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Article

Revealing the Improved Binding Interaction of Plant Alkaloid Harmaline with Human Hemoglobin in Molecular Crowding Condition

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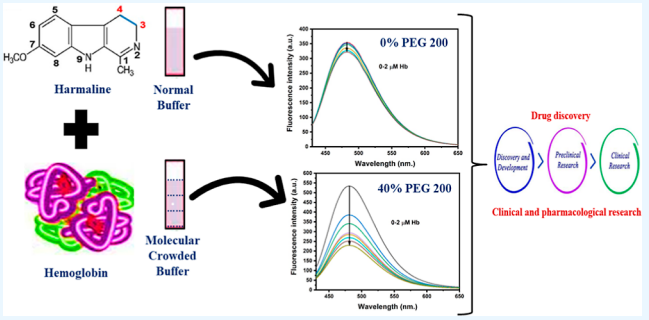
ABSTRACT: Harmaline and harmine are two structurally similar β-carboline alkaloids with several therapeutic activities, such as anti-inflammatory, antioxidant, neuroprotective, nephroprotective, antidiabetic, and antitumor activities. It has been previously reported that the interaction between harmaline and hemoglobin (Hb) is weak in buffer media compared to harmine. Crowding agents induce a molecular crowding environment in the *ex vivo* condition, which is almost similar to the intracellular environment. In this present study, we have investigated the nature of the interactions of harmaline and harmine with Hb by increasing the percentage of the crowding agent in buffer solution. The results of the UV−vis and fluorescence spectroscopy analysis have showed that with an increasing proportion of crowding agents, the interaction between harmaline and Hb is steadily improving in comparison to harmine. It has been found that the binding constant of Hb−harmaline reaches 6.82×10^5 M^{-1} in the 40% polyethylene glycol 200-mediated crowding condition, indicating high affinity compared to very low interaction in buffer media. Steady-state fluorescence anisotropy along with fluorescence lifetime measurements further revealed that the rotational movement of harmaline is maximally restricted by Hb in high crowding environments. Stoichiometry results represent that Hb and harmaline interacts in a 1:1 ratio in different percentages of the crowding agent. The circular dichroism spectroscopic results predict stronger interaction of harmaline with Hb (secondary structure alterations) in a higher crowding environment. From the melting study, it was found that the reactions between Hb and harmaline in crowding environments are endothermic ($\Delta H > 0$) and disordering ($\Delta S > 0$) in nature, indicating that hydrogen bonding and van der Waals interactions are the main interacting forces between Hb and harmaline. Harmaline molecules are more reactive in molecular crowding conditions than in normal buffer condition. This study represents that the interaction between harmaline and Hb is stronger compared to the structurally similar harmine in a molecular crowding environment, which may enlighten the drug discovery process in cell-mimicking conditions.



INTRODUCTION

Hemoglobin (Hb) is the major protein in the blood. Hb consists of a protein part, globin, and an iron-containing porphyrin ring, heme. Globin consists of two alpha (146 amino acids) and two beta (141 amino acids) chains.[1] A heme prosthetic group is present in every one of these single chains, and it is attached to the polypeptide sequence that is just next to the protein surface.[2] The main function of Hb is to carry oxygen in the vascular system of all vertebrates.[3] Besides that, it also helps in regulating the pH of the blood, transporting electrons throughout the body, and dispersing hydrogen peroxide.[4] Hb remains safe and inert, as it remains inside erythrocytes, but upon hemolysis, it becomes free. Hence, it might be expected that some of the free Hb gets exposed to some small molecules in the plasma, resulting in feasible interactions with them. Studies on the binding of Hb to specific compounds, including endogenous or exogenous molecules such as 2,3-bis phosphor glycerate,[5] flavonoids,[6] alkaloids,[7] and food colorants,[8] have been widely documented. The process of the binding of Hb with drugs and small peptides was also recently studied.[9,10] Therefore, the interaction between Hb and various small molecules can be possible with varying binding efficiencies. Harmine and harmaline (Figure 1) are the most representative naturally occurring β-carboline alkaloids and were first isolated from the *Peganum harmala* plant. *P. harmala*, a plant belonging to the Nitrariaceae family, is indigenous to Yemen, sections of Iran,

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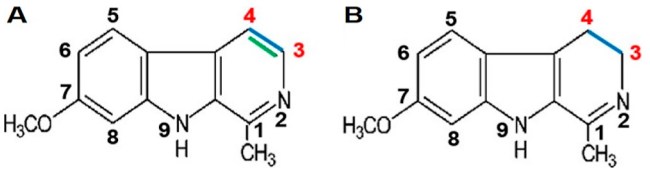

Figure 1. Structure of (A) harmine and (B) harmaline.

Manchuria, Mongolia, Asia, and the Middle East.[11] Harmine and harmaline possess a number of medicinal benefits, including antioxidant,[12] neuroprotective,[13] nephroprotective,[14] anticancer,[15] and anti-inflammatory effects.[16] The β-carboline alkaloids are interesting from both biophysical and pharmacological standpoints because of their many *in vitro* and *in vivo* actions, which are concealed by their simple structural configuration. The methyl group is located at C-1 and the methoxyl group is located at C-7 in the tricyclic plant alkaloids, harmine and harmaline. The existence of the C3═C4 double bond in harmine and partial aromaticity in harmaline is the only structural distinction between the two compounds (Figure 1).

Macromolecular crowding in biological systems is the general concentration of macromolecules inside the cells that take up a significant portion of the volume and are thus inaccessible to other molecules. Crowding is better described as the excluded volume effect, which shows that steric repulsion is the only physical cause of this nonspecific phenomenon.[17] Molecular crowding conditions are observed in all living organisms from lower protists to higher animals containing soluble and insoluble molecules, such as proteins, nucleic acids, saccharides, lipids, and metabolites. The total concentration of biomolecules was found to be 50−400 g L^{-1} inside a cell.[18] Hence, crowding agents occupy a significant fraction (20−40%) of the cellular volume, creating the crowded intracellular environment that has allowed biomolecules to evolve and operate over millions of years. All biochemical studies of biomolecules are conducted under diluted conditions. Besides that, various cellular environmental variables—like temperature, pressure, pH, ion species and concentrations, and redox potential—are precisely maintained in an experimental setting to mirror the physiological condition. Molecules interact and react with a large number of background molecules and ions in the cells. In addition to directly influencing biomolecular reactions through steric hindrance, coexisting components may also cause particular interactions with biomolecules.[19] Furthermore, intracellular water has physical characteristics different from those of a diluted aqueous solution. To assess the impact of macromolecular crowding on proteins and nucleic acids, crowding agents such as polyethylene glycol (PEG), dextran, poly(vinyl alcohol) or polyvinylpyrrolidone, and polysaccharides are frequently utilized.[18]

Understanding the effects of the intracellular environment is crucial for comprehending how biomolecules behave in cells. However, the molecular crowding effects on the properties of biomolecules are unclear. To understand how biomolecules behave when cell-mimicking circumstances are met, there is growing interest in the impact of molecular crowding on the stability and structure of biomolecules. The interaction between small molecules and proteins is influenced by the energy and distance scales of the forces that arise in intracellular condition.

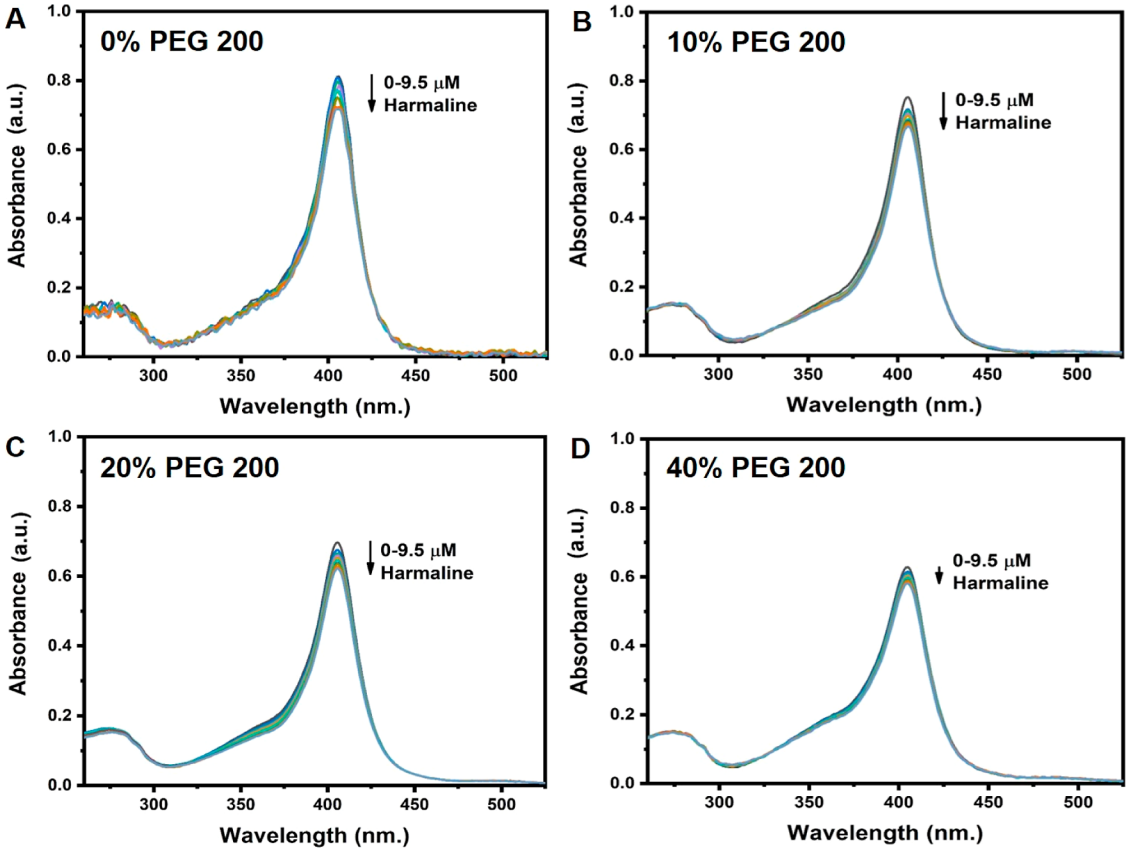

Figure 2. Absorption spectra of Hb (2.5 μM) in the absence and presence of successive additions of harmaline in different percentages of PEG 200-containing solutions. (A) 0 (control), (B) 10, (C) 20, and (D) 40%.

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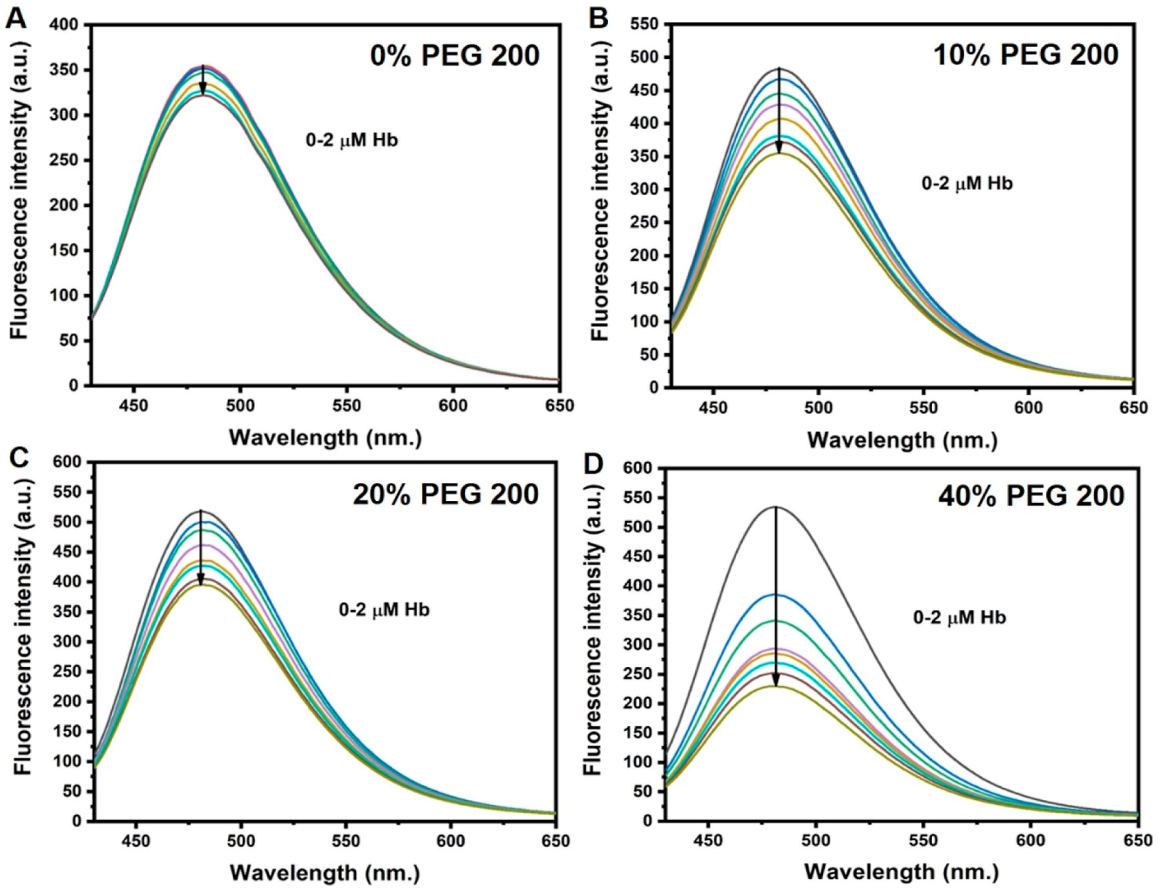

Figure 3. Fluorescence emission spectra of harmaline (1 μM) with increasing concentrations of Hb in different percentages of PEG-200 containing solutions. (A) 0 (control), (B) 10, (C) 20, and (D) 40%.

All living organisms performed their biological activities by interacting between the molecules. The most important physiological feature of Hb in an *in vivo* system is its ability to bind and carry many ligands for effective delivery to their intended sites. The understanding of the drug transport mechanism and the distinct behaviors of these alkaloids in biological systems could be aided by the study. To completely comprehend the manner of interaction in biological environments, it will become more crucial to research the interactions between small molecules and proteins in their native environments. Since only the free drug is pharmacologically active, the process by which medicines bind to Hb has significant pharmacokinetic and pharmacodynamic implications.

Molecular docking is a useful process to understand the mode of interaction between a ligand and a macromolecule through *in silico* processes.[20−22] Molecular simulation provides the effect of a ligand on the structure of a macromolecule over the time after their interaction.[23] According to previous study, we have found that Hb interacts with harmine and harmaline with similar binding pockets, and the interaction mode is also similar (interacts with similar amino acids through hydrogen bonding). Besides that, it was also revealed that the binding energy between Hb and harmaline (−6.39 kcal/mol) was high compared to harmine (−5.93 kcal/mol).[24]

In this work, we used various spectroscopic techniques, including UV−visible absorption studies, steady-state fluorescence studies, steady-state anisotropy studies, lifetime anisotropy, circular dichroism (CD) spectroscopy, and UV-melting studies to examine the nature of the interaction between harmaline and Hb as well as explored the impact of harmaline on Hb stability and conformation in molecular crowding environment. Here, in this investigation, we have used PEG as a crowding agent. Interestingly, we have found that in the molecular crowding environments that is in cell-mimicking conditions, harmaline interacts strongly with Hb as compared to harmine (earlier, our group reported that harmine interacts strongly with Hb in normal buffer conditions).[24] Besides that, the mode of interaction is also altered by a small structural difference (in a group) between ligands and/or interaction with different proteins.[25,26] So, a harmaline molecule is more reactive in the molecular crowding environment, whereas it is not that much reactive in normal buffer conditions.

RESULTS AND DISCUSSION

Impact of Alkaloids on the UV−Visible Spectra of Hb in Various Crowding Conditions. The Hb exhibits two characteristic peaks at 269 nm due to $\pi \rightarrow \pi^*$ transition of the aromatic amino acids and at 405 nm called the Soret band. The Soret band arises due to $\pi \rightarrow \pi^*$ electronic transition in the porphyrin ring of the heme moiety found in the hydrophobic pocket.[27] The absorption spectra of harmaline overlap with the absorption spectra of Hb. To understand the binding interaction between Hb and harmaline in a molecular crowding environment (PEG 200) using UV−vis spectroscopy, the spectrum of harmaline was subtracted from that of the Hb−harmaline system.[24] With the gradual addition of harmaline, the Soret band intensity displayed hypochromicity in different percentages of PEG 200 (Figure 2). Such a result suggests that the interaction

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Table 1. Fluorescence Decay Parameter of Harmaline (Harl) in Aqueous and Different Crowding Environments in the Absence and Presence of Hb

0% PEG 200-containing buffer							
samples	anisotropy (r)	I_{HH}	I_{HV}	I_{VH}	I_{VM}	I_{VV}	G factor
1 μM Harl	0.00318	1042590.00	1053340.00	800598.93	808760.93	817976.93	1.01031
1 μM Harl + 1 μM Hb	0.00357	1345850.00	1366580.00	1034170.00	1050410.00	1063920.00	1.0154
1 μM Harl + 2 μM Hb	0.00362	811651.67	815925.33	625599.33	630,831	633,493	1.00527
10% PEG 200-containing buffer							
samples	anisotropy (r)	I_{HH}	I_{HV}	I_{VH}	I_{VM}	I_{VV}	G factor
1 μM Harl	0.00835	1,950,530	2,248,200	1,705,390	1,813,390	2,018,870	1.15261
1 μM Harl + 1 μM Hb	0.00904	1,445,450	1,684,080	1,283,300	1,373,780	1,536,280	1.1651
1 μM Harl + 2 μM Hb	0.00918	1,676,100	1,928,000	1,476,730	1,572,420	1,747,100	1.15029
20% PEG 200-containing buffer							
samples	anisotropy (r)	I_{HH}	I_{HV}	I_{VH}	I_{VM}	I_{VV}	G factor
1 μM Harl	0.01106	1,948,340	2,235,890	1,711,590	1,816,280	2,025,920	1.14759
1 μM Harl + 1 μM Hb	0.0128	1,654,880	1,895,660	1,449,720	1,542,590	1,724,730	1.1455
1 μM Harl + 2 μM Hb	0.01232	1,518,380	1,742,500	1,334,130	1,422,610	1,588,820	1.1476
40% PEG 200-containing buffer							
samples	anisotropy (r)	I_{HH}	I_{HV}	I_{VH}	I_{VM}	I_{VV}	G factor
1 μM Harl	0.02166	2,065,990	2,305,160	1,777,510	1,887,340	2,127,160	1.11577
1 μM Harl + 1 μM Hb	0.0265	1,470,610	1,635,440	1,266,120	1,347,500	1,522,210	1.11208
1 μM Harl + 2 μM Hb	0.02661	1,188,190	1,320,180	1,021,730	1,087,520	1,225,420	1.11109

between Hb and harmaline in different crowding environments is stronger and maintained. The intensity of the Soret band of Hb (2.5 μM) was decreasing with increasing harmine concentration (0−9.5 μM) in different percentages of PEG 200 (Figure S1). This hypochromicity in the Soret band intensity of Hb by harmine is less compared to harmaline in different PEG 200 environments, which indicates that the interaction between harmine and Hb is reducing with increasing PEG 200 compared to harmaline. These data suggest that the interaction of harmaline with Hb in increasing crowding environments is maintained, whereas the interaction with harmine is decreased. Besides that, a slight red shift has been observed in the Hb absorption curves in the presence of PEG. These shifts in the absorption spectra could be the outcome of PEG-induced alterations in the polarity of the solution. The effect of such polarity change is also found in the α-helical structure of Hb which is reflected by the results of CD.[28]

Effect of Hb on Harmaline Fluorescence Property. Harmaline is a good fluorophore and exhibits emission maxima at 483 nm after excitation at 365 nm, which is far away from the fluorescence maxima of aromatic amino acids present in Hb. Fluorescence emission spectra of harmaline were monitored in the absence and presence of increasing concentration of Hb in different PEG 200 environments. The gradual addition of Hb induces quenching of the fluorescence property of harmaline (Figure 3). Fluorescence quenching refers to the reduction of the quantum yield of fluorescence from a fluorophore induced by a quencher molecule due to several reasons such as excited-state reaction, molecule rearrangement, energy transfer, ground-state complex formation, and collision quenching.[29] For fluorescence quenching, molecular contact between the fluorophore and quencher is necessary. Here, to correct the inner filter effect, the appropriate method is applied as mentioned in the experimental section.

The nature of quenching of the fluorescence of harmaline by Hb is static. This result implies that strong interaction between harmaline and Hb is possible in *in vivo* condition.[30,31] At the highest concentration of Hb, the quenching of the highest fluorescence intensity of harmaline was found to be ∼1.08-fold in the 0% PEG 200 (control group), ∼ 1.35-fold for 10% PEG 200, ∼ 1.31-fold for 20% PEG 200, and ∼1.82-fold for 40% PEG 200 condition.

From the modified Stern−Volmer equation, it has been found that the binding constant between Hb and harmaline was 2.86×10^5 M^{-1} in 10% crowding environment, 2.61×10^5 M^{-1} in 20% crowding environment, and 6.82×10^5 M^{-1} in 40% crowding environment (Figure S3). Thus, molecular crowding may induce some changes in the microenvironment around Hb, which enhances the interaction affinity of harmaline toward Hb. The other structurally similar molecule, harmine, also has good fluorescence property. It exhibits an emission maximum at 415 nm after exciting at 365 nm. In buffer condition, at the maximum concentration of Hb, the inner filter-corrected fluorescence spectrum shows around 1.22-fold quenching with a 12 nm blue shift from the only harmine spectra (Figure S2A). In the presence of Hb, the emission spectra of harmine shift blue, indicating a modification in the surrounding microenvironment as a result of binding in the proteinaceous environment as opposed to the free form in the aqueous phase. This nature of quenching has vanished, and blue shifting is reduced in the corrected fluorescence spectra of harmine by Hb as the PEG 200 is increased (Figure S2B,C). In the 40% PEG 200 environment, the quenching of the harmine fluorescence spectrum by Hb is so small; hence, the inner filter effect is not corrected in this data (Figure S2D). From these data, it is clear that the interaction between harmine and Hb is getting reduced as molecular crowding environment is increased. Based on UV−vis absorption and steady-state fluorescence experimentation results, it has been confirmed that harmaline interacts with Hb more preferentially than harmine does in various crowding conditions. Hence, the following experiments were performed to study the interaction between harmaline and Hb only in different crowding environments.

Study of the Interaction between Harmaline and Hb through Steady-State Fluorescence Anisotropy. Essential information about the orientation shift of a small molecule upon interaction with macromolecules can be obtained from steady-state fluorescence anisotropy. The anisotropy values of

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Table 2. Time-Resolved Fluorescence Decay Characteristics of Harmaline (Harl) in Aqueous Buffer and Other Crowding Conditions and in the Presence of Hb

0% PEG 200-containing buffer						
samples	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_{avg} (ns)	$\chi 2$
1 μM Harl	5.47234	1			5.47234	1.08666
1 μM Harl + 1 μM Hb	5.47604	1			5.47604	1.03727
1 μM Harl + 2 μM Hb	5.48491	1			5.48491	1.05325
10% PEG 200-containing buffer						
samples	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_{avg} (ns)	$\chi 2$
1 μM Harl	6.48871	1			6.48871	1.0317
1 μM Harl + 1 μM Hb	6.56707	1			6.56707	1.03527
1 μM Harl + 2 μM Hb	6.52155	1			6.52155	1.05594
20% PEG 200-containing buffer						
samples	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_{avg} (ns)	$\chi 2$
1 μM Harl	0.2667	0.0218	7.1097	0.9782	7.10398	1.00143
1 μM Harl + 1 μM Hb	0.27615	0.0225	7.16285	0.9775	7.15589	1.01327
1 μM Harl + 2 μM Hb	0.30295	0.0237	7.17944	0.9763	7.17239	1.00728
40% PEG 200-containing buffer						
samples	τ_1 (ns)	α_1	τ_2 (ns)	α_2	τ_{avg} (ns)	$\chi 2$
1 μM Harl	0.33133	0.0304	7.5318	0.9696	7.5218	1.00227
1 μM Harl + 1 μM Hb	0.36666	0.0324	7.62407	0.9676	7.6123	1.01817
1 μM Harl + 2 μM Hb	0.3438	0.032	7.59	0.968	7.57917	1.01094

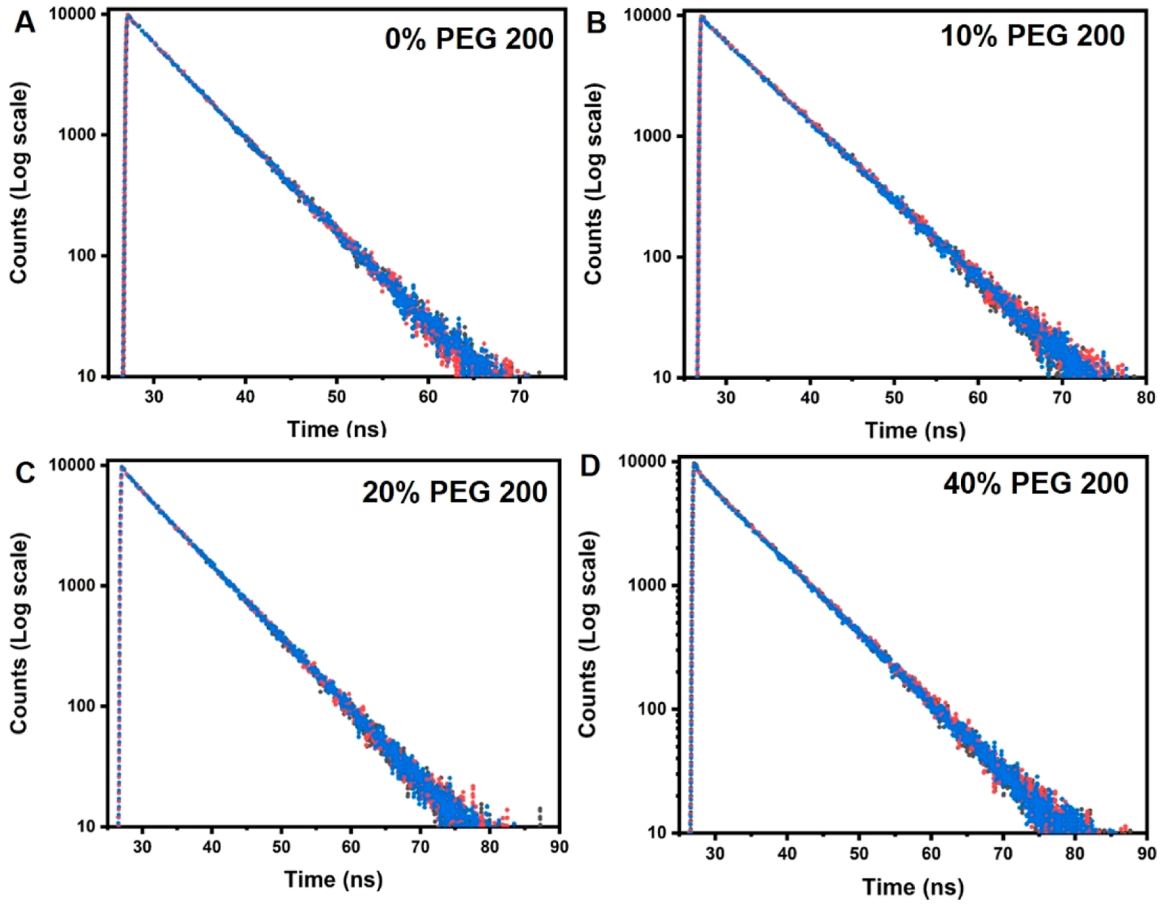

Figure 4. Fluorescence lifetime decay profiles of 1 μM harmaline in different percentages of PEG 200-containing buffers and in the presence of Hb. Black dot = only harmaline, red dot = 1 μM, and blue line = 2 μM in the presence of Hb. (A) 0 (control), (B) 10, (C) 20, and (D) 40%.

fluorophores are very low in a buffer where the fluorophore molecules can freely rotate and increase in motionally constrained environments. The gradual increase in the anisotropy value indicates an interaction between harmaline and Hb in different percentages of PEG 200. The high anisotropy value of the harmaline molecules indicates that these molecules are bound in a motionally restricted environment introduced by Hb in a molecular crowding condition (Table 1).

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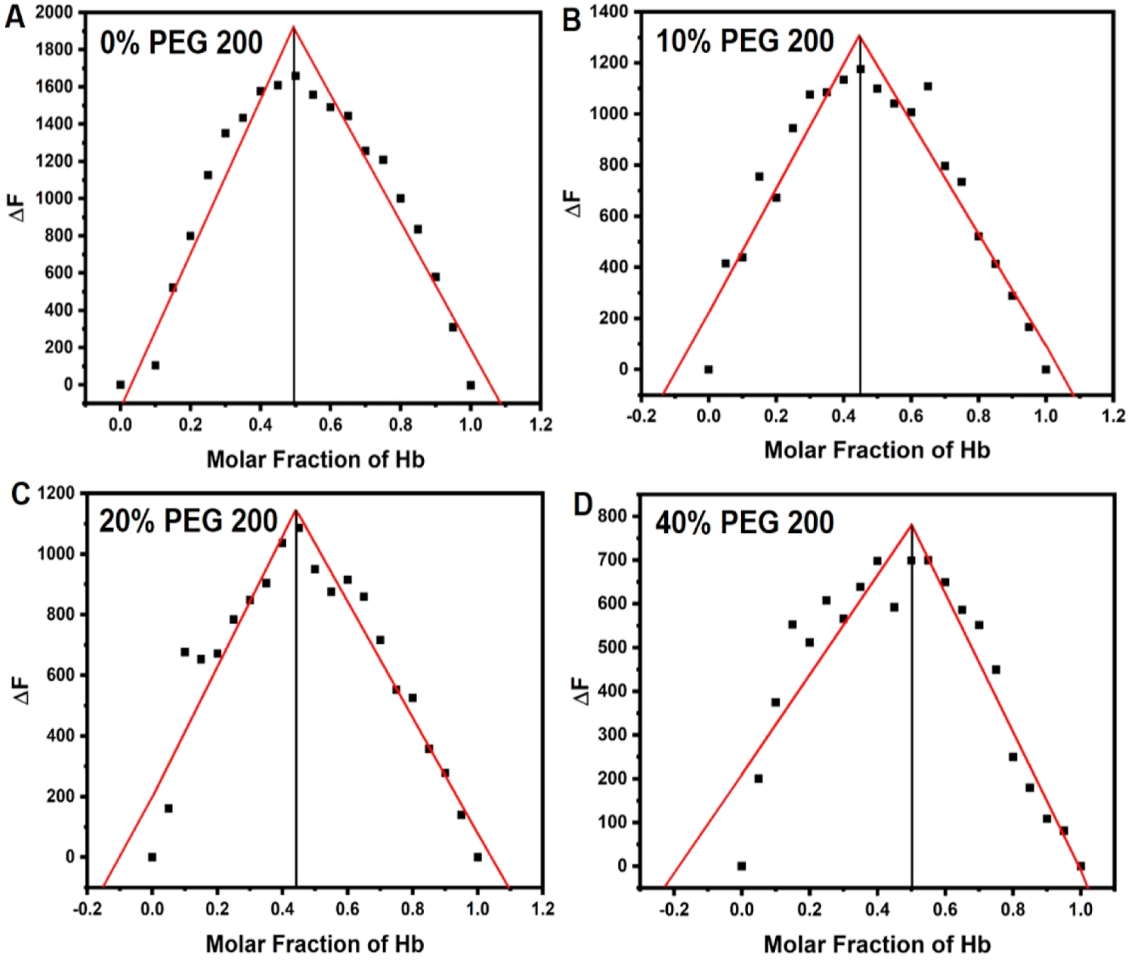

Figure 5. Job plot for the study of interaction between harmaline and Hb in different percentages of PEG 200-containing buffers. (A) 0 (control), (B) 10, (C) 20, and (D) 40%.

Effect of Hb on the Fluorescence Lifetime of Harmaline in Different Crowding Environments. In protein−ligand interaction, time-resolved excited-state lifetime investigation is a highly reliable method for distinguishing between static and dynamic quenching mechanisms. Static quenching is represented by steady average fluorescence lifetime values; in contrast, dynamic quenching causes considerable lifetime value variations as a result of complexation and/or interaction. The fluorescence lifetime of harmaline is 5.4 ns in the buffer, which does not change significantly in different concentrations of Hb indicating that the nature of the quenching is static[32] (Table 2). In the presence of 20 and 40% PEG 200, the lifetime of harmaline turned into two exponentials, but no such significant alteration in the fluorescence lifetime of harmaline was found by Hb (Figure 4). Prompt of the studies are mentioned in Figure S4.

Stoichiometry of Interaction between Hb and Harmaline in Different Crowding Environments. The stoichiometry of harmaline−Hb in different percentages of PEG 200 was performed to evaluate it through the continuous variation process (Job's plot analysis). In this method, the molar fraction of Hb and harmaline was altered, while the total concentration of Hb and harmaline remained constant. Then, the difference in the fluorescence intensity ($\Delta F = F_{\text{harmaline}} - F_{\text{harmaline+Hb}}$) was plotted against the molar fraction of Hb. In the job plot, the maximum fluorescence intensity of harmaline and the harmaline−Hb complex at 483 nm upon excitation at 365 nm was considered. The results indicate that the stoichiometry between harmaline and Hb in different percentages of PEG 200 was remaining 1:1, indicating a stable interaction (Figure 5).

Impact of Harmaline on the Hb Secondary Structure in Various Crowding Conditions. CD is the difference in absorption of right and left circularly polarized light by a chiral compound. It is linked to a transition electric and magnetic dipole moment, which produces a helical charge motion. This motion can discriminate between left and right circularly polarized light, with a positive CD resulting from a right-handed transition and vice versa.

CD has been performed to explore the changes in the secondary conformation in Hb at different percentages of PEG 200 and their interaction with harmaline. In the far UV region of the Hb CD spectra, two negative peaks were found, one at around 208 nm and the other at 222 nm. The former one appears due to amide n → π^* transition and the other for $\pi \rightarrow \pi^*$ transition.[33] The α-helicity of Hb remains almost unaltered by harmaline in the buffer. A decrease in the ellipticity of all wavelengths without significantly affecting the peak displacements indicates that the secondary structure of Hb is predominantly α-helix.[34]

The α-helicity of Hb in the presence of harmaline was increased in 10% of PEG 200, while in 20 and 40% of the PEG 200 buffer, the α-helicity of Hb was decreased. These results indicated that in 0% PEG 200, harmaline interacts with Hb without altering the secondary structure of Hb 28.1 to 28.3%. In

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Table 3. Secondary Structure of Hb Altered by Harmaline (Harl) in the Absence and Presence of Different Percentages of PEG 200

percentage of the crowding agent				
		α-Helix (%)		
	3 μM Hb	3 μM Hb + 3 μM Harl	3 μM Hb + 6 μM Harl	3 μM Hb + 12 μM Harl
0% PEG 200	28.1	28.22	28.26	28.3
10% PEG 200	31	31.1	31.1	32.6
20% PEG 200	33	32.68	32.74	32
40% PEG 200	35.6	34.1	33.87	28
		Antiparallel β-Sheet (%)		
	3 μM Hb	3 μM Hb + 3 μM Harl	3 μM Hb + 6 μM Harl	3 μM Hb + 12 μM Harl
0% PEG 200	15.83	15.76	15.64	15.6
10% PEG 200	18.8	17.28	17.28	17.2
20% PEG 200	18.4	18.8	18.89	18.97
40% PEG 200	15.22	17.94	18	21
		Parallel β-Sheet (%)		
	3 μM Hb	3 μM Hb + 3 μM Harl	3 μM Hb + 6 μM Harl	3 μM Hb + 12 μM Harl
0% PEG 200	14.44	14.56	14.62	14.7
10% PEG 200	15.2	15.4	15.4	15.62
20% PEG 200	16	15.64	15.72	15.54
40% PEG 200	17.4	16.2	15.92	14.36
		β-Turn (%)		
	3 μM Hb	3 μM Hb + 3 μM Harl	3 μM Hb + 6 μM Harl	3 μM Hb + 12 μM Harl
0% PEG 200	12.88	13.24	13.78	14.1
10% PEG 200	15.21	15.24	15.24	15.32
20% PEG 200	15.44	15.36	15.38	15.29
40% PEG 200	15.82	15.76	15.68	12.8
		Random Coil (%)		
	3 μM Hb	3 μM Hb + 3 μM Harl	3 μM Hb + 6 μM Harl	3 μM Hb + 12 μM Harl
0% PEG 200	27.28	27.22	27.17	27.1
10% PEG 200	25	25.14	25.14	23.11
20% PEG 200	22.78	23.08	23.02	23.42
40% PEG 200	21.14	21.87	22.62	27.34

10% PEG 200-containing buffer, the percentage helicity of Hb increases from 31 to 32.6%, which indicates the development of a new H bond during the interaction between Hb and harmaline. Harmaline decreases the α-helicity of Hb from 33 to 32% in 20% PEG-containing buffer and from 35.6 to 28% in 40% PEG 200-containing buffer (Table 3) (Figure 6). This CD data revealed that the interaction between harmaline with Hb leads to the unfolding of the Hb protein by destruction of the H bond of α-helix.[35] Besides that, an increase in the content of α-helix induces a decrease in antiparallel β-sheet and random coil structures and vice versa. Such transition in conformation may be a probable result of an alteration in the hydrogen bonding and the hydrophobic cavities. In this way, harmaline affects the chirality of Hb in different crowding environments.[36]

Effects of Harmaline on Hb Stability in Different Crowding Environments. The effect of harmaline on the melting temperature of Hb is studied through this melting study in different percentages of crowding environments. Harmaline reduces the melting temperature of Hb from 62.5 to 60.36 °C in buffer media. The effect of harmaline on the stability of Hb remains almost unaltered in different percentages of crowding environments (Figure S5) (Table 4).

For the harmaline−Hb complex, the enthalpy change (ΔH) and the entropy (ΔS) values are positive in different crowding conditions, which indicates that the interaction between Hb and harmaline is chiefly for hydrophobic interactions. These results also indicate that the thermal denaturation of Hb in the presence of harmaline is endothermic ($\Delta H > 0$) and disordering ($\Delta S > 0$) in nature. The endothermic nature of the melting of the Hb and Hb−harmaline complex in different crowding conditions indicates that endothermic conditions occur as a result of the intramolecular and/or intermolecular hydrogen bonding, van der Waals interactions, and hydration effects. The positive ΔS values denote the prevalence of the conformational contribution over the contribution due to the hydrophobic hydration of the unfolded protein.[37] Hydrophobic interactions play an important role in stabilizing and preventing the aggregation of Hb.[38]

CONCLUSIONS

Natural products have been considered as a resource of therapeutic drugs, thus helping to synthesize new pharmaceutical products. The interaction between different small biomolecules and Hb has been previously evaluated through different spectroscopic studies. There are several health benefits associated with the plant alkaloids, harmaline and harmine, including anticancer, antibacterial, anti-inflammatory, and antioxidant properties. Generally, the interaction between different small molecules and Hb has been studied in buffer media. To understand the interaction between Hb and the two structurally similar alkaloids, harmaline and harmine, in the *in vivo* mimicking system, a molecular crowding medium is created using a crowding agent, PEG 200.

In this study, the nature of the interaction between harmaline and harmine with Hb was studied using different percentages of PEG 200 to understand the effect of molecular crowding on their interaction. The results represent that the interaction between harmaline and Hb increased with an increasing molecular crowding environment. Absorbance spectra and

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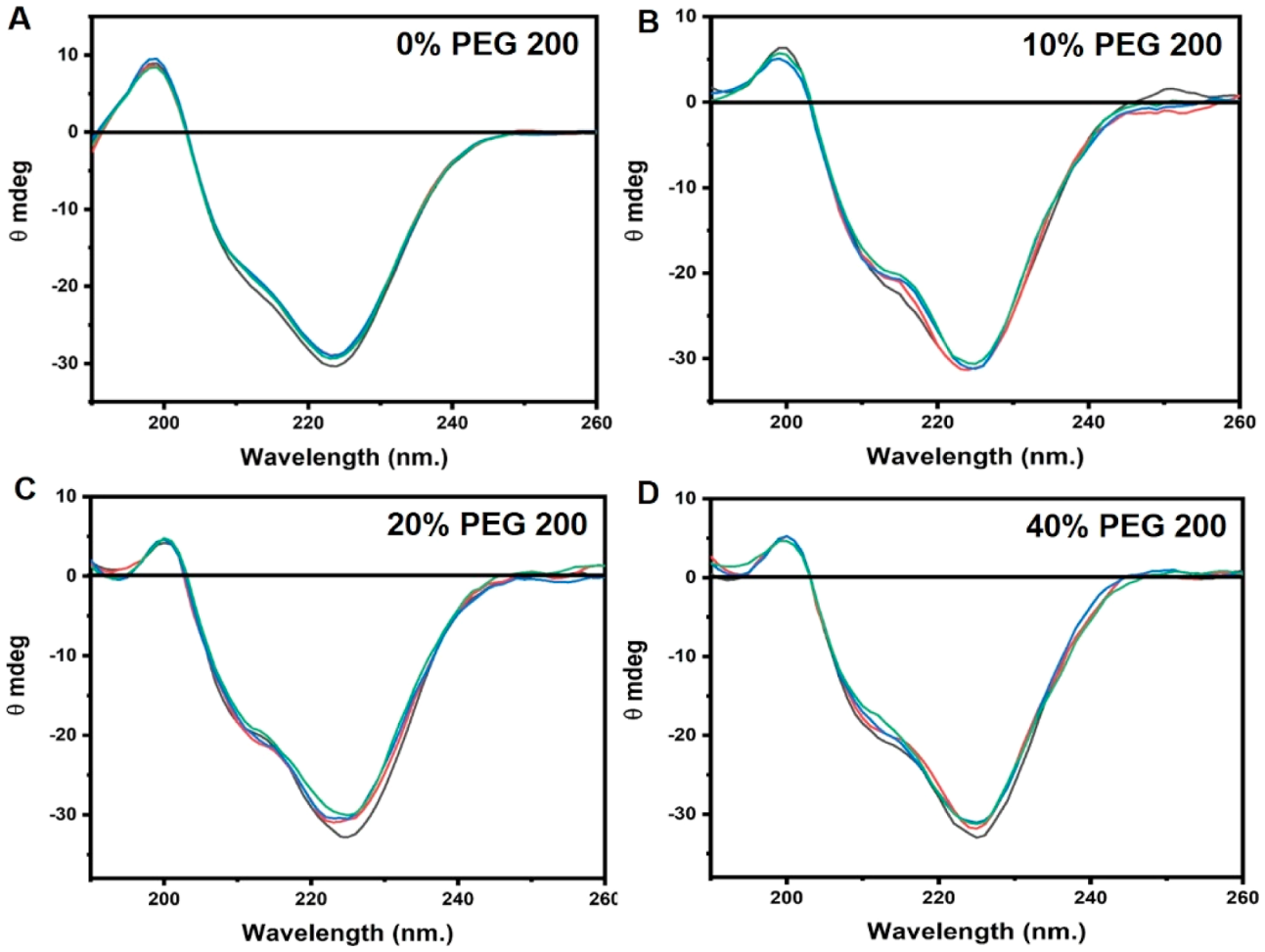

Figure 6. CD spectra of Hb in the absence and presence of 3, 6, and 12 μM harmaline in different percentages of PEG 200-containing buffers. Black line = Hb, red line = 3 μM, blue line = 6 μM, and green line = 12 μM of harmaline in the presence of Hb. (A) 0 (control), (B) 10, (C) 20, and (D) 40%.

Table 4. Effect of Harmaline (Harl) in the Melting Temperature, Enthalpy Change (ΔH), and Entropy Change (ΔS) of Hb in Different Percentages of PEG 200

percentage of PEG 200 (%)	samples	T_m(°C)	ΔH (kJ/mol)	ΔS (kJ/mol·K)
0	Hb 5 μM	61.84	12.976	0.03875
	Hb 5 μM + Harl 5 μM	60.34	12.718	0.03815
10	Hb 5 μM	62.5	12.953	0.03861
	Hb 5 μM + Harl 5 μM	61	12.975	0.03884
20	Hb 5 μM	62.5	12.717	0.03791
	Hb 5 μM + Harl 5 μM	61	12.664	0.03791
40	Hb 5 μM	62.87	12.320	0.03668
	Hb 5 μM + Harl 5 μM	59.03	11.717	0.03529

fluorescence quenching evaluations have confirmed a strong interaction between Hb and harmaline compared to harmine in high crowding environments. Increased hypochromicity of the Soret band by harmaline in different crowding environments compared with harmine indicated that the interaction between Hb and harmaline was enhanced in the highly crowded environment. The close vicinity of the interaction between harmaline and Hb has been well understood through the use of fluorescence techniques. Fluorescence emission data have demonstrated that the binding of harmaline to Hb was caused by the formation of the Hb−harmaline complex compared with harmine in highly crowded environments. From this study, it was found that with increasing crowding in the environment, the interaction between harmaline and Hb is increasing as the binding constant of the interaction between harmaline and Hb is raised gradually. These results indicate that the binding process between harmaline and Hb is possible in *in vivo* condition. Steady-state anisotropy study indicates the interaction between Hb and harmaline due to the increased anisotropy values of harmaline with the addition of Hb in different crowding environments. Time-resolved fluorescence measurements indicate that static quenching appears to be the predominant cause of this reaction process. The stoichiometry results indicated that 1:1 complexation happens between Hb and harmaline, irrespective of the crowding agent percentages. The far-UV CD spectral profiles of Hb indicate the minimum alteration of the secondary structure of Hb with increasing concentrations of harmaline in crowding environments, and the effect of harmaline on the functionality of Hb should also be minimal. UV melting study indicates that the effect of harmaline on the stability of Hb remains unaltered in different crowding environments.

This study provides useful information to compare the interaction of harmaline and harmine with Hb in molecularly crowded environments. These findings indicate the essentiality of considering the effect of intercellular conditions on the

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function and activity of drugs for a rational design. Generally, the interactions between protein and small molecules are studied in normal buffer solution. To understand the nature of the interaction between small molecules and biomolecules, *in vivo* condition is required to understand their actual mode of interaction. It is difficult to mimic the *in vivo* condition in *in vitro* studies. Hence, PEG 200, the inert crowding agent molecule, was used for making the molecular crowding environment in an *in vitro* condition. Usually in molecular crowding environment, the interaction of the ligand is less with biomolecules. But, remarkably, we have found that harmaline interacts strongly with Hb in a molecular crowding environment, which is important for minute understanding in the drug discovery process. From this study, we have also emphasized that the structurally similar molecule, harmine, is not behaving the same (interaction with Hb) in the molecular crowding environment as compared to normal buffer solution. Only C3═C4 bond reduction can change the entire scenario. So, from the molecular point of view, it is also very important. The information gathered here will be crucial for subsequent attempts to rationally design other functional molecules in living cells, in addition to protein−ligand interactions.

Harmaline is a valuable phytoconstituent for food and research, and its versatile use as a traditional medication has given rise to various industrial uses. Hence, this investigation will contribute to the understanding of the structural basis of screening and synthesizing suitable natural compounds, which is crucial to the progress of pharmacological and clinical research. This study also highlights the potential for the use of harmaline in innovative therapeutic medications and sets out the path for further research on the application of plant alkaloids.

MATERIALS AND METHODS

Materials. Lyophilized powders of human hemoglobin, harmine (M.W. 212.25), harmaline (M.W. 214.26), and PEG 200 solution were brought from Sigma-Aldrich. Harmine and harmaline were dissolved in ethanol. The Hb concentration was determined by utilizing spectrophotometric absorbance at 276 nm with an extinction coefficient of 120,808 M^{-1} cm^{-1}.[24,39] The molecular weight of PEG 200 was between 190 and 200 and in this study, we used 0.113 (10%), 0.226 (20%), and 0.452 (40%) g/l of PEG200. Each of the measurements was conducted with a buffer containing 50 mM KCl, 10 mM KH_2PO_4, and 1 mM K_2EDTA (pH 7.4), at room temperature (25 °C). In every experiment, the ethanol concentration was maintained at less than 1% (by volume).

Methods. *UV−Vis Spectroscopy.* The absorption spectra of Hb in the absence and presence of harmine and harmaline in different percentages of PEG 200 were recorded on a Hitachi UH5300 absorption spectrophotometer using a 1 cm cuvette. The spectra were obtained at a data interval of 1 nm and a scanning speed of 400 nm min^{-1}. Spectrophotometric titrations were performed by maintaining the concentration of Hb constant at 2.5 μM and harmine and harmaline have been added gradually to Hb over a concentration range of 0−9.5 μM.

Fluorescence Spectroscopy. The fluorescence emission spectra were recorded by using a BIOBASE BK-F96PRO through a quartz cuvette with a path length of 1 cm. Steady-state fluorescence studies have been performed on the excitation of harmine and harmaline at 365 nm, and the emission wavelength was altered according to the regions of interest. Both excitation and emission band passes were fixed to 10 nm. Emission spectra were recorded with a scanning speed of 300 nm/min and a response time of 0.5 s. The concentrations of harmine and harmaline were kept at 1 μM and Hb concentration was varied from 0 to 2 μM in the buffer containing different percentages of PEG 200. The absorption of light at excitation and emission wavelengths by the compound(s) present in the medium is termed the inner filter effect. The absorbance of a substance greater than 0.1 in the medium induces the inner filter effect. Therefore, the fluorescence intensity of harmine and harmaline was corrected using the following equation[40]

$$F_{cor} = F_{obs} \times 10^{(A_{ex}+A_{em})/2}$$

where F_{cor} is the corrected fluorescence intensity and F_{obs} is the measured fluorescence intensity. A_{ex} and A_{em} are absorbance values at the excitation and emission wavelengths, respectively. The corrected emission spectra were employed to understand the nature of the interaction between harmaline with Hb. To determine the binding constant from the corrected fluorescence spectrum in the process of static quenching, a modified Stern−Volmer equation was employed

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_b + n \log C_q$$

where F_0 means the fluorescence of alkaloids in the absence of Hb and F denotes alkaloid fluorescence in the presence of Hb. K_b is the binding constant between Hb and alkaloids and C_q is the concentration of Hb in the media.

Steady-State Fluorescence Anisotropy. Steady-state fluorescence anisotropy was measured by using a Fluorolog spectrometer (HORIBA Scientific) with polarizer facilities. In this study, harmaline was excited at 365 nm. The experiment was conducted in a 1 cm quartz cuvette. In this study, the concentration of used harmaline was 1 μM and the concentration of Hb was varied from 0 to 2 μM. The excitation and emission bandwidths for anisotropy measurements were both 5 nm. The fluorescence anisotropy (r) values were calculated using the following equation

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

$$G = \frac{I_{HV}}{I_{HH}}$$

where G denotes the sensitivity factor of the detection system. I_{VV}, I_{VH}, I_{HV}, and I_{HH} are fluorescence signals for excitation and emission with the polarizer set at positions (0, 0°), (0, 90°), (90, 0°), and (90, 90°), respectively.

Time-Resolved Fluorescence Measurements. Time-resolved fluorescence decays were recorded on a Fluorolog spectrometer (HORIBA Scientific) utilizing the time-correlated single-photon counting (TCSPC) technique using a 373 nm laser diode as the light source to activate the fluorescence of harmaline. The study was performed as a steady-state anisotropy. In this study, the concentration of harmaline was maintained at 1 μM and the concentration of Hb was gradually increased from 0 to 2 μM. The emission decay data at 483 was analyzed using the DAS-6 (Decay Analysis Software 6) software that came with the TCSPC instrument and was fitted according to the decay function

$$F(t) = \sum_i \alpha_i \exp\left(-\frac{t}{\tau_i}\right)$$

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where $F(t)$ is the fluorescence intensity at time t, and α_i and τ_i are the pre-exponential factor and decay time, respectively, corresponding to the ith decay. The goodness of the fits was evaluated by $\chi 2$ criteria and visual inspection of the residuals of the fitted function to the data. The mean (average) fluorescence lifetimes were estimated using the equation below

$$\tau_{avr} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i}$$

Stoichiometry. The stoichiometry of harmaline with Hb interaction was investigated using the continuous variation method (Job's plot analysis).[41] In this experiment, the overall concentration (C_{Hb} + $C_{harmaline}$) was held constant, while the molar fractions of Hb and harmaline were changed continuously in the buffers containing different percentages of PEG 200. The fluorescence intensity change was calculated ($\Delta F = F_{harmaline} - F_{harmaline+Hb}$) and plotted against the Hb molar fraction. The Job plot was performed considering the maximum fluorescence intensity after exciting at 365 nm.

Circular Dichroism. The CD spectroscopy of Hb in the absence and presence of harmaline in different percentages of PEG 200 buffers were performed in a Jasco J-1500 CD spectrometer with a scan speed of 100 nm/s. In this study, a quartz cuvette with a path length of 1 mm was used. Hb concentration was maintained in this study at 3 μM, while the concentration of harmaline was varied in 1:1, 1:2, and 1:4 ratios. The buffer solution spectrum was subtracted from each spectrum, as it was considered blank. Each CD spectrum of this experiment represents averaged values of three successive measurements in which the scan range was from 190 to 260 nm with an interval of 1 nm. BeStSel (Beta Structure Selection) is a unique method through which secondary structural alteration can be determined from CD spectra of a protein (https://bestsel.elte.hu/index.php).[42]

Melting Study. Thermal denaturation experiments were performed in a Hitachi UH5300 absorption spectrophotometer with a Peltier temperature controller by using a 1 cm path length quartz cuvette. In this study, the effect of harmaline over 5 μM of Hb in a 1:1 ratio was evaluated in the buffers containing different percentages of PEG 200. The absorbance of 5 μM Hb at 280 nm was continuously detected from 30 to 96 °C at a rate of 1 °C/min. The melting temperature was analyzed from the melting curve. The melting of harmaline was also performed to understand the profile as subtraction of this melting profile from the melting profile of the Hb−harmaline complex provides minimal absorbance of harmaline.[24]

Heat denaturates a protein by breaking down its actual conformation. The absorption of a protein is controlled by its surrounding microenvironment. Heat-mediated equilibrium unfolding of Hb can be represented based on a two-state model. The effect of harmaline on the thermal stability of Hb was measured using UV−vis absorption spectrophotometry.[43]

Alteration in the absorbance of Hb at 280 nm with a gradual increase of temperature provides a sigmoidal curve of Hb melting. The fraction of a thermally denatured protein, f_d, at a given temperature can be obtained through following expression

$$f_d = \frac{A - A_N}{A_D - A_N}$$

where A denotes absorbance at a specific temperature, and A_N and A_D indicate absorption values at the native and denaturated state. The apparent equilibrium constant (k) can be determined from the following equation

$$k = \frac{f_d}{1 - f_d} = \frac{A - A_N}{A_D - A_N}$$

The standard Gibbs free energy of denaturation, ΔG, can be estimated using the following equation

$$\Delta G = -RT \ln k$$

Here, R is the gas constant. The corresponding apparent standard enthalpy of denaturation, ΔH, can be evaluated through the following equation

$$\Delta H = 4RT_d^2 \frac{1}{\Delta T}$$

where T_d is the temperature of the half-transition. T_d can be defined as the temperature at which half of the protein molecules are in the native state and half are in the denatured state. ΔT is the interval of temperature in which the two-state approximation of the thermal unfolding is completed.

The entropy value of denaturation (ΔS) can be evaluated from the following equation

$$\Delta S = \frac{\Delta H}{T_d}$$

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02766.

Absorption spectra of Hb; fluorescence emission spectra of harmine; modified Stern−Volmer plot for fluorescence quenching of harmaline by Hb; time-resolved decay curves of prompt of Hb and harmaline interaction study; and UV melting profiles of Hb alone and in the presence of harmaline (PDF)

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Conceptualization: M.D.B. and S. Bhowmik. Experimental design and methodology: M.D.B. and S. Bhowmik. Investigation: M.D.B., S. Bag, S. Ghosal, and M.M. Data acquisition and analysis: M.D.B., S. Bag., S. Ghosal, M.M., G.P., and S. Bhowmik. Manuscript writing: All authors have contributed to write the manuscript. Supervision and funding: S. Bhowmik. The final article was revised and approved by all authors.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Wang, Y.-Q.; Zhang, H.-M.; Zhang, G.-C.; Liu, S.-X.; Zhou, Q.-H.; Fei, Z.-H.; Liu, Z.-T. Studies of the Interaction between Paraquat and Bovine Hemoglobin. *Int. J. Biol. Macromol.* **2007**, *41* (3), 243−250.

(2) Vigneswaran, C.; Ananthasubramanian, M.; Kandhavadivu, P. *Enzyme Technology*; Cambridge University Press: Cambridge, 2014.

(3) Sharma, M.; Hashmi, M. A.; Mahmood, R. Investigating the Binding of Caffeic Acid to Human Hemoglobin Using Multi-spectroscopic and Molecular Docking Approaches. *J. Mol. Struct.* **2024**, *1302*, 137519.

(4) Kaur, L.; Singh, A.; Datta, A.; Ojha, H. Multispectroscopic Studies of Binding Interaction of Phosmet with Bovine Hemoglobin. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2023**, *296*, 122630.

(5) Levitzky, G. M. *Pulmonary Physiology*, 7th ed.; McGraw Hill, 2007.

(6) Yuan, J.-L.; Liu, H.; Kang, X.; Lv, Z.; Zou, G.-L. Characteristics of the Isomeric Flavonoids Apigenin and Genistein Binding to Hemoglobin by Spectroscopic Methods. *J. Mol. Struct.* **2008**, *891* (1−3), 333−339.

(7) Das, A.; Suresh Kumar, G. Binding of the alkaloid aristololactam-β-D-glucoside and daunomycin to human hemoglobin: spectroscopy and calorimetry studies. *J. Biomol. Struct. Dyn.* **2016**, *34* (4), 800−813.

(8) Basu, A.; Kumar, G. S. Interaction of the Dietary Pigment Curcumin with Hemoglobin: Energetics of the Complexation. *Food Funct.* **2014**, *5* (8), 1949−1955.

(9) Sanei, H.; Asoodeh, A.; Hamedakbari-Tusi, S.; Chamani, J. Multi-Spectroscopic Investigations of Aspirin and Colchicine Interactions with Human Hemoglobin: Binary and Ternary Systems. *J. Solution Chem.* **2011**, *40* (11), 1905−1931.

(10) Sadeghzadeh, F.; Entezari, A. A.; Behzadian, K.; Habibi, K.; Amiri-Tehranizadeh, Z.; Asoodeh, A.; Saberi, M. R.; Chamani, J. Characterizing the Binding of Angiotensin Converting Enzyme I Inhibitory Peptide to Human Hemoglobin: Influence of Electromagnetic Fields. *Protein Pept. Lett.* **2020**, *27*, 1007−1021.

(11) Kartal, M.; Altun, M. L.; Kurucu, S. HPLC Method for the Analysis of Harmol, Harmalol, Harmine and Harmaline in the Seeds of Peganum Harmala L. *J. Pharm. Biomed. Anal.* **2003**, *31* (2), 263−269.

(12) Li, S. P.; Wang, Y. W.; Qi, S. L.; Zhang, Y. P.; Deng, G.; Ding, W. Z.; Ma, C.; Lin, Q. Y.; GuanLiu, H. W.; Cheng, X. M.; Wang, C. H. Analogous β-Carboline Alkaloids Harmaline and Harmine Ameliorate Scopolamine-Induced Cognition Dysfunction by Attenuating Acetylcholinesterase Activity, Oxidative Stress, and Inflammation in Mice. *Front. Pharmacol* **2018**, *9*, 346.

(13) Dakic, V.; Maciel, R. d. M.; Drummond, H.; Nascimento, J. M.; Trindade, P.; Rehen, S. K. Harmine Stimulates Proliferation of Human Neural Progenitors. *PeerJ* **2016**, *4* (12), No. e2727.

(14) Salahshoor, M. R.; Roshankhah, S.; Motavalian, V.; Jalili, C. Effect of Harmine on Nicotine - Induced Kidney Dysfunction in Male Mice. *Int. J. Prev Med.* **2019**, *10*, 97.

(15) Hamsa, T. P.; Kuttan, G. Harmine Activates Intrinsic and Extrinsic Pathways of Apoptosis in B16F-10 Melanoma. *Chin. Med.* **2011**, *6*, 11.

(16) Liu, X.; Li, M.; Tan, S.; Wang, C.; Fan, S.; Huang, C. Harmine Is an Inflammatory Inhibitor through the Suppression of NF-KB Signaling. *Biochem. Biophys. Res. Commun.* **2017**, *489* (3), 332−338.

(17) Model, M. A.; Hollembeak, J. E.; Kurokawa, M. Macromolecular Crowding: A Hidden Link between Cell Volume and Everything Else. *Cell. Physiol. Biochem.* **2021**, *55* (S1), 25−40.

(18) Chebotareva, N. A.; Kurganov, B. I.; Livanova, N. B. Biochemical Effects of Molecular Crowding. *Biochem.* **2004**, *69* (11), 1239−1251.

(19) Christiansen, A.; Wang, Q.; Cheung, M. S.; Wittung-Stafshede, P. Effects of Macromolecular Crowding Agents on Protein Folding in Vitro and in Silico. *Biophys. Rev.* **2013**, *5* (2), 137−145.

(20) Murugan Sreedevi, S.; Vinod, S. M.; Krishnan, A.; Perumal, T.; Ali Alasmary, F.; Salem Alsaiari, N.; Govindasamy, M.; Rajendran, K. Molecular Docking Approach on the Effect of Site- Selective and Site-Specific Drugs on the Molecular Interactions of Human Serum Albumin (HSA) - Acridinedione Dye Complex. *Arab. J. Chem.* **2023**, *16* (5), 104701.

(21) Vaidyanathan, R.; Murugan Sreedevi, S.; Ravichandran, K.; Vinod, S. M.; Hari Krishnan, Y.; Babu, L. K.; Parthiban, P. S.; Basker, L.; Perumal, T.; Rajaraman, V.; Arumugam, G.; Rajendran, K.; Mahalingam, V. Molecular Docking Approach on the Binding Stability of Derivatives of Phenolic Acids (DPAs) with Human Serum Albumin (HSA): Hydrogen-Bonding versus Hydrophobic Interactions or Combined Influences ? CA. *JCIS Open* **2023**, *12* (July), 100096.

(22) Vinod, S. M.; Murugan Sreedevi, S.; Krishnan, A.; Ravichandran, K.; Karthikeyan, P.; Kotteswaran, B.; Rajendran, K. Complexity of the Role of Various Site-Specific and Selective Sudlow Binding Site Drugs in the Energetics and Stability of the Acridinedione Dye - Bovine Serum Albumin Complex: A Molecular Docking Approach. *ACS Omega* **2023**, *8*, 5634−5654.

(23) Hu, X.; Zeng, Z.; Zhang, J.; Wu, D.; Li, H.; Geng, F. Molecular Dynamics Simulation of the Interaction of Food Proteins with Small Molecules. *Food Chem.* **2023**, *405*, 134824.

(24) Burman, M. D.; Bag, S.; Ghosal, S.; Karmakar, S.; Pramanik, G.; Chinnadurai, R. K.; Bhowmik, S. Exploring the Structural Importance of the C3=C4 Double Bond in Plant Alkaloids Harmine and Harmaline on Their Binding Interactions with Hemoglobin. *ACS Omega* **2023**, *8* (40), 37054−37064.

(25) Li, M.; Zhou, D.; Wu, D.; Hu, X.; Hu, J.; Geng, F.; Cheng, L. Comparative Analysis of the Interaction between Alpha-Lactalbumin and Two Edible Azo Colorants Equipped with Different Sulfonyl Group Numbers. *Food Chem.* **2023**, *416*, 135826.

(26) Zhang, J.; Wu, D.; Tang, L.; Hu, X.; Zeng, Z.; Wu, W.; Geng, F.; Li, H. Evaluation of the Binding Affinity and Antioxidant Activity of Phlorizin to Pepsin and Trypsin. *Food Sci. Hum. Wellness* **2024**, *13* (1), 392−400.

(27) Soret, J.-L. Analyse Spectrale: Sur Le Spectre d'absorption Du Sang Dans La Partie Violette et Ultra-Violette. *Comptes rendus l'Académie des Sci.* **1883**, *97*, 1269−1270.

(28) Cho, S. Y.; Han, J. H.; Jang, Y. J.; Kim, S. K.; Lee, Y. Binding Properties of Various Cationic Porphyrins to DNA in the Molecular Crowding Condition Induced by Poly(Ethylene Glycol). *ACS Omega* **2020**, *5*, 10459.

(29) Van De Weert, M.; Stella, L. Fluorescence Quenching and Ligand Binding: A Critical Discussion of a Popular Methodology. *J. Mol. Struct.* **2011**, *998* (1−3), 144−150.

(30) Naeeminejad, S.; Assaran Darban, R.; Beigoli, S.; Saberi, M. R.; Chamani, J. Studying the Interaction between Three Synthesized Heterocyclic Sulfonamide Compounds with Hemoglobin by Spectros-

copy and Molecular Modeling Techniques. *J. Biomol. Struct. Dyn.* **2017**, *35* (15), 3250−3267.
(31) Malek-Esfandiari, Z.; Rezvani-Noghani, A.; Sohrabi, T.; Mokaberi, P.; Amiri-Tehranizadeh, Z.; Chamani, J. Molecular Dynamics and Multi-Spectroscopic of the Interaction Behavior between Bladder Cancer Cells and Calf Thymus DNA with Rebeccamycin: Apoptosis through the Down Regulation of PI3K/AKT Signaling Pathway. *J. Fluoresc.* **2023**, *33* (4), 1537−1557.
(32) Mandal, P.; Ganguly, T. Fluorescence Spectroscopic Characterization of the Interaction of Human Adult Hemoglobin and Two Isatins, 1-Methylisatin and 1-Phenylisatin: A Comparative Study. *J. Phys. Chem. B* **2009**, *113* (45), 14904−14913.
(33) Hu, X.; Wu, D.; Tang, L.; Zhang, J.; Zeng, Z.; Geng, F.; Li, H. Binding Mechanism and Antioxidant Activity of Piperine to Hemoglobin. *Food Chem.* **2022**, *394*, 133558.
(34) Khashkhashi-Moghadam, S.; Soleimani, S.; Bazanjani, A.; Hoseinpoor, S.; Taheri, R.; Mokaberi, P.; Saberi, M. R.; Chamani, J. Fabrication of Versatile and Sustainable Cellulose Nanocrystals from Lettuce Stalks as Potential Tamoxifen Delivery Vehicles for Breast Cancer Treatment: Biophysical, Cellular and Theoretical Studies. *New J. Chem.* **2023**, *47* (31), 14768−14791.
(35) Wang, Y. Q.; Zhang, H. M.; Tang, B. P. The Interaction of C.I. Acid Red 27 with Human Hemoglobin in Solution. *J. Photochem. Photobiol. B Biol.* **2010**, *100* (2), 76−83.
(36) Mahaki, H.; Memarpoor-yazdi, M.; Chamani, J.; Reza Saberi, M. Interaction between Ropinirole Hydrochloride and Aspirin with Human Serum Albumin as Binary and Ternary Systems by Multi-Spectroscopic, Molecular Modeling and Zeta Potential. *J. Lumin.* **2013**, *134*, 758−771.
(37) Poklar, N.; Vesnaver, G. Thermal Denaturation of Proteins Studied by UV Spectroscopy. *J. Chem. Educ.* **2000**, *77* (3), 380.
(38) Chamani, J.; Heshmati, M. Mechanism for Stabilization of the Molten Globule State of Papain by Sodium N-Alkyl Sulfates: Spectroscopic and Calorimetric Approaches. *J. Colloid Interface Sci.* **2008**, *322* (1), 119−127.
(39) Ghosal, S.; Bag, S.; Burman, M. D.; Bhowmik, S. Multi-spectroscopic Investigations of the Binding Interaction between Polyethylene Microplastics and Human Hemoglobin. *J. Phys. Chem. Lett.* **2023**, *14* (46), 10328−10332.
(40) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Springer: New York, 2006.
(41) Job, P. Formation and Stability of Inorganic Complexes in Solution. *Ann. Chim.* **1928**, *9*, 113−203.
(42) Micsonai, A.; Wien, F.; Kernya, L.; Lee, Y. H.; Goto, Y.; Réfrégiers, M.; Kardos, J. Accurate Secondary Structure Prediction and Fold Recognition for Circular Dichroism Spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* **2015**, *112* (24), E3095−E3103.
(43) Poklar, N.; Vesnaver, G.; Lapanje, S. Studies by UV Spectroscopy of Thermal Denaturation of P-Lactoglobulin in Urea and Alkylurea Solutions. *Biophys. Chem.* **1993**, *47*, 143−151.

https://doi.org/10.1021/acsomega.4c02766
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