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Exploring the Structural Importance of the C3═C4 Double Bond in Plant Alkaloids Harmine and Harmaline on Their Binding Interactions with Hemoglobin

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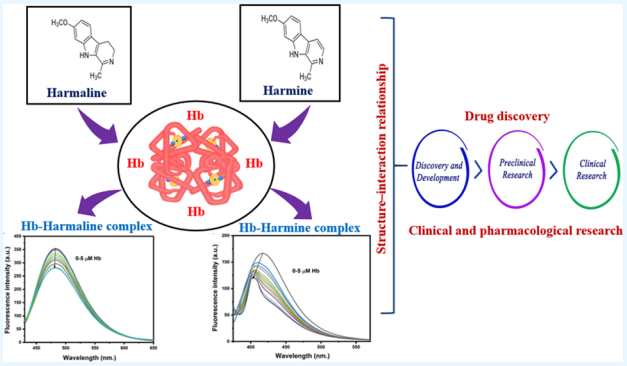
ABSTRACT: Harmine and harmaline are two structurally similar heterocyclic β-carboline plant alkaloids with various therapeutic properties, having a slight structural difference in the C3═C4 double bond. In the present study, we have reported the nature of the interaction between hemoglobin (Hb) with harmine and harmaline by employing several multispectroscopic, calorimetric, and molecular docking approaches. Fluorescence spectroscopic studies have shown stronger interaction of harmine with Hb compared to that of almost structurally similar harmaline. Steady-state anisotropy experiments further show that the motional restriction of harmine in the presence of Hb is substantially higher than that of the harmaline−Hb complex. Circular dichroism (CD) study demonstrates no conformational change of Hb in the presence of both alkaloids, but CD study in 1-cm cuvette path length also demonstrates stronger affinity of harmine toward Hb compared to harmaline. From the thermal melting study, it has been found that both harmine and harmaline slightly affect the stability of Hb. From isothermal titration calorimetry (ITC), we have found that the binding process is exothermic and enthalpy driven. Molecular docking studies indicated that both harmine and harmaline prefer identical binding sites in Hb. This study helps us to understand that slight structural differences in harmine and harmaline can alter the interaction properties significantly, and this key information may help in the drug discovery processes.



INTRODUCTION

The β-carboline alkaloids are a large group of natural and synthetic alkaloids with different degrees of aromaticity, some of which are widely distributed in medicinal plants, foodstuffs, marine creatures, and human tissues and body fluids.[1,2] β-Carboline alkaloids, also known as harmala alkaloids because they were first isolated from *Peganum harmala* (Zygophillaceae) plant, native to dry areas and found in north India, eastern Iran, North Africa, Middle East China, and some regions of western USA.[1] *P. harmala* seeds contain different types of β-carboline alkaloids such as harmine, harmane, harmalol, harmaline, harmalidine, etc. Among these alkaloids, harmine and harmaline are one of the most important representatives with various therapeutic activities such as anti-inflammatory,[2] antioxidant,[3] neuroprotective,[4] nephroprotective,[5] antidiabetic,[6] and anti-tumor activity.[7] The biological implementation (interaction with proteins and other biological targets) of β-carboline alkaloids in different studies has sparked interest in these compounds. The tricyclic plant alkaloids harmine and harmaline (Figure 1), both have a methoxyl group at the C-7 position and a methyl group at C-1. The only structural difference between harmine and harmaline is the presence of full aromaticity in harmine (presence of the C3═C4 double bond) and partial aromaticity in harmaline.

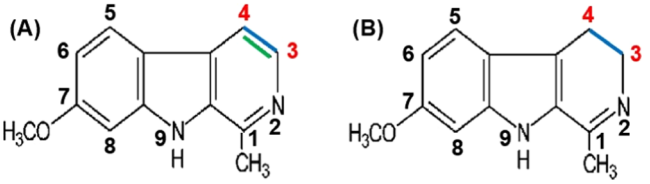


Figure 1. Structure of alkaloids (A) harmine, and (B) harmaline.

Hemoglobin (Hb) is an important component of the human body. It consists of two parts, globin protein, and an iron-containing porphyrin ring, heme. In adult Hb, the globin part consists of four amino acid chains, two α chains, and two β

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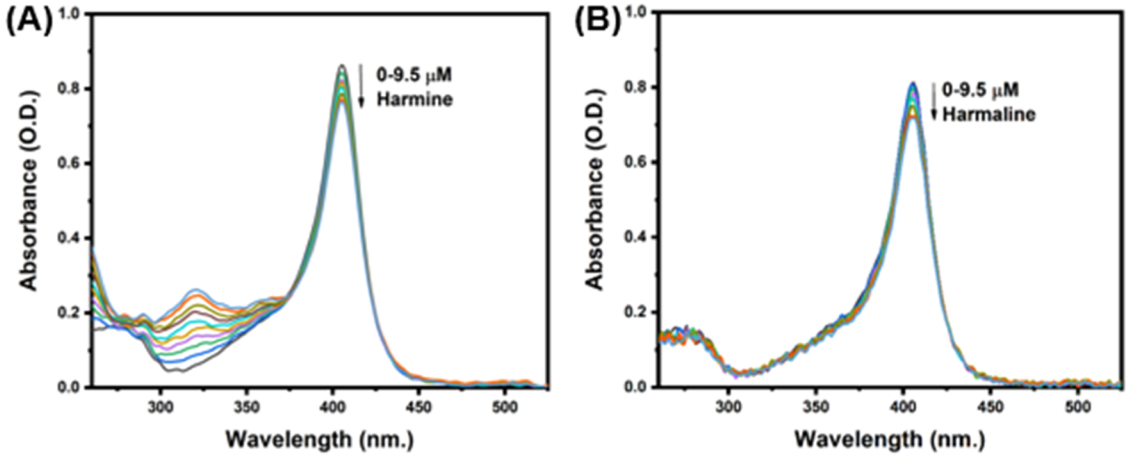

Figure 2. UV−Vis absorption spectra of Hb (2.5 μM) in the absence and presence of (0−9.5 μM) (A) harmine and (B) harmaline (0−9.5 μM) (subtracted absorbance).

chains.[7] Each α chain is made up of 141 amino acids and β chain is made up of 146 amino acids.[8] The tetrameric nature of Hb is crucial to its biological function. The heme part is made up of an iron atom inside a porphyrin ring.[9] Normal Hb concentration in an adult man and woman is 13.2−16.6 and 11.6−15 g/dL, respectively. Hb functions as a two-way respiratory carrier; it carries oxygen from the lungs to the tissues and carbon dioxide in the reverse direction. Hb's strong affinity for oxygen and lower affinity for carbon dioxide, hydrogen ions, chloride ions, and organic phosphates in arterial circulation are characteristics that distinguish it from others. Hb molecules can reversibly bind endogenous or exogenous molecules, such as 2,3-bis phosphoglycerate,[10] flavonoids[11] alkaloids,[12] and food colorants, etc.[13] Hence, free Hb can be exposed to various small molecules which may interact with different binding efficiencies.

The association of small molecules with proteins results in functional and structural alterations. The binding procedure and antioxidant properties of alkaloids with Hb have been discussed in recent works utilizing spectroscopic and computational approaches.[14,15] The binding mechanism of Sorafenib with c-MYC G-quadruplexes was explored at the molecular scale using computer-aided methods.[16] In a recent investigation, it has been found that fisetin seems to be an effective HRAS1 i-motif DNA ligand, whereas morin appeared to be an effective HRAS2 i-motif DNA ligand.[17] Scientists have discovered that the number of particular groups of edible azo colorants may impact the relationship of azo dyes with proteins by investigating the mechanism of interaction among proteins and food azo colorants.[18] Biological activities in all living organisms are primarily governed by interactions between molecules. In the *in vivo* system, the most significant physiological characteristic of Hb is the binding and transportation of many ligands for efficient delivery to their target locations. Hb may be considered as a good carrier for the transportation of these two β-carboline alkaloids harmine and harmaline. The interaction studies may shed light on the differential behaviors of these alkaloids in biological systems and help to understand the drug transport mechanism. Several important activities of Hb such as signal transmission may also be mediated by the binding of such alkaloids.

A lot of studies on the binding of small molecules with Hb have recently been investigated.[19,20] Numerous small molecules' affinity for Hb is directly related to how they are metabolized, distributed, and act *in vivo*. It is essential and crucial to conduct research on how these small molecules interact with Hb. Understanding the medicinal potential of these studied alkaloids (harmine and harmaline) requires a thorough grasp of its binding properties with Hb. The spectroscopic and thermodynamic characterization of the alkaloid−Hb complex will allow us to gain a thorough understanding of the molecular components of the interaction as well as correlate the structural features, which may be useful in implementing Hb's full therapeutic potential.

In this study, by utilizing different spectroscopic methods such as UV−Vis absorption studies, steady-state fluorescence studies, steady-state anisotropy studies, circular dichroism spectroscopy, UV-melting studies, and isothermal titration calorimetry along with molecular docking studies, we have explored the interaction of Hb with two structurally similar two β-carboline alkaloids, harmine and harmaline (Figure 1) and described how the little structural difference of these two alkaloids (double bond between C3 and C4 for harmine) impacts their mode of interaction. From this investigation, it has been revealed that harmine has a greater affinity toward Hb than almost structurally identical harmaline.

RESULTS AND DISCUSSION

UV−Vis Absorption Spectroscopy. The Soret band is a strong peak in the visible spectrum's blue wavelength range. The shifting of an electron dipole which enables $\pi-\pi^*$ transitions, which are most common in porphyrin substances, leads to the formation of the Soret band.[21] Several investigations of porphyrin-containing moieties may be performed using UV−Vis spectroscopy at the corresponding Soret band wavelength (405 nm). The absorption spectra of Hb (2.5 μM) show two peaks, one at 269 nm due to the phenyl group of tryptophan and tyrosine residues, and the other at 405 nm (Figure S1A). The former peak is primarily formed due to the $\pi-\pi^*$ transition of the carbonyl groups of the amino acid residues, while the latter is the porphyrin-Soret band ($\pi-\pi^*$ transition), which originates from the heme moiety embedded in the hydrophobic pocket formed by the protein's backbone through appropriate folding.

In the presence of increasing concentration of the harmine (0−9.5 μM), a decrease in the intensity of the Soret band of Hb (2.5 μM) was observed (Figure 2A). This result suggests the formation of a complex between harmine and Hb. Two isosbestic points were noticed at 380 and 430 nm. The appearance of isosbestic points may denote equilibrium between

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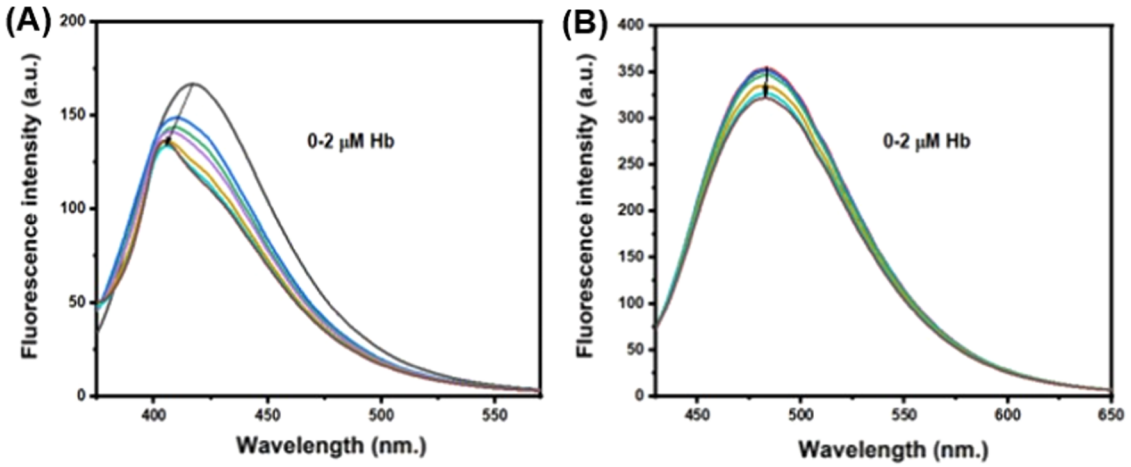

Figure 3. Change in the fluorescence spectrum of (A) harmine (1 μM) and (B) harmaline (1 μM) upon the addition of increasing amounts of Hb (0−2 μM).

Table 1. Fluorescence Anisotropic Parameters of Harmine and Harmaline (1 μM) in buffer and in the Presence of Hemoglobin (1 & 2 μM)[a]

sample	anisotropy (r)	I_{HH}	I_{HV}	I_{VH}	I_{VM}	I_{VV}	G factor
1 μM harmine	0.00271	146188.33	357347.33	113263	150827.33	279458	2.44
1 μM harmine +1 μM hemoglobin	0.00380	83982.1	203119.1	64308.1	84797.43	157277.43	2.41
1 μM harmine +2 μM hemoglobin	0.00442	48342.1	116911.76	36365.76	48358.76	89673.1	2.41
1 μM harmaline	0.00318	1042586.26	1053338.26	800598.93	808760.93	817976.93	1.01
1 μM harmaline +1 μM hemoglobin	0.00356	1345853.9	1366575.23	1034169.9	1050410.56	1063919.9	1.01
1 μM harmaline +2 μM hemoglobin	0.00361	811651.6	815925.33	625599.33	630831	633493	1.00

[a]Where I_{VV}, I_{VH}, I_{HV}, and I_{HH} represent fluorescence signals for excitation and emission with the polarizer set at positions (0°,0°), (0°,90°), (90°,0°), and (90°,90°), respectively. G is the sensitivity factor of the detection systems.

protein-bound and free ligands in the complex between harmine and Hb. The decrease in the 405 nm absorbance reveals that the heme group of Hb may be exposed by the interaction of harmine molecules, inducing a change in its environment leading to the access of the aqueous medium. From the results, it may be assumed that harmine showed differential behavior in UV-absorbance spectroscopy due to the noncovalent molecular association between the harmine and Hb. Harmaline shows an absorption peak at around 375 nm, which is merged with the absorbance of Hb. Therefore, the absorbance of harmaline (Figure S1C) was subtracted from the absorbance of the Hb−harmaline complex to get the exact results. It was found that after the gradual addition of harmaline (0−9.5 μM) in Hb (2.5 μM), the Soret band intensity of Hb was decreased (Figure 2B), though no such isosbestic points were noticed. These results indicate that a stronger complex formation between Hb & harmine compared to Hb & harmaline.

Steady-State Fluorescence Spectroscopy. The intrinsic fluorescence of proteins is mainly due to tryptophan and tyrosine residues (mostly tryptophan) and is very sensitive to the change in the microenvironment. Hence, a study of the alteration in the intrinsic fluorescence property of Hb in the presence of the alkaloids may give information about the local environment of the tryptophan moiety on interaction. Fluorescence emission spectra of harmine (1 μM) and harmaline (1 μM) were monitored in the absence and presence of increasing concentrations of Hb (0−2 μM). The addition of Hb in an increasing concentration (0−2 μM) leads to quenching of the fluorescence property of both harmine and harmaline. Such quenching might appear due to excited-state reactions, molecular recognition, inner filter effect, energy transfer, and collisional quenching.[22] Hence, the inner filter effect was corrected by applying the appropriate method described in the Experimental Section.

Fluorescence emission spectra of both of these alkaloids (harmine and harmaline) on titration with Hb were recorded. Harmine has the emission spectral peak at 417 nm while harmaline has a fluorescence maximum at 483 nm when exited at 365 nm. Two distinctly different fluorescence maxima for the two forms of alkaloids make it easy to understand the whole binding phenomenon from the fluorescence study. At the highest concentrations of added Hb (0−2 μM), the quenching of the fluorescence emission intensity is found to be ∼1.22-fold for harmine (Figure 3A) with a 12 nm blue shift and ∼1.08-fold for harmaline with no detectable shifting (Figure 3B). These changes in the fluorescence intensity of both alkaloids upon the addition of Hb indicate the stronger interaction between Hb and harmine. The alterations of the emission spectra (blue shifting) of harmine in the presence of Hb reflect a change in the microenvironment around harmine due to binding in the proteinaceous environment compared to the free form in the aqueous phase. It has been frequently observed that a molecule with a significant excited-state dipole has an emission spectrum that is very sensitive to solvent relaxation. The binding of Hb to harmine not only quenched the fluorescence intensity of harmine but also displayed a substantial blue shift in the emission maxima as the Hb concentration increased. It can be predicted that the blue shift of emission maxima of harmine in the presence of Hb is due to the reorientation of its solvent molecules toward the excited-state dipole. From the modified Stern−Volmer equation, the calculated binding constant (K_b) for the harmine−Hb complex is 1.08×10^4 M^{-1}. For the

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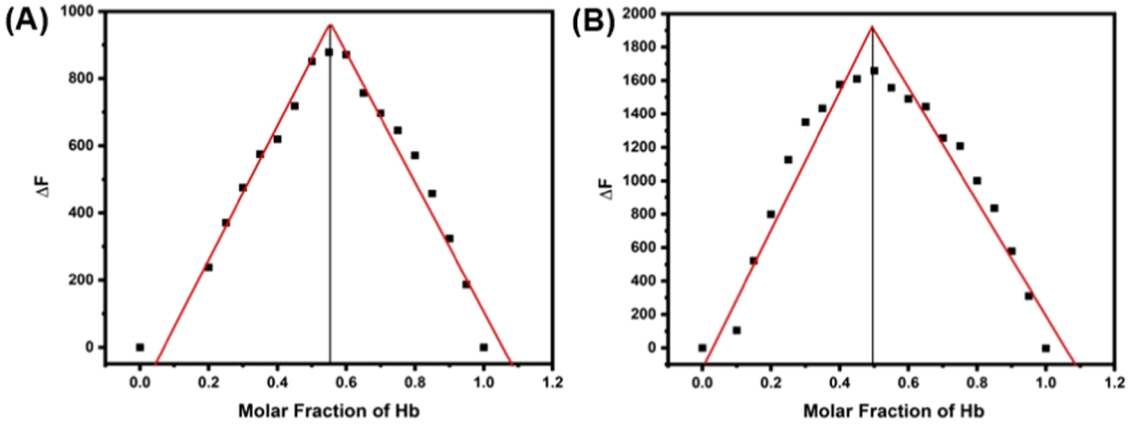

Figure 4. Continuous variation plot (Job plot) using spectrofluorometric data for (A) harmine/Hb and (B) harmaline/Hb complex. λ excitation = 365 nm and λ emission = 420 nm for harmine and 480 nm for harmaline, at room temperature.

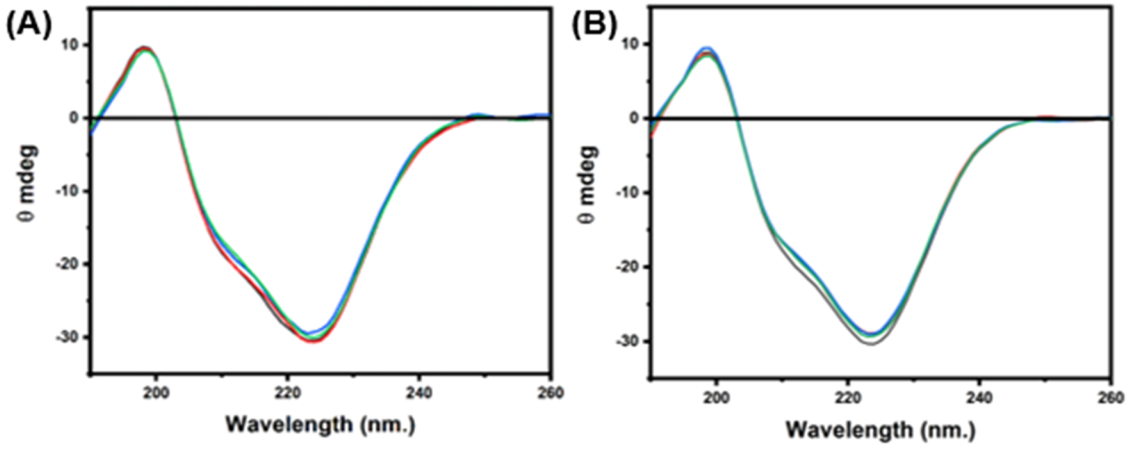

Figure 5. CD spectral changes of Hb (3 μM) in the absence and presence of (3, 6, and 12 μM) (A) harmine and (B) harmaline. Black line = Hb, red line = 3 μM, blue line = 6 μM, and green line = 12 μM of harmine and harmaline in the presence of Hb.

harmaline−Hb complex, it is not possible to calculate the binding constant through the modified Stern−Volmer equation as the observed fluorescence quenching was quite small. The calculated standard deviation for the K_b value of the harmine−Hb complex is 0.0021×10^4 (Figure S2).

Steady-State Fluorescence Anisotropy Measurements. Steady-state fluorescence anisotropy measurements are used to track and collect further information in favor of alkaloid's (harmine and harmaline) interaction with Hb. The increase in anisotropy is most likely due to some sort of interaction between the alkaloids and the Hb molecules, which inhibits alkaloid's rotational flexibility. In pure buffer solution (pH 7.4) the anisotropy value of harmine (1 μM) was found to be 0.00271, which increases to 0.00442 in the presence of Hb (2 μM) (Table 1). This result demonstrates that harmine has significantly lower rotational flexibility when attached to Hb than the free form in solution. This rise in anisotropy indicates that harmine is bound to Hb and is occupying a motionally restricted environment. For harmaline, the increase of anisotropy value is much lower as compared to harmine (Table 1).

Stoichiometric Analysis. The stoichiometry of alkaloid−Hb binding is analyzed by the continuous variation method (Job's plot analysis). In the experiments, the total concentration ($C_{Hb} + C_{Alkaoids}$) is maintained constant while the molar fractions of Hb and alkaloids are altered. Then, the changes in the fluorescence intensity ($\Delta F = F_{Hb} - F_{Hb+Alkaoids}$) are plotted versus the Hb's molar fraction (Figure 4). The Job's plots for Hb−harmine and Hb−harmaline are performed considering the maximum fluorescence emission intensity upon excitation at 365 nm. It was found that the stoichiometry of the Hb:harmine and Hb:harmaline complex at room temperature are approximately 1:1 (Figure 4).

Circular Dichroism (CD) Spectroscopy. CD has been used to investigate the conformational changes in the secondary structure of proteins in different environments and different protein−ligand complexes.[23] In the far UV region (260 to 190 nm) the Hb exhibits two negative peaks around 208 and 222 nm that are mainly due to protein secondary structure, which is predominantly α-helical in nature. The 208 nm band corresponds to $\pi-\pi^*$ transition of the α-helix and the 222 nm band is due to $n \rightarrow \pi^*$ transition for both the α-helix and random coil complexes; both are contributed by the transition of the peptide bond of the α-helix.[24,25] Upon successive addition of harmine and harmaline (3−12 μM) to a fixed concentration of Hb (3 μM), we did not find any perturbation in the CD spectral profiles of Hb (Figure 5). We have also performed CD spectra of hemoglobin with 1% ethanol, which shows negligible structural change of Hb (Figure S3). Interestingly, when we are using a 1-cm path length cuvette for CD measurements, Hb exhibits a negative peak at around 234 nm. Upon successive addition of harmine (3−12 μM) to a fixed concentration of Hb (3 μM), we

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found significant changes in the CD spectral profile of Hb indicating stronger interaction of harmine with Hb (Figure S4A). But in the case of harmaline, the spectral change is quite less which indicates less interaction (Figure S4B).

We also quantified the α-helicity change in Hb due to the binding of alkaloids. The calculated percentage of α-helicity in Hb was ~ 28.1%. After the addition of alkaloids at increasing concentrations (3, 6, and 12 μM), the percentage of α-helicity increases to ~ 30.1% for harmine and ~ 28.3% for harmaline, respectively. In the case of harmine, the slight increase in the α-helical content caused by harmine binding might be related to the newly developing H-bonds. Because a protein's biological activity is largely determined by its secondary structure, this increase in helical content suggests that Hb may be regaining part of its biological activity as a result of harmine binding.[26]

UV-Melting Analysis. The interactions of alkaloids, harmine, and harmaline with Hb were studied by UV-melting experiments. In this experiment, Hb (5 μM) exhibits a melting temperature (T_m) of around 62.58 °C (Figure 6). In the presence of alkaloids, the T_m of Hb (Hb/alkaloid−1:1) was decreased to 60.51 and 60.36 °C for harmine and harmaline, respectively. We have recorded the melting profile of harmine and harmaline alone to make sure that their UV absorbance would not affect the melting profile of Hb (Figure S5). Hence, it can be stated that harmine and harmaline induce very little destabilization effect in Hb.

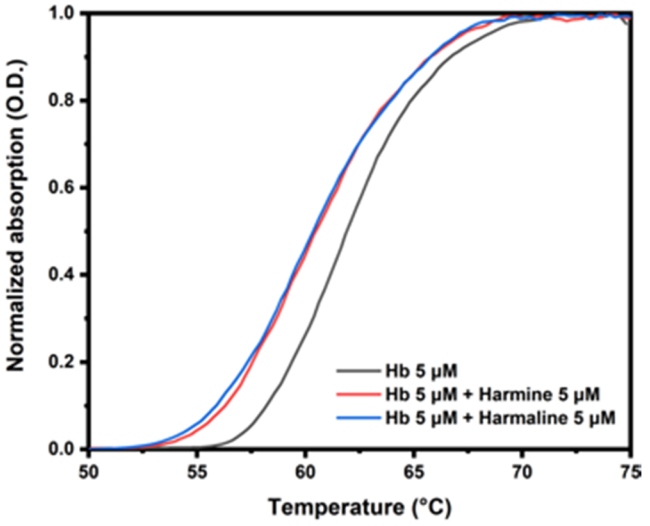

Figure 6. Melting graph of Hb in the absence and presence of alkaloids, harmine, and harmaline.

Isothermal Titration Calorimetry (ITC) Studies. The reactions' entropy and enthalpy dynamics along with the complexes' stoichiometry and the binding constants can be calculated effectively from ITC.[27] The thermodynamic parameters and energetics of binding between harmine and harmaline with Hb were studied by ITC. The amount of heat generated during the complexation process between harmine and harmaline with the Hb complex is shown by the area of each peak. We verified that there was no reasonable influence of background data measured by dilution of alkaloids with a comparable experimental setup. The process of binding of harmine and harmaline is exothermic in nature (Figure 7A and Supporting Table 1).

For the harmine−Hb complex, the enthalpy change (ΔH is −100 kJ mol^{-1}) for the association process is more negative than the entropy change ($T\Delta S$ is −76 kJ mol^{-1}), indicating the binding process to be enthalpy driven. The Gibbs free energy change (ΔG = −24 kJ mol^{-1}) is also negative, implying this binding reaction is spontaneous. The ITC data of harmine and Hb complexation yielded a binding constant (K_b value) of 1.42 × 10^4 M^{-1}. In the case of harmaline−Hb complex (Figure 7B and Supporting Table 1), the enthalpy change (ΔH is −100 kJ mol^{-1}) for the association process is more negative than the entropy change ($T\Delta S$ is −76.69 kJ mol^{-1}), indicating this binding process to be enthalpy driven. The Gibbs free energy change (ΔG is −24.31 kJ mol^{-1}) is also negative. From ITC, the obtained K_b value of harmaline−Hb complex was 1.67 × 10^4 M^{-1}. The calculated confidence levels of ΔH, $T\Delta S$ and ΔG are found to be 95%. The Gibbs free energy changes and the binding constant (K_b) values are more or less similar for the harmine−Hb complex and harmaline−Hb complex (Supporting Table 1).

Molecular Docking-Based Binding Site Prediction of Harmine and Harmaline. The amino acid residues involved in the binding of harmine and harmaline with Hb are given along with their binding energy in Supporting Table 2. From the table, it is evident that the binding site of harmine and harmaline remains identical in Hb. Both harmine and harmaline form hydrogen bonds with Tyr42(A) and Asn97(A) of Hb. In addition, harmine and harmaline interact with Thr38(A), Thr41(A), Asp94(A), Val96(A), Arg40(D), Phe41(D), Leu96-(D), His97(D), and Asp99(D) through hydrophobic interactions (Figure 8B,C).

Unlike residual interactions, the harmine and harmaline binding with Hb differs in terms of their binding energy. Upon analysis, the difference in the binding energy of harmaline and harmine with Hb was found to be very less i.e., 0.46 kcal/mol, and warrants further investigations using the molecular dynamics-based approach to understand the stability of the ligand-protein interaction. The reliability of our docking procedure was evaluated by redocking 2-[(2-methoxy-5-methylphenoxy)methyl]pyridine with Hb. The analysis revealed that our docking procedure accurately predicts the binding site of 2-[(2-methoxy-5-methylphenoxy)methyl]pyridine upon re-docking with Hb. Based on our docking analysis, 2-[(2-methoxy-5-methylphenoxy)methyl]pyridine interacts with Hb with a binding energy of −4.35 kcal/mol, and the interacting residues were found to be Lys99(A), Ser102(A), Asp126(A), His103(A), Asn108(B), Leu105(B), Trp37(B), Tyr35(B), and Glu101(B). Out of 9, 6 residues were found to be identical with the crystal structure (PDBID 3IC0) namely, Lys99(A), Ser102(A), Asp126(A), Leu105(B), Trp37(B), and Glu101(B). The mode of interaction between the 2-[(2-methoxy-5-methylphenoxy)methyl]pyridine and amino acid residues was also found to be quite similar to that of the crystal structure (Figures 8A and S6). Furthermore, the root-mean-square deviation (RMSD) analysis of the docked and crystal structure conformer of 2-[(2-methoxy-5-methylphenoxy)methyl]-pyridine with Hb was found to be only 4.252 Å. Thus, based on these observations both harmine and harmaline prefer identical sites in Hb for their binding.

CONCLUSIONS

Natural products have long been an important source of therapeutic drugs, which promotes new approaches in organic synthesis.[28] Several recent investigations have reported the molecular interaction between protein and ligand through

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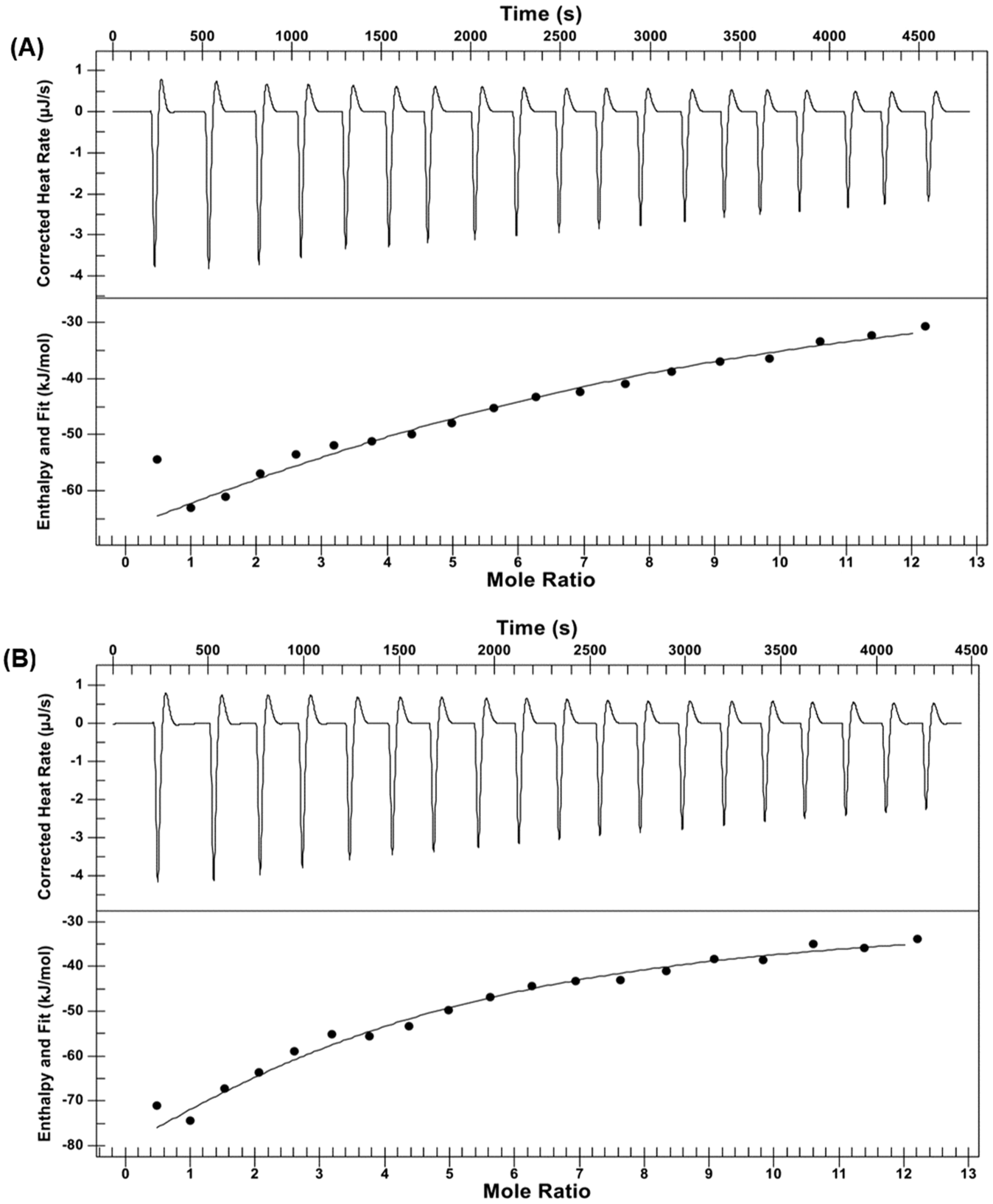

Figure 7. ITC profiles of interactions between (A) harmine and (B) harmaline with Hb. The upper panel represents the isothermal plot of the interaction between Hb and alkaloids. The lower panel shows the integrated heat results from the integration of peak area as a function of the molar ratio of Hb/alkaloids. The data were best fitted using the "one site binding model", and the fitting line was represented by a solid line.

advanced multispectroscopic methods.[29−32] The plant alkaloids, harmine and harmaline have different health-beneficial activities such as antioxidant, anti-inflammatory, antimicrobial, and antitumor activities. Various small molecules have been reported, which show potential to interact with Hb. Hence, studying the interactions of two structurally similar plant

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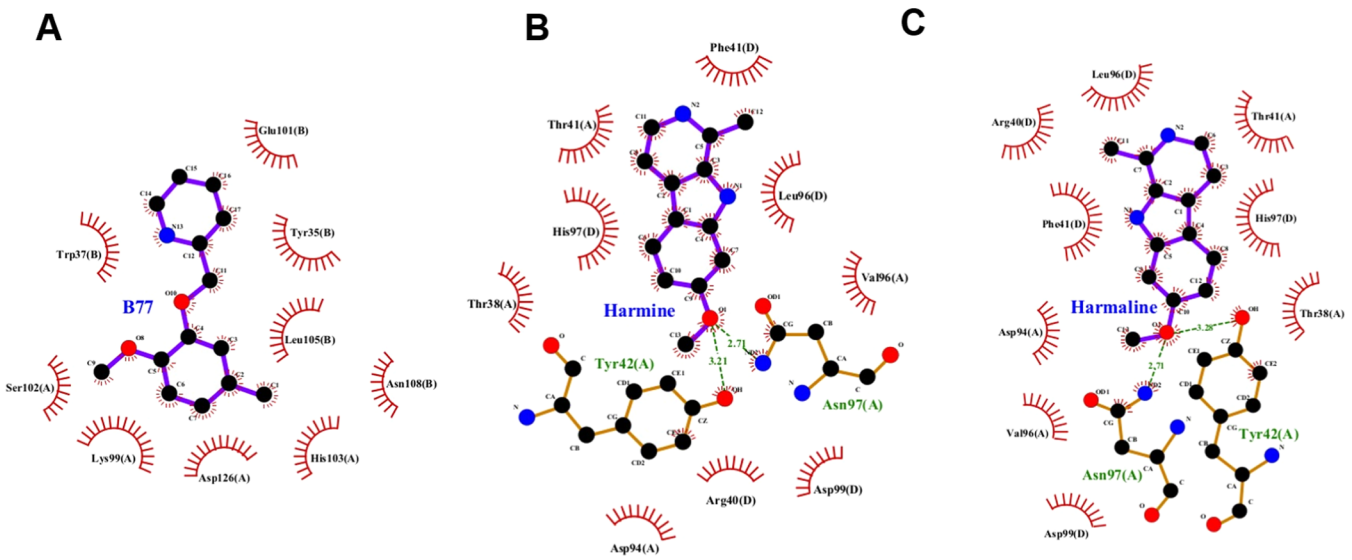

Figure 8. LIGPLOT analysis of ligands complexed with hemoglobin. The amino acids involved in the binding 2-[(2-methoxy-5-methylphenoxy)methyl]pyridine (A), harmine (B), and harmaline (C) with hemoglobin were represented in 2D. The residues interacting with the ligands through hydrogen bonds were represented as green colored dotted line. The hydrophobic interaction between the ligand and amino acid residues was represented as arcs.

alkaloids harmine and harmaline with Hb will provide us a clear scenario in terms of drug design and actions. The spectrophotometric titrations of Hb showed hypochromic changes in the presence of both alkaloids, harmine and harmaline, indicating that both of them form complexes with Hb. The hypochromicity of harmine with Hb is higher compared to harmaline depicting strong interaction with harmine. Regarding the fluorescence analysis, the fluorescence spectrum of harmine quenched more with a large blue shift than harmaline upon interactions with Hb. This result implies that harmine form stronger complex with Hb compared to harmaline. From stoichiometry, it has been found that both harmine and harmaline interact with Hb in 1:1 ratio. Steady-state anisotropy experiments further demonstrated that the motional restriction of harmine in the presence of Hb is significantly higher than the harmaline−Hb complex. Furthermore, CD data indicated that in the presence of harmine and harmaline there was no significant changes in the conformation of Hb but a slight increase in the α-helicity for the Hb−harmine complex. Interestingly, by using a 1-cm path length cuvette, the CD data showed significant changes for harmine toward Hb compared to harmaline, suggesting stronger affinity for the harmine−Hb complex. Melting experiments showed that both the alkaloids slightly affect Hb stability. The binding energetics of harmine and Hb were investigated using ITC, and it revealed that the binding mechanism is exothermic and enthalpy-directed. The calculated binding constants from ITC are more or less similar for Hb−harmine and Hb−harmaline complex. From molecular docking studies, it has been observed that both harmine and harmaline have similar binding sites in Hb. The results suggest that harmine forms a more stronger complex with Hb compared to harmaline. So, a structure−interaction relationship was observed: the polycyclic planar full N-heteroaromatic structure of harmine was shown to be more reactive toward Hb than the partial aromatic harmaline. It can be concluded that the binding of planar compounds (harmine with full aromaticity) with Hb is stronger than that of nonplanar compounds (harmaline with partial aromaticity). By decreasing the planarity of the β-carboline skeleton, the binding affinity to Hb decreases. The current study indicates that the structural variations between harmine and harmaline cause these alkaloids to interact differently with Hb.

This study provides useful information to understand that a small difference in the structure (presence of the C3═C4 double bond in harmine) can significantly change the interaction process, which may be useful for drug design purposes. Harmine's application as a multipurpose traditional drug has turned into several industrial uses, and it is a precious phytoconstituent for food and research area. Hence, this study will help to interpret the structural basis for screening and designing appropriate natural molecules that will be important in the advancement of clinical and pharmacological research. This research also underlines the potential of harmine to be used in novel therapeutic drugs and provides the basis for additional investigation of the use of plant alkaloids.

EXPERIMENTAL SECTION

Materials. Lyophilized powder of human Hb, harmine, and harmaline were purchased from Sigma Aldrich. Harmine and harmaline were dissolved in ethanol. The Hb concentration was measured by spectrophotometric absorbance considering the molar extinction coefficient of Hb at 276 nm (120,808 M^{-1} cm^{-1}).[33−35] The experimental stock concentrations of alkaloids (harmine and harmaline) and Hb are 5 mM and 100 μM, respectively. All experiments were performed using a buffer composed of 50 mM KCl, 10 mM KH_2PO_4, and 1 mM K_2EDTA (pH 7.4) at room temperature (25 °C). The concentration of ethanol was always kept <1% (by volume) in all experiments.

UV−Vis Absorption Spectroscopy. UV−Vis absorption spectra of Hb in the absence and presence of harmine and harmaline were measured with a Hitachi UH5300 using a quartz cuvette with 1-cm path length. Spectrophotometric titrations were performed by maintaining the concentration of Hb constant at 2.5 μM and titrated by elevating the concentrations (1.5−9.5 μM) of harmine and harmaline.

Fluorescence Spectroscopy. The fluorescence emission spectra were recorded with a Biobase BK-F96Pro using a quartz cuvette with 1-cm path length at room temperature (25 °C). For

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the emission spectral measurements, both the excitation and emission spectral slit widths were set to 10 nm and the excitation wavelength for harmine and harmaline were set to 365 nm. In the experiments, the concentrations of harmine and harmaline were maintained constant to 1 μM and titrated by elevating concentrations of Hb up to 2 μM.

The inner filter effect can be defined as the absorption of light at excitation and emission wavelengths by the compound(s) present in the medium. The inner filter effect occurs when the absorbance by a substance is higher than 0.1.[36] Hence, the emission spectra were corrected using the following equation[36]

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

where F_{cor} and F_{obs} are the observed and corrected fluorescence emission intensities, A_{ex} and A_{em} are the alterations in the absorbance at excitation (365 nm) and emission wavelengths. The corrected emission spectra were used to understand the binding character between Hb and alkaloids. The fluorescence emission data were analyzed by the modified Stern−Volmer equation to determine the binding constant.

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

where F_0 is the peak of fluorescence intensity of alkaloids (harmine and harmaline) alone and F is the fluorescence intensity of the solution to be measured after the addition of Hb. K_b is the binding constant and C_q is the concentration of Hb.

Steady-State Fluorescence Anisotropy Measurements. Steady-state fluorescence anisotropy measurements were performed with a Fluorolog spectrometer (Horiba Scientific) equipped with polarizers. The solutions containing the sample were taken in a quartz cell with a 1-cm path length. Emission spectra of harmine and harmaline were recorded in the presence of increasing concentrations of Hb at an excitation wavelength of 373 nm, and emission spectral band passes at 5 nm. All these spectrofluorometric measurements were performed in buffer containing 50 mM KCl, 10 mM KH_2PO_4, and 1 mM K_2EDTA (pH 7.4) at room temperature (25 °C).

Steady-state fluorescence anisotropy (r) values were calculated using the expression

$$r = \frac{(I_{VV} - GI_{VH})}{I_{VV} + 2GI_{VH}};\ G = \frac{I_{HV}}{I_{HH}}$$

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

Stoichiometry. The stoichiometry between alkaloids harmine and harmaline with Hb binding was analyzed by the method of continuous variation (Job's plot analysis).[37] This is one of the useful methods for characterizing complexes formed between two compounds. During the experiments, the total concentration ($C_{Hb} + C_{Alkaloid}$) was kept constant, while the molar fractions of Hb and alkaloids were varied. Then, the fluorescence intensity changes ($\Delta F = F_{alkaloid} - F_{Hb+alkaloid}$) were recorded and plotted against the Hb molar fraction. The Job's plot for Hb−harmine and Hb−harmaline was calculated by using maximum fluorescence emission upon excitation at 365 nm.

CD Spectroscopy. The CD measurements of Hb in the presence or absence of harmine and harmaline were performed in the range of 190−260 nm with a scan speed of 100 nm/min using a Jasco J-1500 CD spectrometer. Cuvettes with 1-mm and 1-cm path lengths were used in this study. Hb concentration was kept at 3 μM while the molar ratios of harmine or harmaline to Hb concentration were 1:1, 2:1, and 4:1. The buffer solution was considered as the blank and subtracted from each scanned spectrum. Each sample was scanned three times to average the CD spectrum. The CD spectra of Hb in the presence of ethanol (1%) were also recorded. BeStSel (β Structure Selection) is a novel method for secondary structure determination and fold recognition from protein circular dichroism spectra (https://bestsel.elte.hu/index.php).[38] By using this method, the percentage changes in the α-helical structure after adding harmine and harmaline to Hb was determined in our study.

UV Melting. Melting profiles (absorbance change against temperature curves) of 5 μM Hb in the absence and presence of 5 μM alkaloids (harmine and harmaline) were performed on a Hitachi UH5300 equipped with a Julabo F12 water circulator and a 3J1−0104 water circulated cell holder device. A single chambered quartz cuvette with 1-cm path length was used. The samples were heated at a rate of 1 °C/min, and the change in absorbance at 280 nm was monitored. The measurement of melting temperature (T_m) was performed by analyzing the melting curves. The melting profiles of harmine and harmaline alone were also measured at 280 nm. The melting profile of harmine was subtracted from the melting profile of the Hb−harmine complex melting profile to minimize the absorbance of harmine. A similar method was also performed in the case of harmaline.

Isothermal Titration Calorimetry (ITC). In order to demonstrate the binding activity of the alkaloid−Hb complex, we performed ITC and monitored the heat response using AITC (TA Instruments). A fixed concentration of alkaloids (harmine and harmaline, 175 μM) was injected into Hb (10 μM). During measurements, the temperature was kept constant at 25 °C. After each injection, the power output was integrated, and the molar binding enthalpies were calculated. The isotherms were analyzed using Nanoanalyze software (TA Instruments). To assess the heat of dilution, control experiments were performed. The binding isotherms obtained in the titrations were analyzed with model equations. The data points obtained best fitted the "one set of sites" models. The standard molar entropy (ΔS) and the standard molar Gibbs energy (ΔG) were calculated using standard thermodynamic relationship $\Delta G = -RT \ln K$. where R (1.9872041 cal K^{-1} mol^{-1}) is the gas constant and T is the temperature in kelvins. Both Hb and alkaloid solutions were prepared in the same buffer and filtered through a 0.22 μm filter membrane to avoid blocking.

Binding constants (K_b), change in enthalpy (ΔH), and change in entropy (ΔS) associated with alkaloid−protein complex formation were evaluated after fitting the isotherms. The Gibbs free energy (ΔG) was calculated using the relationship: $\Delta G = \Delta H - T\Delta S$.

Molecular Docking. The binding site and amino acid residues involved in the binding of harmine and harmaline were predicted with a molecular docking-based approach using AutoDock 4.2.[39] The Cartesian coordinates of human hemoglobin were taken from the protein data bank (PDBID: 3IC0).[40] The structural conformation for the ligands 2-[(2-methoxy-5-methylphenoxy)methyl]pyridine, harmine, and harmaline were obtained from the PubChem database and converted into PDBQT using AutoDock Tools (ADT). Polar hydrogens were added to the amino acid residues of the Hb protein using ADT. Water molecules, sulfate ion, and 2-[(2-

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methoxy-5-methylphenoxy)methyl]pyridine were removed from the protein structure, and Gasteiger charges were added. The autogrid program was used to generate a grid covering the entire protein for docking analysis. The grid points along the *X*, *Y*, and *Z* coordinates were kept at a maximum with a spacing of 0.4 Å. Molecular docking of ligands against human Hb was performed using the autodock program, keeping the protein as a rigid molecule. Lamarckian genetic algorithm (LGA) was chosen to predict the binding site of the ligands with the population size, energy evaluations, and number of generations and run set at 600, 250,000, 10,000, and 100, respectively. The docked ligand conformer was selected from the cluster with the maximum number of conformers for each ligand. The docking simulation was validated by redocking 2-[(2-methoxy-5-methylphenoxy)methyl]pyridine with the protein, and the ligand RMSD between the predicted and crystal structure was measured using Chimera.[41] The residual interaction analysis was performed using Ligplot+ V2.2.[42]

Statistical Analysis. The investigations in this study were carried out in triplicate. ANOVA was used to assess the experimental outcomes, and data were analyzed at a significance level of $p < 0.05$. The plots were processed and exported using the OriginPro program.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c04432.

UV−Vis absorption spectra of (A) Hb (2.5 μM), (B) harmine, and (C) harmaline (5 μM) alone as a control; CD spectra of 3 μM Hb with 1% ethanol. [Black: 3 μM Hb; Red: 3 μM Hb + 1% Ethanol]; modified Stern−Volmer plot for determining standard deviation of Harmine; CD spectral change (1-cm path length cuvette) of Hb (3 μM) in the absence and presence of (3, 6, and 12 μM) (A) harmine and (B) harmaline [black line = Hb, red line = 3 μM, blue line = 6 μM, and green = 12 μM of harmine and harmaline in the presence of Hb]; melting graph of 5 μM (A) harmine and (B) harmaline; Supporting Table 1: thermodynamic parameters determined from calorimetric measurements of temperature-dependent interaction of harmine−hemoglobin and harmaline−hemoglobin complex. Supporting Table 2: binding energy and residual interaction analysis of ligands complexed with hemoglobin. The amino acid residues involved in the binding of 2-[(2-methoxy-5-methylphenoxy)methyl]pyridine with hemoglobin in the crystal structure (PDBID: 3IC0) are highlighted in bold. The residues were mentioned along with their respective chain information within the brackets; Ligplot analysis of 2-[(2-methoxy-5-methylphenoxy)methyl]-pyridine interaction with hemoglobin. Amino acids involved in the binding of 2-[(2-methoxy-5-methylphenoxy)methyl]pyridine with human hemoglobin as reported in the crystal structure (PDBID: 3IC0) were represented as arcs (PDF)

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‖M.D.B. and S.Bag. contributed equally to this work. M.D.B., S.B., and S.Bag. designed the research. M.D.B. and S.Bag. conducted the research; S. Bag., S.G., S.K., and G.P. analyzed the ITC and spectroscopic data and R.K.C. performed the docking studies. M.D.B, S.Bag., S.G., and S.B. wrote the paper. S.B. is the corresponding author of this work. The final article was revised and approved by all authors.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) 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.

(2) 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.

(3) Li, S. P.; Wang, Y. W.; Qi, S. L.; Zhang, Y. P.; Deng, G.; Ding, W. Z.; Ma, C.; Lin, Q. Y.; Guan, H. Da.; Liu, 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* (APR), No. 346.

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

(5) 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* (1), No. 97.

(6) Wang, P.; Alvarez-Perez, J.-C.; Felsenfeld, D. P.; Liu, H.; Sivendran, S.; Bender, A.; Kumar, A.; Sanchez, R.; Scott, D. K.; Garcia-Ocaña, A.; Stewart, A. F. A High-Throughput Chemical Screen Reveals That Harmine-Mediated Inhibition of DYRK1A Increases Human Pancreatic Beta Cell Replication. *Nat. Med.* **2015**, *21* (4), 383−388.
(7) Wang, Y. Q.; Zhang, H. M.; Zhou, Q. H. Studies on the Interaction of Caffeine with Bovine Hemoglobin. *Eur. J. Med. Chem.* **2009**, *44* (5), 2100−2105.
(8) Yuan, Y.; Simplaceanu, V.; Lukin, J. A.; Ho, C. NMR Investigation of the Dynamics of Tryptophan Side-Chains in Hemoglobins. *J. Mol. Biol.* **2002**, *321* (5), 863−878.
(9) Vigneswaran, C.; Ananthasubramanian, M.; Kandhavadivu, P. *Enzyme Technology*; Cambridge University Press: Cambridge, 2014. DOI: 10.1016/b978-93-80308-42-5.50001-9.
(10) Levitzky, G. M. *Pulmonary Physiology*, 7th ed.; McGraw Hill, 2007.
(11) 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), 333−339.
(12) Das, A.; Kumar, G. S. 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.
(13) Basu, A.; Kumar, G. S. Interaction of the Dietary Pigment Curcumin with Hemoglobin: Energetics of the Complexation. *Food Funct.* **2014**, *5* (8), 1949−1955.
(14) 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* (June), No. 133558.
(15) 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* (October 2022), No. 134824, DOI: 10.1016/j.foodchem.2022.134824.
(16) Wu, W.; Hu, X.; Zeng, Z.; Wu, D.; Li, H.; Li, H. Characterization of the Binding Properties of Sorafenib to C-MYC G-Quadruplexes: Evidence for Screening Potential Ligands. *J. Phys. Chem. B* **2023**, *127* (4), 874−883.
(17) Bag, S.; Ghosal, S.; Karmakar, S.; Pramanik, G.; Bhowmik, S. Uncovering the Contrasting Binding Behavior of Plant Flavonoids Fisetin and Morin Having Subsidiary Hydroxyl Groups (−OH) with HRAS1 and HRAS2 i-Motif DNA Structures: Decoding the Structural Alterations and Positional Influences. *ACS Omega* **2023**, *8* (33), 30315−30329.
(18) 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* (October 2022), No. 135826, DOI: 10.1016/j.foodchem.2023.135826.
(19) Das, S.; Hazarika, Z.; Sarmah, S.; Baruah, K.; Rohman, M. A.; Paul, D.; Jha, A. N.; Singha Roy, A. Exploring the Interaction of Bioactive Kaempferol with Serum Albumin, Lysozyme and Hemoglobin: A Biophysical Investigation Using Multi-Spectroscopic, Docking and Molecular Dynamics Simulation Studies. *J. Photochem. Photobiol. B Biol.* **2020**, *205* (July 2019), No. 111825.
(20) Hazra, S.; Hossain, M.; Suresh Kumar, G. Binding of Isoquinoline Alkaloids Berberine, Palmatine and Coralyne to Hemoglobin: Structural and Thermodynamic Characterization Studies. *Mol. Biosyst.* **2013**, *9* (1), 143−153.
(21) Soret, J.-L. Analyse Spectrale: Sur Le Spectre d'absorption Du Sang Dans La Partie Violette et Ultra-Violette. *Comptes rendus l'Acaďemie des Sci.* **1883**, *97*, 1269−1270.
(22) 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.
(23) Panchal, S.; Sehrawat, H.; Sharma, N.; Chandra, R. Biochemical Interaction of Human Hemoglobin with Ionic Liquids of Noscapinoids : Spectroscopic and Computational Approach. *Int. J. Biol. Macromol.* **2023**, *239* (March), No. 124227.
(24) Shao, Q.; Wu, P.; Gu, P.; Xu, X.; Zhang, H.; Cai, C. Electrochemical and Spectroscopic Studies on the Conformational Structure of Hemoglobin Assembled on Gold Nanoparticles. *J. Phys. Chem. B* **2011**, *115* (26), 8627−8637.
(25) Peng, W.; Ding, F.; Peng, Y. K.; Sun, Y. Molecular Recognition of Malachite Green by Hemoglobin and Their Specific Interactions: Insights from in Silico Docking and Molecular Spectroscopy. *Mol. Biosyst.* **2014**, *10* (1), 138−148.
(26) Bhattacharjee, P.; Sarkar, S.; Shmatova, O. I.; Nenajdenko, V. G.; Pandya, P.; Bhadra, K. Synthetic Carboline Compounds Targeting Protein: Biophysical and Biological Perspective. *J. Biomol. Struct. Dyn.* **2021**, *39* (10), 3703−3720.
(27) Bastos, M.; Abian, O.; Johnson, C. M.; Ferreira-da-Silva, F.; Vega, S.; Jimenez-Alesanco, A.; Ortega-Alarcon, D.; Velazquez-Campoy, A. Isothermal Titration Calorimetry. *Nat. Rev. Methods Prim.* **2023**, *3* (1), No. 17.
(28) Astrain-Redin, N.; Sanmartin, C.; Sharma, A. K.; Plano, D. From Natural Sources to Synthetic Derivatives: The Allyl Motif as a Powerful Tool for Fragment-Based Design in Cancer Treatment. *J. Med. Chem.* **2023**, *66*, 3703.
(29) Al-Shabib, N. A.; Khan, J. M.; Malik, A.; Tabish Rehman, M.; AlAjmi, M. F.; Husain, F. M.; Hisamuddin, M.; Altwaijry, N. Molecular Interaction of Tea Catechin with Bovine β-Lactoglobulin: A Spectroscopic and in Silico Studies. *Saudi Pharm. J.* **2020**, *28* (3), 238−245.
(30) Al-Shabib, N. A.; Khan, J. M.; Malik, A.; Rehman, M. T.; AlAjmi, M. F.; Husain, F. M.; Ahmed, M. Z.; Alamery, S. F. Molecular Interactions of Food Additive Dye Quinoline Yellow (Qy) with Alpha-Lactalbumin: Spectroscopic and Computational Studies. *J. Mol. Liq.* **2020**, *311*, No. 113215.
(31) Khan, J. M.; Malik, A.; Husain, F. M.; Alkaltham, M. S. Molecular Interaction of Sunset Yellow with Whey Protein: Multi-Spectroscopic Techniques and Computational Study. *J. Mol. Liq.* **2022**, *345*, No. 117838.
(32) Khan, J. M.; Malik, A.; Ahmed, M. Z.; Ahmed, A. SDS Modulates Amyloid Fibril Formation and Conformational Change in Succinyl-ConA at Low PH. *Spectrochim. Acta, Part A* **2022**, *267*, No. 120494.
(33) Chugh, H.; Kumar, P.; Kumar, N.; Gaur, R. K.; Dhawan, G.; Chandra, R. Ex Vivo Binding Studies of the Anti-Cancer Drug Noscapine with Human Hemoglobin: A Spectroscopic and Molecular Docking Study. *New J. Chem.* **2021**, *45* (3), 1525−1534.
(34) Gratzer, W. B.; Kollias, N. http://omlc.ogi.edu/spectra/hemoglobin/index.html.
(35) Sengupta, B.; Chakraborty, S.; Crawford, M.; Taylor, J. M.; Blackmon, L. E.; Biswas, P. K.; Kramer, W. H. Characterization of Diadzein-Hemoglobin Binding Using Optical Spectroscopy and Molecular Dynamics Simulations. *Int. J. Biol. Macromol.* **2012**, *51* (3), 250−258.
(36) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 3rd ed.; Springer, 2006.
(37) Job, P. Formation and Stability of Inorganic Complexes in Solution. *Ann. Chim.* **1928**, *9*, 113−203.
(38) 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.
(39) Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J. Comput. Chem.* **2009**, *30*, 2785−2791.
(40) Abdulmalik, O.; Ghatge, M. S.; Musayev, F. N.; Parikh, A.; Chen, Q.; Yang, J.; Nnamani, I.; Danso-Danquah, R.; Eseonu, D. N.; Asakura, T.; Abraham, D. J.; Venitz, J.; Safo, M. K. Crystallographic analysis of human hemoglobin elucidates the structural basis of the potent and dual antisickling activity of pyridyl derivatives of vanillin. *Acta. Crystallogr. D. Biol. Crystallogr.* **2011**, *67*, 920−928.
(41) Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E. UCSF Chimera−A

visualization system for exploratory research and analysis. *J. Comput. Chem.* **2004**, *25*, 1605−1612.
(42) Laskowski, R. A.; Swindells, M. B. LigPlot+: Multiple Ligand-Protein Interaction Diagrams for Drug Discovery. *J. Chem. Inf. Model.* **2011**, *51*, 2778−2786.

https://doi.org/10.1021/acsomega.3c04432
ACS Omega 2023, 8, 37054−37064