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Research Article

Spectroscopic insights on the interaction between 4-(3,4 dimethoxyphenyl)-6-hydroxy-2-methylpyrimidine-5-carbonitrile and tryptophan.

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ABSTRACT
The interaction between 4-(3,4-dimethoxyphenyl)-6-hydroxy-2-methylpyrimidine-5-carbonitrile (DMPHMPC) and tryptophan (Trp) has been explored by fluorescence and Uv-visible studies. The formation of DMPHMPC-Trp complex leads to the fluorescence quenching of Trp was induced by DMPHMPC and evaluated by spectrofluorimetry. The fluorescence mechanism of Trp due to DMPHMPC is static in nature as analyzed by temperature effect study. Stern-Volmer quenching constants (Ksv) between DMPHMPC and Trp at three different temperatures 303, 308 and 313 K were obtained to be 9.12 x 10^{-3}, 4.07 x 10^{-3} and 1.63 x 10^{-3} L mol^{-1}, respectively which detected that the binding mechanism between DMPHMPC and Trp is predominantly static in nature. The calculated binding constants were 3.96 x 10^{-4}, 3.61 x 10^{-4} and 2.85 x 10^{-4} L mol^{-1}, respectively. The numbers of binding sites were 1.01, 0.92 and 0.91. Enthalpy change (ΔH) and Entropy change (ΔS) has positive value which indicates that hydrophobic interactions played a major role in the binding mechanism. Synchronous fluorescence suggesting that DMPHMPC bonded to Trp and placed in the close vicinity of Trp. Whereas the binding distance (r) between DMPHMPC and Trp was obtained to be 4.46 nm by Forster's theory of nonradiative energy transfer .

KEYWORDS
DMPHMPC; Trp; Fluorescence; FRET; UV-visible study.

1. INTRODUCTION

Fluorescence spectroscopy is broadly used for study of peptides and proteins. The aromatic amino acids like tryptophan, tyrosine and phenylalanine give intrinsic fluorescent probes of protein conformation, dynamics, and intermolecular interactions. Out of three, tryptophan is the most popular probe. Tryptophan (Trp) occurs in one or a few residues in most proteins and biologically active peptides. The fluorescence of indole chromophore is highly sensitive to environment, making it an ideal choice for reporting protein conformation changes and interactions with other molecules [1]. Tryptophan and its derivatives are certainly the most important source of emission in proteins. Tryptophan fluorescence wavelength is widely used as a tool to monitor changes in protein folding, and protein−protein or protein−peptide interactions and as a probe of the local environment within a protein [2].

Trp is an important amino acid in the human nutrition fundamental for life activity of living being. D-Trp is barely consumed by organisms while the L-Trp is required for life action of creature and used in structural or enzyme proteins playing an active role in protein biosynthesis and also used as a precursor for serotonin, melatonin and quinolic acid. It is extensively usefull in medicine, food and feed stuff and acts as a natural therapy for depression, sleeplessness, ache, hyperactivity and eating disorders [3]. Milk, dried dates, yogurt, cheese, oats, red meat, eggs, fish, sesame, almonds, sunflower seeds, pumpkin seeds, and peanuts, chocolate contains Trp. Trp control the natural mood by improving the body's ability to produce and balance certain hormones obviously. By taking Trp rich food or Trp supplements helps to induce sleep, fights anxiety, and burn more body fat. It stimulates the release of growth hormone. 5HTP (5-hydroxytryptophan) is one of the byproduct of Trp works in the central nervous system which boost the feelings of well-being, safety by increasing the feel good hormone serotonin. It also helps to control appetite which contributes to maintenance or weight loss. It useful in sleep and mood disorder, migraines and tension headaches, menopausal symptoms, fibromyalgia. Trp deficient diet lead to reduction in serotonin level which lead to depression, anxiety, emotional imbalance and weakened neural health. Also depletion of Trp causes motion sickness, memory weakening, activates violent behavior. Molecular formula of Trp is $C_{11}H_{12}N_2O_2$ having molecular weight 204.23 $gmol^{-1}$. Its chemical structure is shown in figure 1.

The fluorescence properties of the Trp and its parent indole ring system have been mostly studied. Indole functional group of Trp plays a major task in the light sensitivity of biological systems [4]. Being extremely responsive to environment used to investigate the interaction with added molecule and trace the alterations [5]. Studying the interaction of drugs and L-Trp is important to know about the passage and dispersal of drugs in body, for understanding the action mechanism and drugs pharmacodynamics [6].

The chemistry of pyrimidine and its derivatives has been widely studied because of the pharmacological and physical properties of these important heterocycles. Pyrimidine analogous including uracil, thymine, cytosine, adenine, and guanine are basic building blocks of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA). Vitamin B1 (thiamine) is a well known example of a naturally occurring pyrimidine that is embedded in our daily lives. Synthetic pyrimidine derivatives are used in the pharmaceutical industry as potent drugs. For example, pyrimethamine is used as an antimalarial and antiprotozoal drug that is applied in combination

with sulfadiazine [7,8]. Pyrimidine also plays a role of analgesic, antihypertensive, antipyretic, anti-inflammatory, antineoplastic, antibacterial, antiprotozoal, antifungal, antiviral and antifolate drugs and as pestisides, herbicides and plant growth regulators [9-11].

Pyrimidine analogous compounds were extensively investigated as electroluminescent materials in the past and as a two-photon absorption organic chromophores [12,13]. There is investigation on effect of pyrimidine additives on the dye sensitized solar cell performance [14]. The influence of solvents on the spectral properties of molecules called as slvatochromism has been investigated for many years [15]. Fluorescent probe has been widely used in the field of biological and organic material science. Some pyrimidine derivatives are fluorescent materials that possess many valuable photophysical properties. In recent years considerable efforts have been given to design and synthesis of functional molecules that could serves as sensitive sensors for the analytical detection of chemically and biologically important species [16]. Therefore we plan to study the determination of binding affinity of pyrimidine derivative (DMPHMPC) especially for Trp molecule using steady state fluorescence and UV-visible spectroscopic techniques to understand the carrier role of Trp. The pyrimidine derivative i.e. 4-(3,4-dimethoxyphenyl)-6-hydroxy-2-methylpyrimidine-5-carbonitrile serves as 80% anti-inflammatory activity which was synthesized by Dr. S. S. Undre and et al via three-component condensation of 2,3 dimethoxy benzaldehyde, ethyl cyanoacetate and acetamidine hydrochloride in ethanol at 80 ^{0}C. [17].

In the present article, we reported a spectroscopic analysis of the interaction between Trp and DMPHMPC at three temperatures 303, 308 and 313 K using stable Trp concentration and various DMPHMPC compositions. The aim of the present examination was to study the binding affinity of DMPHMPC towards Trp. The evidence of interaction between Trp and DMPHMPC concerning quenching mechanism, binding factors and thermodynamic parameters were discussed. Also we reported herein the FRET study between Trp and DMPHMPC.

2. MATERIALS AND METHODS

2.1 Materials

The Trp is obtained from Himedia Chemical Company and their solutions were prepared at concentration 2 x 10^{-5} M in doubly distilled water. The solutions of DMPHMPC in ethanol-water (1: 9) mixture were prepared at concentration 1 x 10^{-4} M.

2.2 Equipments and spectral measurements

All fluorescence quenching spectra were recorded on PC based fluorescence spectrophotometer (JASCO Japan FP-8300) equipped with a xenon lamp and 1.0 cm quartz cell. Fluorescence spectra were recorded at three different temperatures. An excitation wavelength of 280 nm was chosen. Synchronous fluorescence measurements were made at emission range between 320 – 360 nm and $\Delta\lambda$ was set at 60 nm. The UV-Visible absorption spectra were measured at room temperature on Specord Plus-210 UV-Visible spectrophotometer.

3. RESULTS AND DISCUSSION

3.1. UV-visible absorption spectral studies

UV-visible absorption measurement is a general method to explore the structural change and obtain information about the complex formation [18, 19]. Figure 2 shows UV-visible absorption spectra of Trp in the absence and presence of DMPHMPC drug. It shows that a band in the near-UV region with a maximum 278 nm, which appears due to phenyl group of tryptophan. The UV-visible spectra of donor was not altered only when excited-state of fluorescent molecule influenced by quenchers for dynamic quenching; but for static quenching a complex is appeared between fluorophore and quencher [20]. The absorption spectrum of Trp was changed due to formation of ground state complex. So type of quenching was static in nature, indicating that there is existence of interaction between DMPHMPC and Trp.

3.2 Trp fluorescence characteristics and quenching mechanism

Fluorescence quenching is a most usable method for understanding binding interactions between donor and acceptor. Fluorescence intensity of a substance decreases by a variety of molecular interactions viz., excited-state reactions, molecular rearrangements, energy transfer, ground state complex formation and collisional quenching. Such reduction in intensity is called as quenching. In the experimental work concentration of Trp were fixed at $2x10^{-5}$ mol.L^{-1} and the concentration of DMPHMPC varied from 0 to 6.0 x 10^{-4} mol.L^{-1}. Figure 3 display the fluorescence emission spectra of Trp in presence of various concentration of DMPHMPC at 303 K upon excitation is 280 nm. The intensity of Trp fluorescence is decreases along with successive addition of DMPHMPC accompanied by an increase of wavelength emission maxima λ_{em} (red shift 353 to 356 nm). It suggested that polarity around Trp was increased [21]. Under the same condition, fluorescence of DMPHMPC was observed with iso emissive point at 397 nm. The results indicated that binding of DMPHMPC to Trp quenches intrinsic fluorescence of Trp. The fluorescence quenching effect was due to formation of complex between Trp and DMPHMPC [22].

Fluorescence quenching can occur by two types of mechanisms, generally classified as either dynamic or static quenching, which can be distinguished by their contrary dependence on temperature and viscosity and fluorescence lifetime [23]. As higher temperatures result in large diffusion coefficients for dynamic quenching, and the quenching constants are increases with rising temperature. In reverse, a higher temperature may bring about the decrease in the stability of the complexes; resulting in lower quenching constant for the static quenching [24]. The Stern–Volmer equation is applied to explain the quenching mechanism,

$$F_0/F = 1 + k_q\tau_0[Q] = 1 + K_{sv}[Q] \quad (1)$$

Where, F_0 and F are the fluorescence intensities in the absence and presence of quencher respectively, *[Q]* is the concentration of quencher, K_{sv} is the Stern-Volmer dynamic quenching constant, k_q is the bimolecular quenching rate constant, τ_0 is the lifetime of the fluorophore. The formation of complex was further confirmed from the values of quenching rate constant k_q, which are evaluated using the equation:

$$k_q = K_{sv} / \tau_0 \quad (2)$$

Figure 4 depicted the plot of F_0/F of Trp verses *[Q]* of DMPHMPC at three different temperatures and the calculated K_{sv} and k_q values were listed in Table 1. The plots indicate that results exhibited a good linear relationship. Table 1 show K_{sv} values were inversely proportional to temperatures. Cause of an influence of fluorophore absorption spectra, the ground state complex is formed between donor and acceptor for static quenching [25]. It also persistent that from the table 1, k_q values were much greater than the maximum scatter collision quenching constant (2.0×10^{10} M^{-1} S^{-1}) for various quenchers with biomolecule [26], so it recommended that the static quenching was dominant in Trp-DMPHMPC interaction.

The fluorescence data was again examined by using modified Stern-Volmer equation.

$$F_0/(\Delta F) = 1/(f_a\ K_a) \times 1/[Q] + \ 1/f_a \qquad (3)$$

Table 1. Stern-Volmer quenching constants and bio-molecular quenching rate constants for Trp-DMPHMPC at various temperatures.

T (K)	10^{-2} Ksv /(L mol^{-1})	10^{-10}Kq /(Lmol-1 s^{-1})	R
303	9.63	9.63	0.9979
308	9.12	9.12	0.9964
313	8.41	8.41	0.9948

R = correlation constant

Where, f_a is the fraction of the initial fluorescence which is accessible to quencher, K_a is the Stern-Volmer quenching constant of the accessible fraction and [Q] is the concentration of quencher. Figure 5 showed that modified Stern-Volmer plots; the result indicates the dependence of $F_0/\Delta F$ on the reciprocal values of the quencher concentration $[Q]^{-1}$ is linear. Within the exhibited concentration the plot detected that, the results exhibited a good linear relationship. These results further confirm that, the quenching mechanism between pyrimidine derivative DMPHMPC and Trp is static quenching.

3.3 Analysis of binding constant and binding site

The fluorescence measurements can give information about the binding of donor and acceptor such as, binding mechanism, binding mode, binding constant, number of binding sites, intermolecular distances, etc. [27]. The equilibrium between free and bound molecules can be expressed by following equation,

$$\log\ (F_0 - F)\ /\ F\ = \log\ K + \ n \log\ [Q] \qquad (4)$$

Where K and n are binding constant and number of binding sites respectively. Figure 6 displays a plot of log (F_0-F)/F versus log [Q] whose slopes equal to n and intercepts on Y-axis equal to log K. Table 2 represent values of K and n obtained for drug-Trp interactions at 303, 308 and

313 K. The values of (*n*) were calculated as 1.01, 0.92 and 0.91 for Trp, showing there is approximately one binding site in Trp for DMPHMPC during their interactions.

Table 2. Binding constants (K) and number of binding sites (n) of competitive experiment of Trp- DMPHMPC system.

T(K)	10^{-3} K/(Lmol^{-1})	n	R
303	9.120	1.01	0.9979
308	4.073	0.92	0.9996
313	1.63	0.91	0.9816

R = correlation constant

3.4 Study of fluorescence lifetime

The time-resolved fluorescence has been studied successfully, for the confirmation of static quenching and its measurements were carried out in absence and presence of quencher and displayed in figure 7. There was no significant change in lifetime of Trp after addition of DMPHMPC. Thus, the possibility of dynamic quenching was ruled out; therefore, sole mechanism of quenching was that of static quenching [28].

3.5 Thermodynamic Parameters and Nature of Binding forces

Basically, there are four types of non-covalent interactions that could play a key role in binding of drug and biomolecule. These are hydrogen bonding, van der waals forces, electrostatic and hydrophobic interactions [29]. The forces accountable for binding are determined from the signs and magnitude of thermodynamic parameters which were determined by using van't Hoff equation.

$$ln\, K_T = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \qquad (5)$$

K is the binding constant at temperature *T* and R is gas constant. The enthalpy change (*ΔH*) is calculated from slope of Van't Hoff relationship. The free energy change (*ΔG*) is predicted from the following equation,

$$\Delta G = \Delta H - T\Delta S \qquad (6)$$

The temperature chosen were 303, 308 and 313 K. Thermodynamic parameters were determined from linear Van't Hoff plot showing in figure 8 and presented in Table 3. Ross and Subramanian [30] have characterized sign and magnitude of the thermodynamic parameters associated with various individual types of interactions.

Table 3. Thermodynamic parameters of Trp- DMPHMPC interaction.

T (K)	ΔH (KJ mol^{-1})	ΔG (KJ mol^{-1})	ΔS (Jmol^{-1}K^{-1})	R
303		-2802.29		

308	332.06	-2852.66	10.34	0.9963
313		-2904.36		

R = correlation constant

Negative value of ΔG indicates that the interaction process between these molecular pair is spontaneous. Positive ΔS shows entropy driven binding between Trp and DMPHMPC and positive ΔH reveals that endothermic process of binding. According to sign and magnitude of ΔS and ΔH hydrophobic forces and hydrogen bonding plays significant role in the binding between Tryptophan and DMPHMPC [31].

3.6 Energy transfer from Trp to DMPHMPC

Fluorescence resonance energy transfer is an important nondestructive method which can be used extensively to calculate molecular distances and to investigate the structure and spatial distribution of protein complexes. Generally efficiency of energy transfer depends on three factors. 1) Proper orientation of transition dipole of the donor and acceptor, 2) fluorescence emission spectra of donor must overlap with an absorption spectrum of acceptor, 3) Distance between donor and acceptor must be lower than 7 nm [32]. The overlap of excitation spectrum of DMPHMPC with fluorescence emission spectrum of Trp is shown in figure 9.

By using FRET, the distance r between DMPHMPC and Trp could be calculated by using the equation:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r_0^6} \qquad (7)$$

Where R_0 is the critical distance when efficiency of excitation energy transferred to the acceptor is 50 %, r is binding distance between donor and acceptor. It can be calculated by using Forster formula:

$$R_0^6 = 8.79 \times 10^{-25}\, K^2\, n^{-4}\, \emptyset\, J \qquad (8)$$

Where n is the refractive index of medium, K^2 is spatial orientation factor of the dipole, *ø* is fluorescence quantum yield of the donor, *J* is overlap integral of the fluorescence emission spectrum of donor with excitation spectrum of the acceptor, which can be calculated by using the equation:

$$J = \frac{\int_0^\infty F(\lambda)\, \varepsilon(\lambda)\, \lambda 4\, d\lambda}{\int_0^\infty F(\lambda)\, d\lambda} \qquad (9)$$

Where ε (λ) is the molar absorption coefficient of acceptor at wavelength λ, F (λ) is the fluorescence intensity of donor at wavelength λ to $\lambda + \Delta\lambda$. In this case K^2 = 2/3, n = 1.336 and ø = 0.12. Hence from equations (7) – (9) we could calculate that R_0 = 3.67 nm, E = 0.2390 and r = 4.46 nm. The binding distance r is smaller than 8 nm and 0.5 $R_0 < r < 1.5\ R_0$. These observations illustrate the conclusion that there is high probability of energy transfer from Trp to DMPHMPC [33].

3.7 Synchronous fluorescence Spectra

In order to investigate the structural change of Trp by adding DMPHMPC as quencher the synchronous fluorescence spectra were obtained. The synchronous fluorescence spectra give information about the molecular environment in the vicinity of chromophore molecules. These spectra can be obtained by scanning the two monochromators simultaneously with a fixed difference between emission and excitation wavelength ($\Delta\lambda$). For $\Delta\lambda$ = 60 nm synchronous fluorescence is characteristic of Trp molecule [34]. The effect of DMPHMPC on Trp synchronous fluorescence was shown in Figure 10.

It could be seen that the fluorescence intensity of Trp with maximum emission wavelength at 340 nm decreased regularly along with addition of quencher suggesting that DMPHMPC bonded to Trp and placed in the close proximity of Trp.

4. CONCLUSION

Binding interaction between Trp and DMPHMPC was investigated by spectroscopic methods. The absorption spectrum of Trp was altered due to formation of ground state complex. Again fluorescence spectra shows binding of Trp and DMPHMPC. Temperature dependence of quenching indicates the decrease of Stern-Volmer quenching constant with temperature detects that static quenching mechanism. Again static type quenching is confirmed by time resolved spectra. Values of binding site indicates the presence of only one binding site for DMPHMPC on Trp. Thermodynamic parameters signifies that the binding process is spontaneous, endothermic and entropy driven. Hydrophobic force and hydrogen bonding plays an important role in the interplay between Trp and DMPHMPC. Distance between donor and acceptor is 4.46 nm reveals the energy transfer with high probability.

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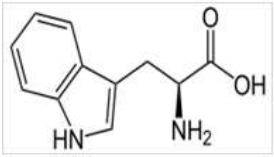

Figure 1. Molecular structure of Tryptophan.

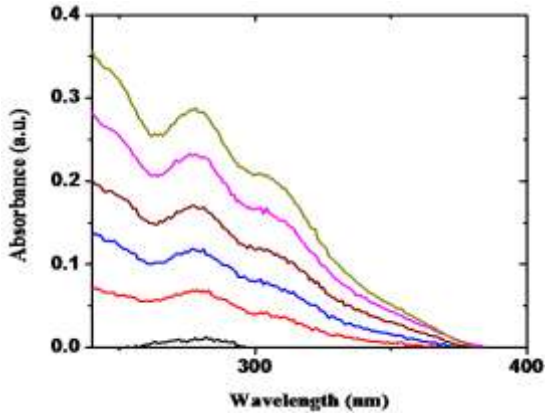

Figure 2. The UV-visible absorption spectra of Trp with increasing concentration of DMPHMPC, C_{Trp} = 2x10^{-5} mol L^{-1}, $C_{DMPHMPC}$/(10^{-4} mol L^{-1}) a-f: 0, 1, 2, 3, 4, 5, (T = 303 K).

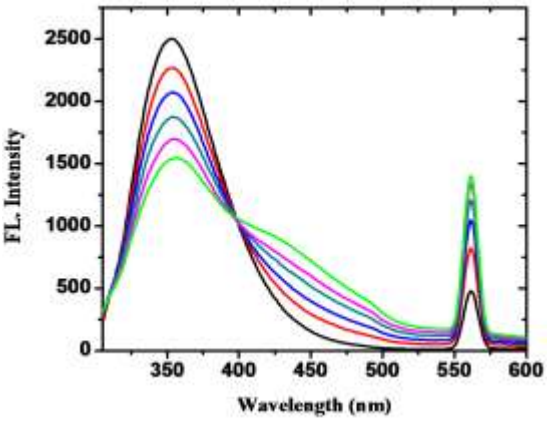

Figure 3. The fluorescence emission spectra of Trp with increasing concentration of DMPHMPC, C_{Trp} and $C_{DMPHMPC}$ are same as those in Figure 2.

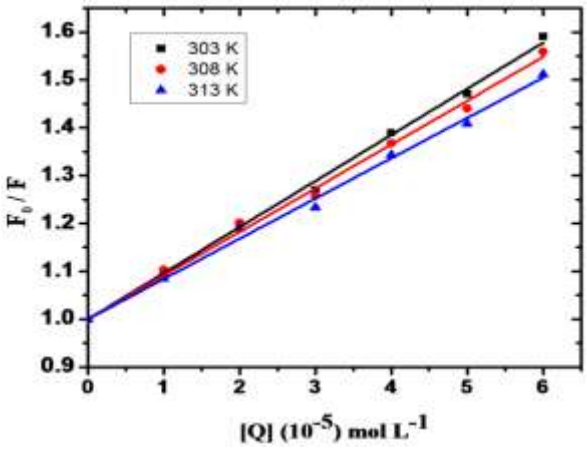

Figure 4. Stern-volmer plots at three different temperatures. C_{Trp} and $C_{DMPHMPC}$ are same as those in Figure 2.

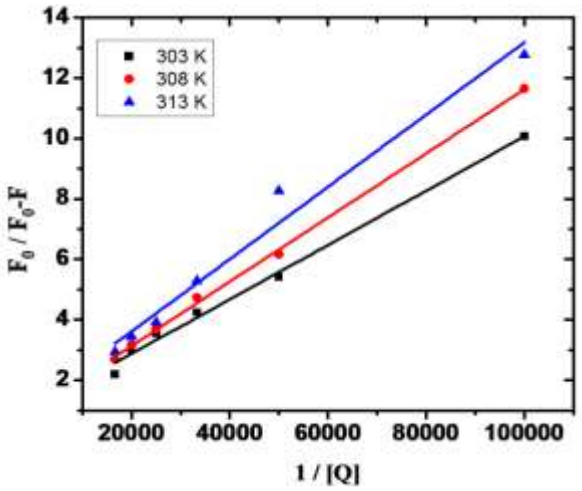

Figure 5. Modified Stern-Volmer Plot for the binding of Trp-DMPHMPC**.**

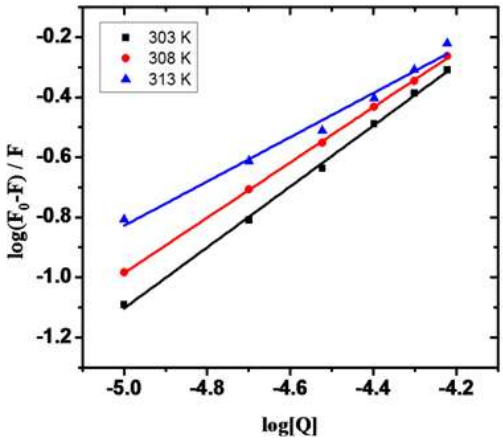

Figure 6. The plots of log (F_0 – F) / F versus log [Q] at three different temperatures C_{Trp} and $C_{DMPHMPC}$ are the same as those in Figure 2.

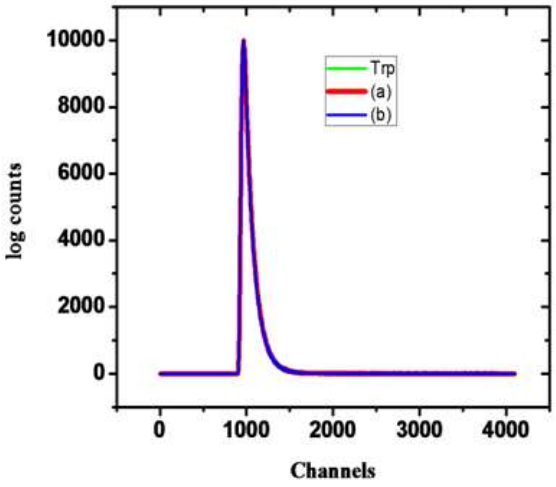

Figure 7. Time-resolved fluorescence spectra of Trp in presence and absence of DMPHMPC. C_{TRP} = 2x10^{-5} mol L^{-1} and $C_{DMPHMPC}$ /(10^{-4} mol L^{-1}) a-b: 3, 6.

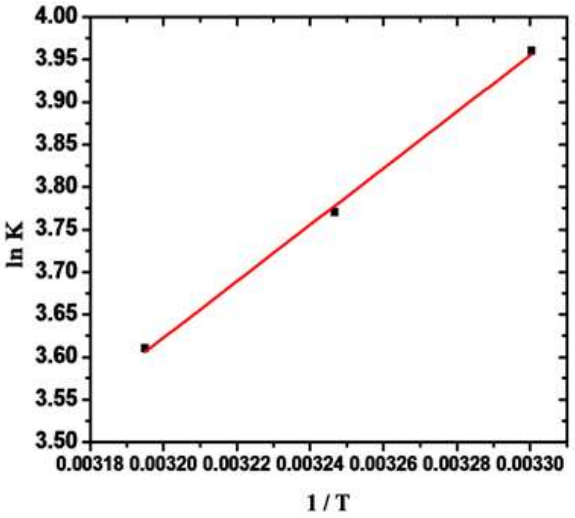

Figure 8. Van't Hoff plot for the binding of Trp to DMPHMPC.

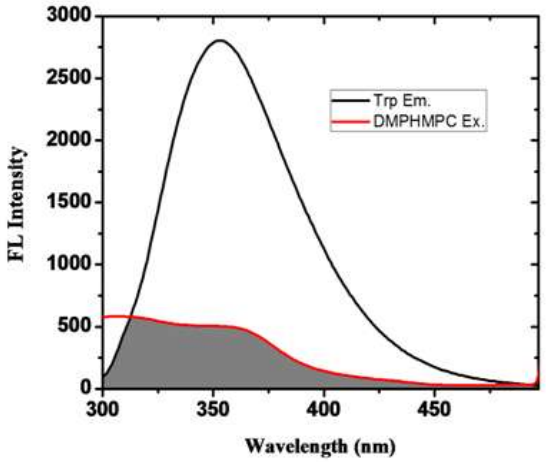

Figure 9. Spectral overlay of DMPHMPC excitation with Trp emission; T = 303 K, C_{Trp} = 2x10^{-5} mol L^{-1} and $C_{DMPHMPC}$ = 2 x 10^{-3} mol L^{-1}.

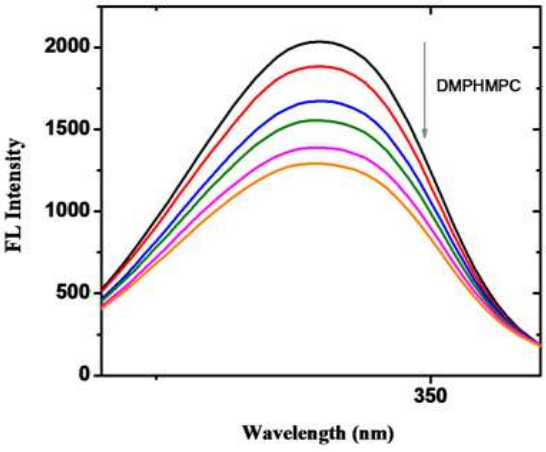

Figure 10. Synchronous fluorescence spectrum of Trp (T = 303 K), C_{Trp} and $C_{DMPHMPC}$ are the same as those in figure 2, $\Delta\lambda$ = 60 nm.