different concentrations of 0-250 μM Diuron with UV-Vis Varian spectrophotometer model Cary 100 Bio equipped with a temperature control system made in Australia in the wavelength range of 200-700 nm in the PBS (PH = 7.4) at 298 K. All spectra were obtained by titrating Diuron on the Hb solution (3 μM) in the ambient temperature.

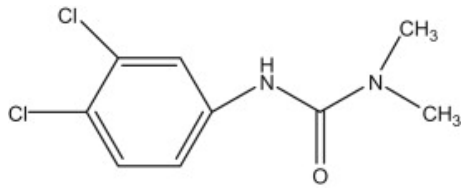

Fig. 1. The Molecular structure of Diuron.

Aggregation Measurements

The aggregation of hemoglobin (3 μM) in the presence and absence of Diuron (0-250 μM) was measured by UV-Vis Varian spectrophotometer model Cary 100 Bio equipped with a temperature control system at different temperatures of 288, 290, 293 and 298 K. The absorbance at a wavelength of 360 nm was recorded for 1000 s in each temperature.

Thermal Denaturation

The appropriate amount of Diuron was tittered to Hb solution (3 μM) and the absorbance at wavelength 280 nm was recorded while temperature raised from 25 °C to 90 °C at a rate of 1 °C min^{-1}. The midpoint of spectra suggested the t_m.

Fluorescence Spectral Studies

Fluorescence emission spectra of Hb with a concentration of 3 μM were recorded by the device fluorescence spectrophotometer model carry eclipse made in the Varian Corporation of Australia equipped with a temperature control system. Spectra were measured by titrating 0-250 μM of Diuron solution and exiting the protein solution at 280 nm. This scan was carried out using 1.0 cm quartz cell and keeping the excitation and emission slit widths 5nm and 10 nm, respectively. Scan was carried out in the range of 300-600 nm at 298, 303 and 310 K.

Mechanism of Fluorescence Enhancement

The mechanism of fluorescence enhancement analogous

to quenching is classified into dynamic or static. The mechanism of fluorescence enhancement is related to temperature and viscosity for elucidating this mechanism. Fluorescence experiments were carried out at 293, 303 and 310 K in which Hb does not undergo any thermal denaturation. Fluorescence enhancement data was analyzed by the Stern Volmer equation:

$$\frac{F_0}{F} = 1 - K_D[E] = 1 - K_B\tau_0[E] \quad (1)$$

Where F and F_0 are the fluorescence intensities in the presence and absence of enhancer, respectively. K_D is the dynamic enhancement constant like the dynamic quenching constant, K_B is the enhancement rate constant of biomolecule, E is the molar concentration of enhancer and τ_0 is the average lifetime of the fluorophore without enhancer.

Mechanism of the Association Constant and the Number of the Binding Sites

The fluorescence enhancement due to the binding of Diuron with Hb was used to determine the association constant and the number of the binding sites. The binding parameters can be assessed using the following modified Hill equation:

$$\log\left[\frac{(F_0 - F)}{F}\right] = \log K_a + n\log[Q] \quad (2)$$

Where the F and F_0 are the fluorescence intensities in the presence and absence of ligand, respectively. The K_a is the association constant and n is the number of the binding sites. But when the fluorescence intensity increased, F is larger than F_0 and the Eq. (2) is replaced by:

$$\log\left[\frac{(F - F_0)}{F}\right] = \log K_a + n\log[E] \quad (3)$$

By plotting $\log[(F\text{-}F_0)/F]$ against log[E] can determine the K_a and n values [14].

Thermodynamic Parameters

The enthalpy change (ΔH°) and entropy change (ΔS°) are important thermodynamic parameters which can determine the interaction forces between ligand and protein. Thus, the temperature-dependency of the binding constant was studied. The thermodynamic parameters were obtained from the plot of $\ln K_a$ *vs*. 1/T by using the Van't Hoff equation:

$$LnK_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

where R and K_a are the gas constant and the binding constant, respectively. By assuming that the enthalpy change (ΔH°) is constant in the three considering temperature, the free energy change (ΔG°) can be calculated from the following equation:

$$\Delta G = \Delta H - T\Delta S = -RT\ln K_a \quad (5)$$

Synchronous Fluorescence Spectra

Synchronous fluorescence spectra were measured in interval Δλ of 15 nm and 60 nm for Tyr and Trp residues, respectively. Spectra were scanned in the wavelength range of 220-600 nm while excitation and emission slit were kept 5 nm and 10 nm, respectively. The concentration of Hb in all of the 3 sample preparations in the 50 mM buffer phosphate was 3 μM. Diuron was tittered in all samples to the concentration of 0-250 μM and incubated with Hb for 10 min.

Heme Degradation Analysis

For the detection of heme degradation products during the interaction of Diuron with Hb fluorescence spectrophotometer model Cary Eclipse made in Varian company was used.

The protein solution was exited and scanned in 321 nm and 460 nm the wavelength range of 330-500 nm and from 470-600 nm, respectively. All of the sample solutions consisted 3 μM of Hb and variant concentration of Diuron from 0-250 μM.

Determination of Protein Surface Hydrophobicity

The variations in the surface hydrophobicity of protein perchance is displayed using convenien probes such as ANS. For this purpose, the stock of the ANS solution was prepared with (mM) concentration in the phosphate buffer.

It was titrated to 3 µM Hb solution in the presence and absence of Diuron. The final concentration of ANS was 250 µM in any 1000 µl samples. The fluorescence spectra were recorded using fluorescence spectrophotometer model carry eclipse equipped by 1.0 cm quartz cell while keeping the excitation and emission slit widths 5 nm and 10 nm, respectively. A scan was carried out in the range of 400-600 nm with excitation of wavelength at 380 nm.

Circular Dichroism Spectropolarimetry

The effects of Diuron on the secondary structure of Hemoglobin were studied using the JASCO-810 spectropolarimeter equipped with cell holder and the process was thermostatically controlled. The CD spectra of Hb were recorded in the Far-UV CD region at the wavelength range of 200-250 nm. All the spectra were measured by titrating the Diuron concentrations from 0 up to 255 µM on the 3 µM Hb solution. The results were presented in unites of deg cm^2 $dmol^{-1}$ and analyzed with the following equation:

$$MRE = \theta_{obs}(C_p nl \times 10) \quad (6)$$

where θ_{obs} is the measured ellipticity with device, C_p is the molar concentration of the Hb, n is the number of the amino acid residue of protein and l is the path length in centimeters.

Molecular Modeling Studies

Molecular docking study used for deciphering the binding location of a ligand to a biomicromolecules. Also, molecular modeling exploited to corroborate the experimental observation in recent years. The docking studies were carried out using the Molegro Virtual Docker software (MVD 2008 Version 3.2.1). The crystal structure of Hb (PDB: 2D60) was downloaded from protein Data Bank. The third structure of Diuron (CID: 3120) was resumed from PubChem. Molecular docking result was exhibited by Molegro molecular viewer.

RESULTS AND DISCUSSION

Absorption Spectral Studies

UV-Vis absorption spectroscopy is a very simple method and applicable to investigation of conformational changes in protein and complex formation [9,15]. Absorption spectra of Hb in the presence of a different concentration of Diuron from 0-250 µM have been presented in Fig. 2. The alteration of the soret band suggested variation in the microenvironment around the heme site [10]. The intensity of the soret band at 407 nm and the absorption peak at 280 nm increases with rising the concentrations of Diuron without any shift. It indicates the formation of a complex between Diuron and Hb [16]. The absorbance intensity of Q band at 541 nm, 576 nm and 630 nm decreases with the enhancement of concentration of Diuron which shows that the interaction between Diuron and hemoglobin induced the alteration in the binding site and function of hemoglobin (Fig. 3).

Thermal Denaturation

The thermal denaturation curves give some information about the effect of ligand binding on the stability of the protein. For studying stability and denaturation of protein, the changes in A_{280} *versus* the temperature were investigated. The maximum absorption wavelength of aromatic amino acids tryptophan, phenylalanine and tyrosine in various proteins are near to 280 nm [8]. So, any changes in the spectrum suggest the alterations of the microenvironment of these amino acids. The melting temperature of protein was calculated by using the thermal denaturation curves in the presence and absence of Diuron (Fig. 4). The T_m values of Hb were obtained 56.12 °C. The stability of Hb decreased with titration of Diuron and T_m shifted to a lower temperature.

Aggregation Measurements

The thermal aggregation study of protein is a useful technique for mensuration of the rate of resistance of protein versus thermal denaturation. The thermal aggregation spectra possess three regions of lag phase, logarithmic phase and plateau phase. A low slope of the logarithmic phase and a long leg phase suggest that the protein is more stable [17]. In the high concentration of Diuron, the leg phase was disappeared and the slope of the logarithmic phase was fast (Fig. 5). So in the presence of Diuron, aggregation potential of Hemoglobin increased at each temperature which demonstrated that Hb is undergoing denaturation in its

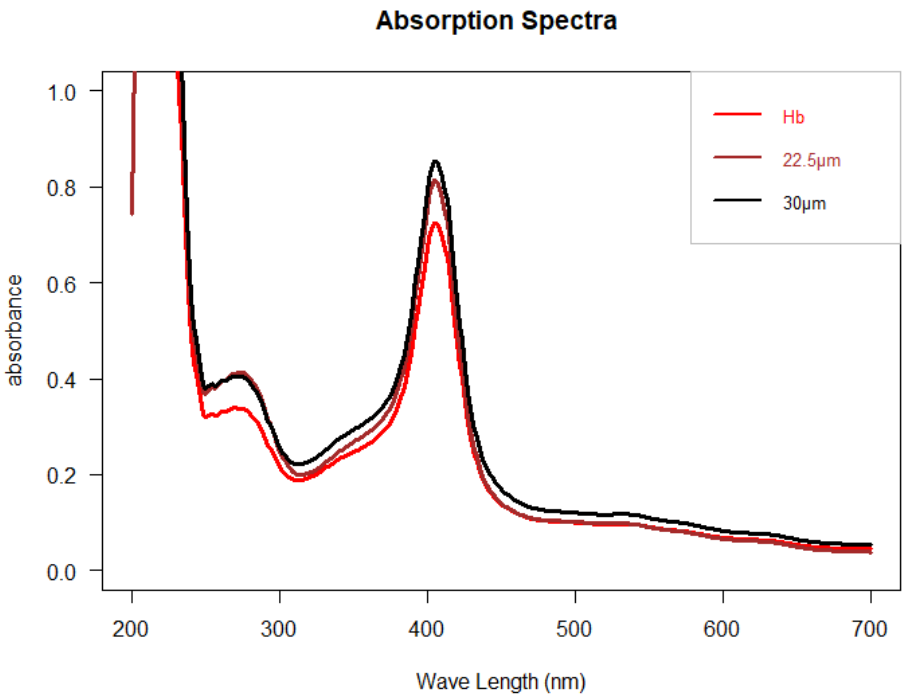

Fig. 2. Absorption spectra of Hb in the presence of Diuron at PH = 7.4. [Hb] = 3 µM.

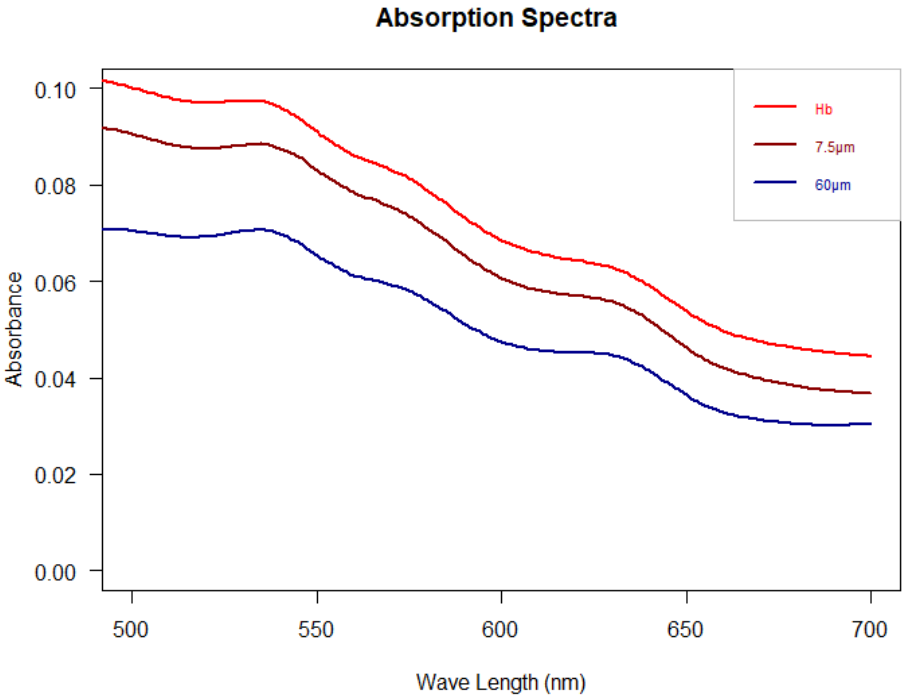

Fig. 3. Partial absorption spectra of Hb in the presence of different concentrations of Diuron (0-250 µM).

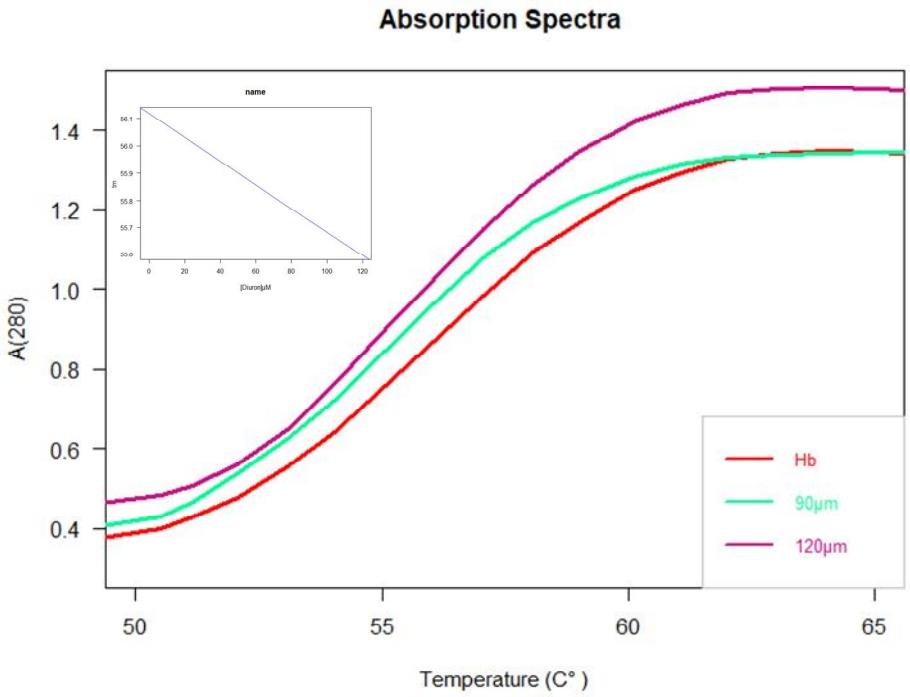

Fig. 4. Thermal denaturation of Hb (3 µM) in the presence and absence of Diuron.

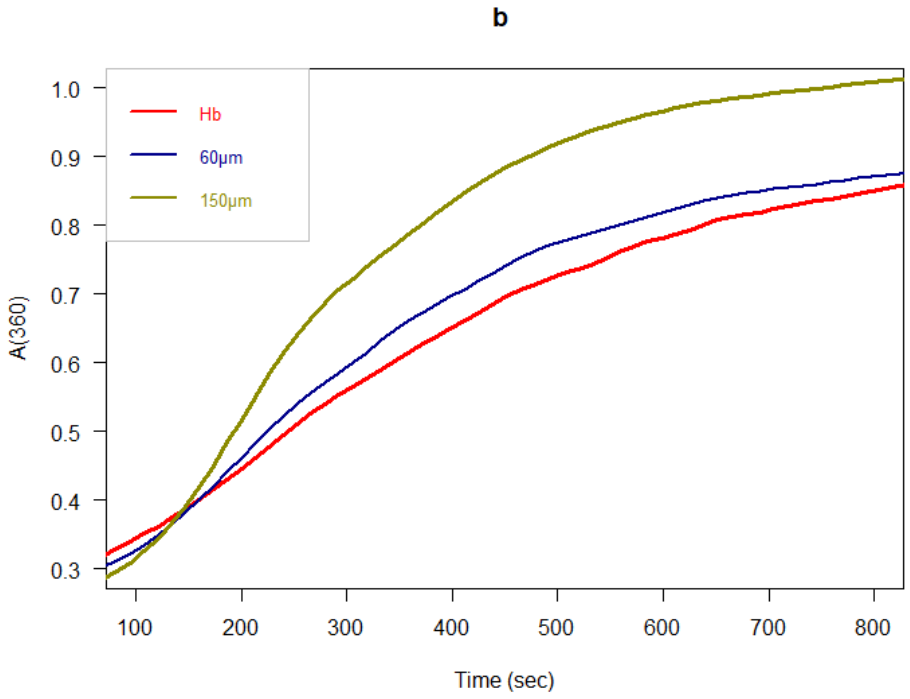

Fig. 5. Aggregation measurements in the presence and absence of Diuron.

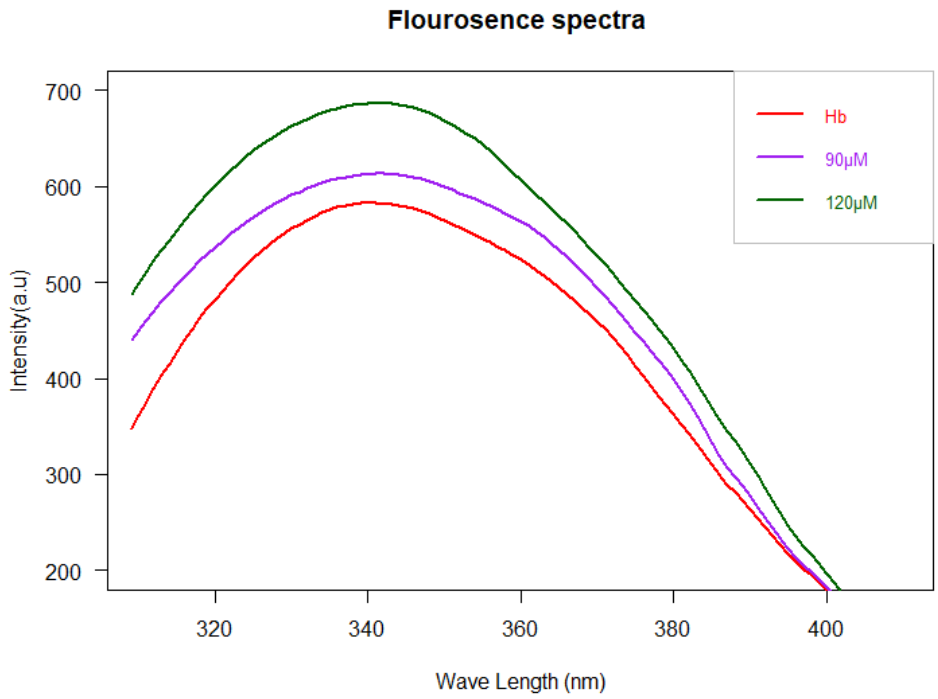

Fig. 6. The intrinsic fluorescence spectra of Hb in various concentrations of Diuron from 0 up to 255 µM.

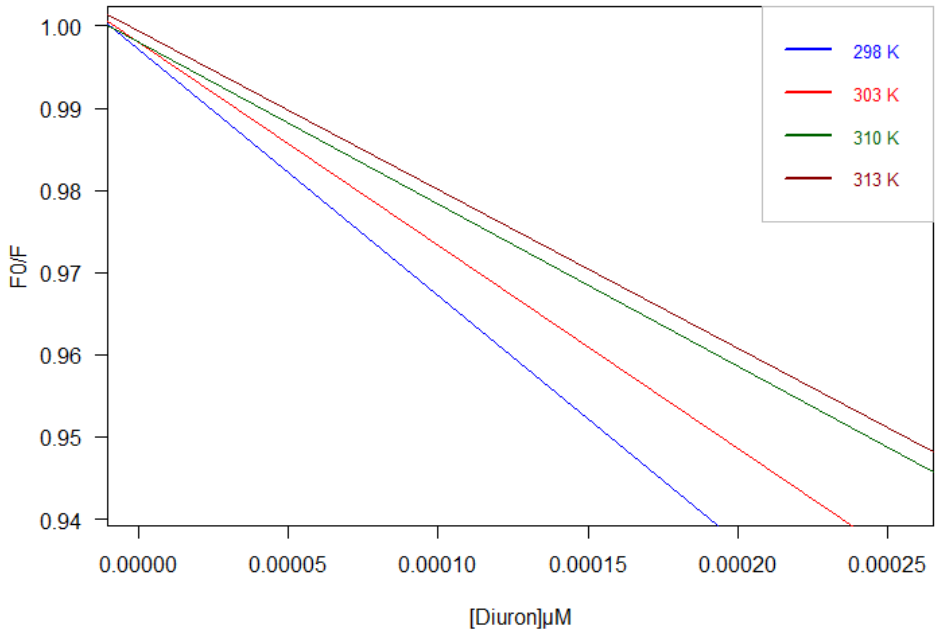

Fig. 7. The Stern-Volmer plots for fluorescence enhancement of Hb by Diuron at 298, 303 and 310 K.

Table 1. Dynamic and Bimolecular Enhancement Constant the Binding Diuron Binding to Hb at Different Temperatures

T (K)	K_D (M^{-1})	K_B ($M^{-1} s^{-1}$)	R^2
298	300.4	3×10^{10}	0.918
303	285.3	2.85×10^{10}	0.927
310	273	2.73×10^{10}	0.902
313	230.6	2.30×10^{10}	0.910

Table 2. The Association Constant and Nnumber of the Binding Sites for Diuron-HHb System at Different Temperatures

T (K)	N	K_a (M^{-1})	R^2
298	1.140	4572.03	0.971
303	1.130	1144.19	0.970
310	1.085	239.27	0.905
313	0.981	164.399	0.900

Table 3. The Thermodynamic Parameters for the Binding of Diuron to HHb

T (K)	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})	ΔG° (kJ mol^{-1})	R^2
298	-174.173	-514.977	-20.709	0.991

structure.

Intrinsic Fluorescence

The intrinsic fluorescence spectra of Hb increased with the enhancement of Diuron concentration with a red shift (Fig. 6). This observation indicates the formation of a complex and alteration of the polarity of the microenvironment of Hb fluorophores. Hb has a six tryptophan residue. Intrinsic fluorescence of Hb arises from β-37 Trp located at the $\alpha_1\beta_2$ interface near the heme group. The enhancement of fluorescence intensity of Hb may be attributed to two factors: 1) Movement of fluorophore toward the core of protein 2) the rising distance between the tryptophan and heme group [9].

Mechanism of Fluorescence Enhancement

The increment of the fluorescence intensity of Hb in the presence of Diuron can be described through the Stern-Volmer equation (Eq. (1)). The mechanism of fluorescence enhancement can be determined by dependency on viscosity and temperature. Weakly bound complexes dissociation with rising of temperature and due to the rate of static enhancement decreased. The increasing of enhancement constant with temperature suggested the dynamic enhancement and on the contrary, shows the static enhancement [14,15]. So, the measurement of the K_D values in different temperatures suggests that the involving mechanism is an static enhancement (Fig. 7) and (Table 1). The Stern-Volmer plots are linear at three different

temperatures which indicate the occurrence of a single type of enhancement. The bimolecular enhancement constant values (K_B) are 9.42×10^{10} M^{-1} s^{-1} which are greater than the 2×10^{10} M^{-1} s^{-1} values of K_B of the biomolecules in aqueous solutions. So, Hb formed a complex with Diuron by the static enhancement.

Mechanism of the Association Constant and the Number of the Binding Sites

$\log\left(\frac{F-F_0}{F}\right)$ is plotted *versus* log[E] and the values of the association constant (K_a) and the number of the binding sites (n) were determined from the double logarithmic curve at different temperatures (Table 2) (Fig. 8) [18]. The value of R square is equal to 0.96 suggesting that the interaction between Diuron and Hb is possible. The values of n and binding constant decreased with temperature, suggesting that the Diuron-HHb system was destabilized with rising temperature. Also, the reduction of n showed that the alteration in protein structure during the binding of Diuron occurs with temperature.

Thermodynamic Parameters

The ligand binds to protein with noncovalent interacting forces such as Van der Waals, Hydrogen bonding, electrostatic and hydrophobic interaction [19]. According to Ross and Subramanian's report, thermodynamic parameters were used to determine the binding mode. If $\Delta H < 0$ and $\Delta S > 0$ presented the electrostatic interaction, $\Delta H > 0$ and $\Delta S > 0$ suggested the hydrophobic forces and $\Delta H < 0$ and $\Delta S < 0$ indicated hydrogen bonds and Van der Waals interaction. These parameters were calculated according to the Van't Hoff equation (Fig. 9) (Table 3) [20]. The negative values of entropy change and enthalpy change show that the Van der Waals and hydrogen bonding play a major role in the interaction of Diuron with HHb. Also, the negative values of ΔG suggested that the binding process is spontaneous.

Synchronous Fluorescence

The synchronous fluorescence spectra were introduced by Llyod and Evett in 1971 [21]. Synchronous fluorescence technique was used for determination of conformational changes of protein and it gives information about the vicinity of the chromophores of protein by measuring the shift in wavelength emission spectra. This technique simultaneously scans the excitation and emission spectra while maintaining a constant wavelength interval between them. The wavelength intervals were maintained at 15 nm and 60 nm for Tyr and Trp, respectively [22]. The synchronous fluorescence of Hb increased with the titrating of Diuron (Fig. 10). The emission maximum of Trp and Tyr possesses a considerable red shift and blue shift, respectively. These results indicate that the polarity around the tryptophan and tyrosine change amino acid residue. Thus, the hydrophobicity around Tyr increased and the polarity decreased while the hydrophobicity around of Trp decreased and the polarity increased. The interaction of Diuron with Hb can be affected by the physiological function of Hb by conformational alteration.

Heme Degradation

In effect of non-enzymatic degradation of the heme prosthetic groups two fluorescence products were generated with a non-protein structure. The fluorescence spectra of these products were recorded at different excitation wavelengths of 321and 460 nm in the emission wavelength range 330-600 nm and 470-700 nm, respectively (Fig. 11) [23]. The fluorescence intensity increased with an enhancement of the concentration of Diuron at both excitation wavelengths which suggests the formation of fluorescence products obtained from Heme degradation. This observation indicates that the structure and function of Hb induced significant alterations.

ANS as a hydrophobic probe was used in the detection of surface hydrophobic patches on protein. The fluorescence intensity of ANS in a hydrophobic medium is more than water. Thus, ANS was used to investigate the changes in surface hydrophobicity of protein during the ligand binding [24]. The fluorescence intensity of ANS was measured at a certain concentration of Hb in the presence and absence of Diuron. The fluorescence intensity increased with the enhancement concentration of Diuron (Fig. 12). Thus, the number of surface hydrophobic sites of the Hb increased. This result is in agreement with the result of UV-Vis and intrinsic fluorescence spectroscopy which suggests the alteration of the conformation of Hb in the presence of Diuron.

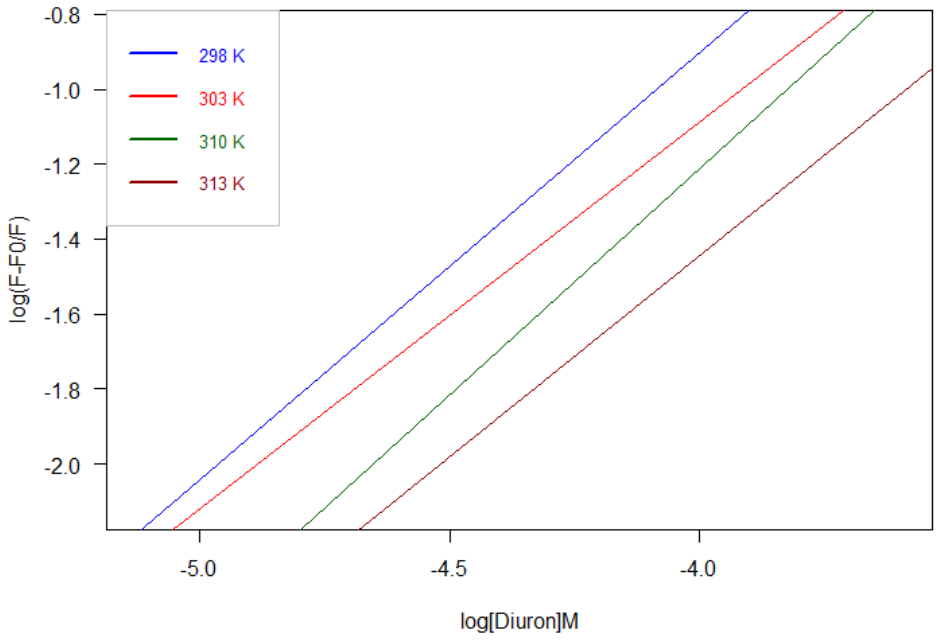

Fig. 8. The plots of log(F - F_0)/F *vs*. log[E] for enhancement of HHb by Diuron at different temperatures.

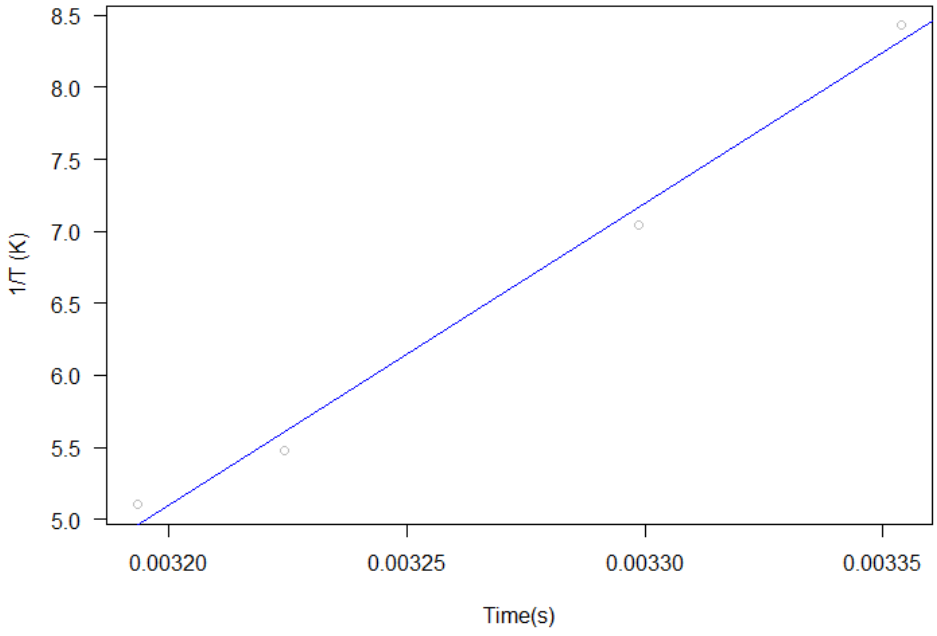

Fig . 9. Van't Hoff plots for determination of binding mode between the Diuron and HHb at different temperature.

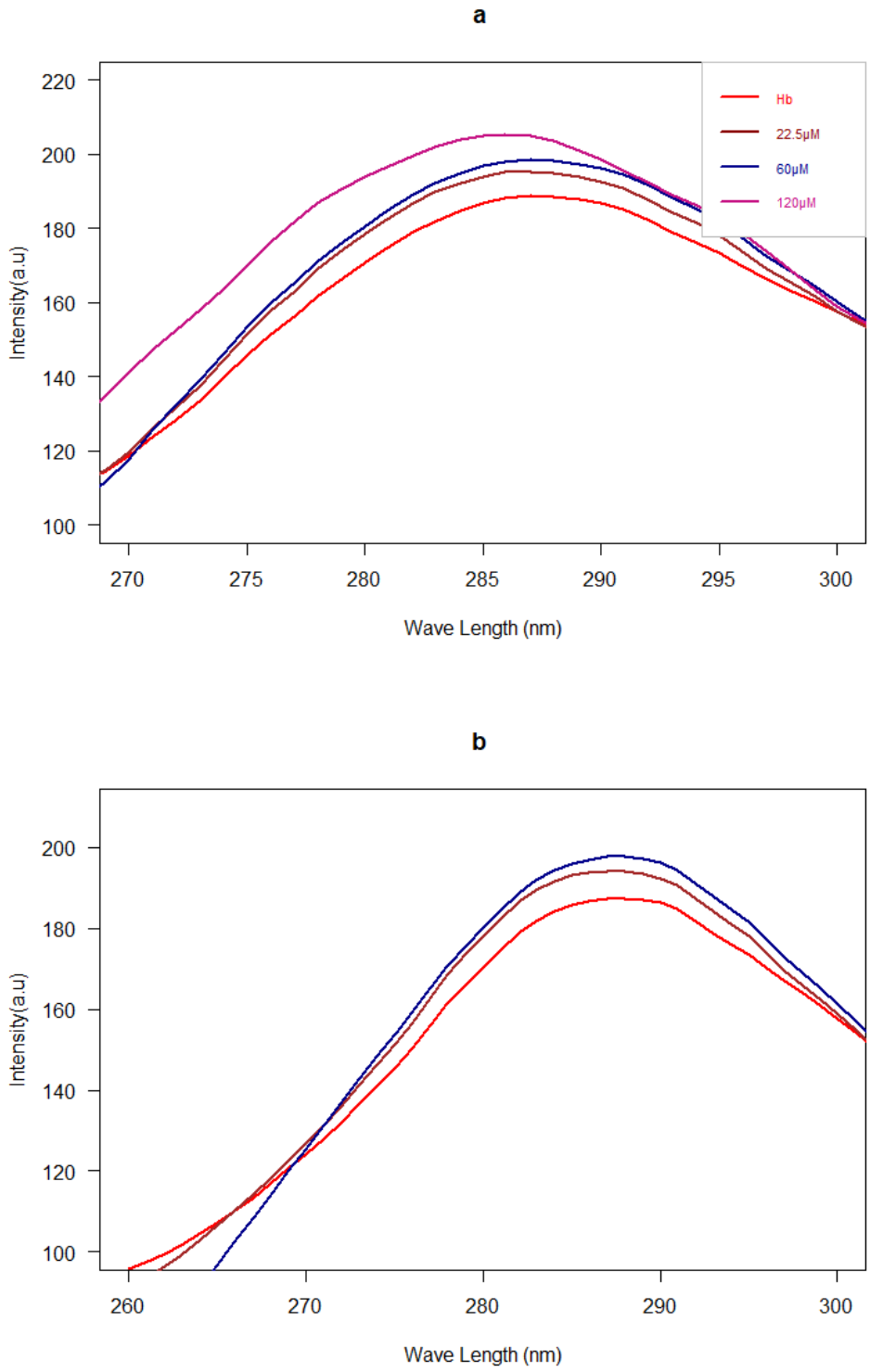

Fig. 10. The synchronous fluorescence of the Diuron-HHb system. a) $\Delta\lambda$ = 15 nm and b) $\Delta\lambda$ = 60 nm.

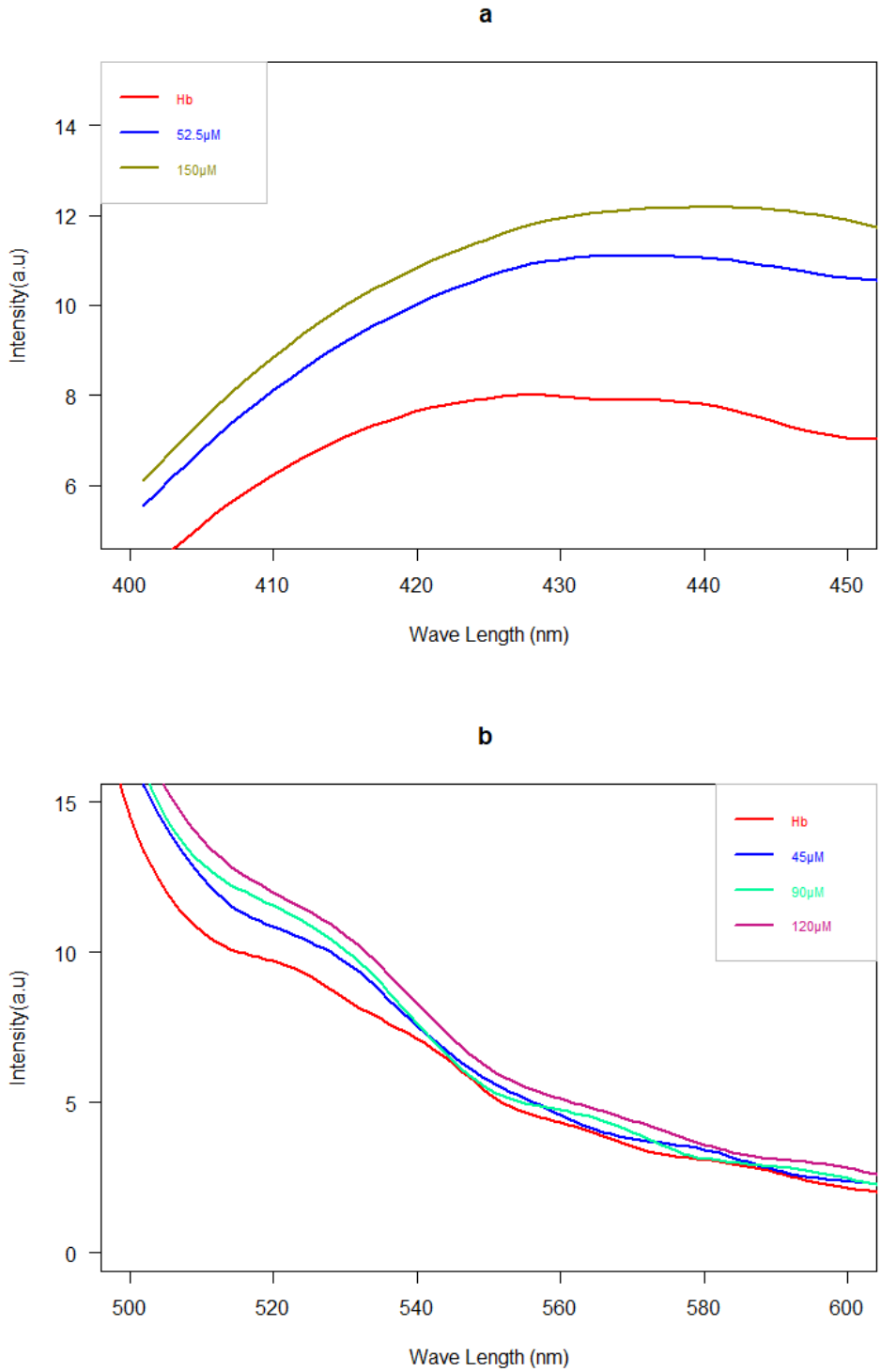

Fig. 11. The heme degradation of HHb (3 μM) in the presence of a different concentration of Diuron. a) the excitation wavelength is 321 nm and b) 460 nm.

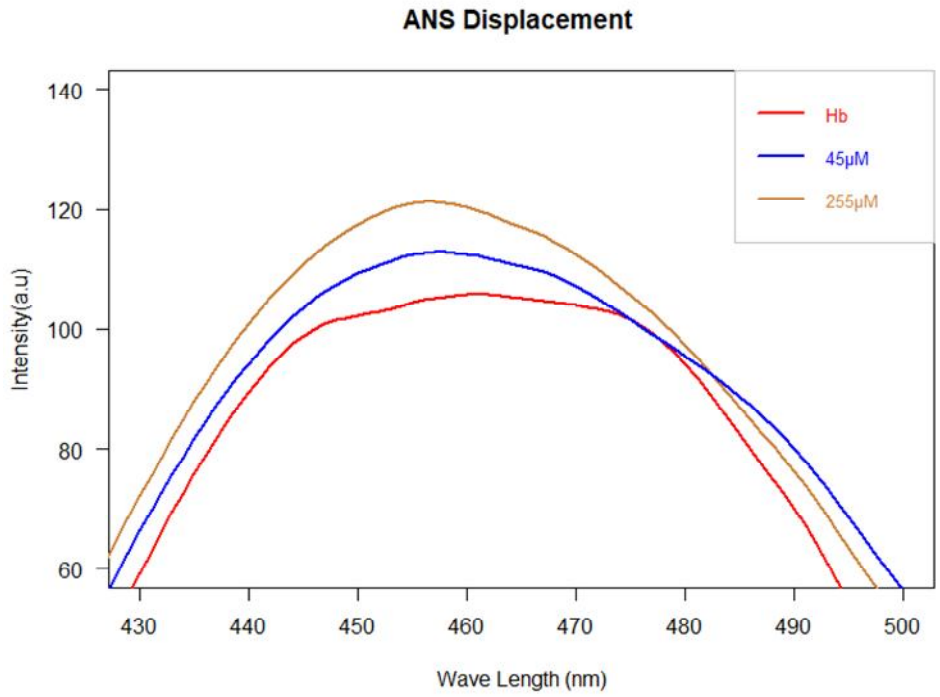

Fig. 12. The fluorescence emission spectra of binding of ANS to HHb in the absence and presence of Diuron.

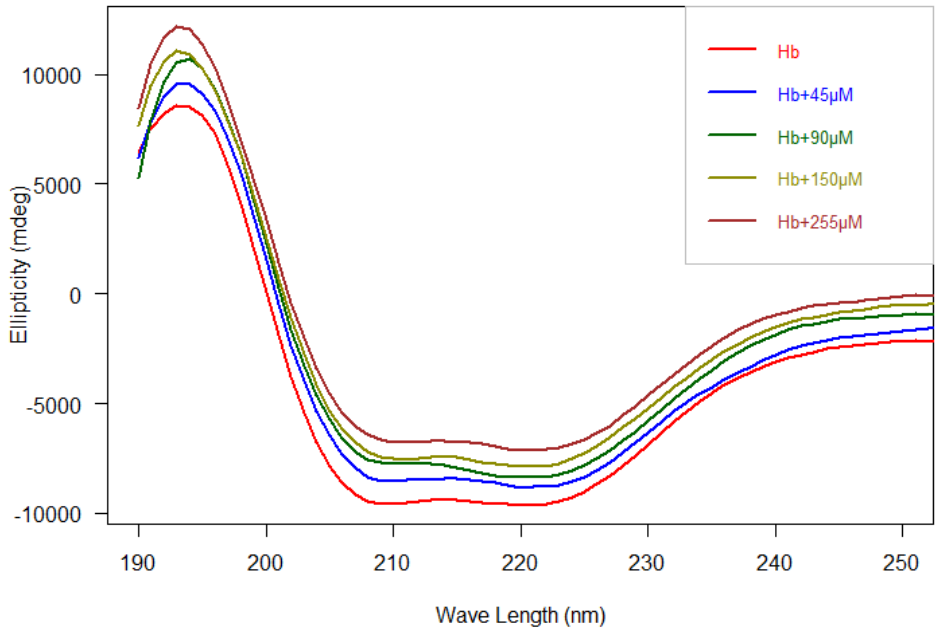

Fig. 13. CD spectra of Hb (3 μM) in phosphate buffer with different concentrations of Diuron at 298 K.

Table 4. The Percentage of Secondary Structure of Hb at Different Concentration of Diuron

	Hb	Hb + 45 µM Diuron	Hb + 90 µM Diuron	HB + 150 µM Diuron	Hb + 255 µM Diuron
Helix	28.2%	27.2%	26.1%	26%	25.4%
Antiparallel	12.7%	13.1%	13.8%	13.9%	14.2%
Parallel	9.8%	10.3%	10.8%	10.8%	11.1%
Beta-turn	17.8%	18.0%	18.2%	18.2%	18.3%
Rndm.coil	34.1%	36%	37.7%	37.5%	38.5%

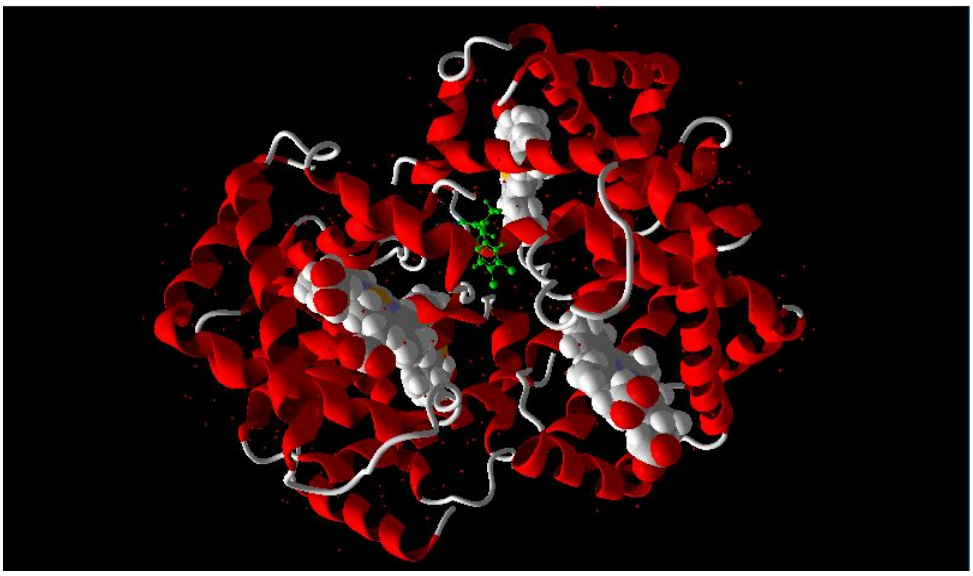

Fig. 14. Molecular modeling of Diuron docked to Hb.

Circular Dichroism Spectropolarimetry

Circular dichroism (CD) spectroscopy is a valuable research technique that has been widely used for gathering information about the structure of proteins. The secondary structure alteration of protein in presence and absence of Diuron was monitored by Far-UV CD experiments (Fig. 13). The CD spectra of Hemoglobin in the far-UV region shows two negative streaks at 208 and 222 nm, which are the characteristic of the α-helical structure of the proteins [25]. The CD data were analyzed with CDNN software. The binding of Diuron to HHb decreases the negative ellipticity at 208 and 222 nm which clearly indicates the decrease in the α-helical content of Hb. The diminish of α-helicity leads to unfolding of protein. The α-helicity of Hb-Diuron complex during the interaction decreased from 28.2% to 25.4% (Table 4). This result suggests that the aromatic residue exposure to the solvent changes the conformation of Hb.

Molecular Modeling Studies

Molecular docking is a benefit tool for predict the probable binding site of ligand to a biomicromolecules. It helps to identify types of interaction involved in the complexation of ligand and protein. Molecular modeling was carried out for determination probable binding region of Diuron at the active site of human hemoglobin (HHb)

employing the Molegro Virtual Docker tools. According to molecular docking results, Diuron binds within the cavity constituted by helices of Hb. The lowest binding energy has been obtained to be -70.7281. The Diuron binds near the hydrophobic pocket of Hb which hydrophobic residue was located in this region. The Diuron interacted with Asp 94, Pro 95, Trp 37, Tyr 35 and Val 34 residues through of Hydrogen bonds and steric interaction which it causes structural alteration in Hb (Fig. 14). This observation conforms that the experimental results.

CONCLUSIONS

Diuron is a biologically active pollutant belonging to phenylurea herbicide which remains in the soil, water and sediment more than two weeks to one year and its penetration into the human body causes diseases like: spleen, depression, anemia and *etc*. In this study, the Diuron effect on the structure and stability of human hemoglobin was investigated using the spectroscopic methods. The UV-Vis result suggested complexation and the melting temperature decreased with titrating of Diuron. So, the Diuron induced the denaturation of Hb. Moreover, aggregation accelerated during the binding of Diuron to HHb. The fluorescence intensity of Hb increased by static enhancement mechanism. The reduction of the association constant with temperature indicated the destabilization of complex and it is in agreement with thermal denaturation test. The binding forces in this system were hydrogen bonds and Van der Waals forces. According to the result of synchronous fluorescence, the secondary structure of Hb changed. Also, heme degradation results suggested some Diuron effects on the function of hemoglobin in addition of structure. The unfolding of protein in the presence of Diuron approved the determination surface hydrophobicity test results. So, the Diuron interacted with porphyrin ring and leads to heme destruction, elimination of heme filtering group and the reduction of the oxy form of Hb with change in the conformation of Hb.

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