Supplementary Information

Inhibition of amyloid formation of bovine hemoglobin by bioactive phenolic acids: An elaborate investigation into their binding properties with the protein using multi-spectroscopic and computational techniques

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1. Experimental methodologies

1.1 Ground state complexation

The ground state complexation process of BHb and BHb in presence of phenolic acids, FA, CA, and CGA was investigated using UV-vis absorption studies. The measurements were executed on a PerkinElmer Lambda 365 spectrophotometer using quartz cuvette. 1.5 µM native BHb was dissolved in 20 mM phosphate buffer (pH 7.4) and its absorption spectra was recorded in the wavelength range of 200-600 nm. Similarly, the absorption spectra of BHb complexed with 4.5 µM FA/CA/CGA was also recorded in the same range. Finally, the absorption spectra of only the phenolic acids were also recorded in this range.

1.2 Inner filter effect correction

The emission profiles of BHb complexed with FA/CA/CGA were required to be corrected because these phenolic acids possess some amounts of absorbance at the λ_{ex} (295 nm) and λ_{em} of BHb (337 nm). Equation 1 was used to carry out the necessary.[1,2]

$$F_{corr} = F_{obs} \times 10^{\frac{(A_{em} + A_{ex})}{2}} \quad (1)$$

Where F_{corr} and F_{obs} are the corrected and observed fluorescence intensities, respectively. A_{em} and A_{ex} are the absorbance at the emission and excitation wavelengths, respectively. The corrected fluorescence emission spectra were used to determine certain parameters such as quenching mechanism and binding forces between BHb and the phenolic acids.

1.3 Steady state fluorescence measurements to determine quenching mechanism, binding constant and thermodynamic parameters

Steady state fluorescence of BHb in presence of increasing concentration of FA/CA/CGA in a 1 cm path length quartz cuvette, was carried out in a Cary Eclipse Fluorescence Spectrophotometer (Agilent technologies, USA). A concentration of 3 µM BHb was used which was titrated with successive additions of FA/CA/CGA (0-16.4 µM) at three

temperatures, 293, 300 and 307 K. The protein was excited at 295 nm and the emission spectra was recorded in the wavelength range of 305-500 nm, maintaining a slit width of 5/5 nm. Prior to addition of phenolic acids, the protein was equilibrated for 10 minutes at each temperature. An average of three titrations were used to determine various parameters established through steady state fluorescence.

The fluorescence emission spectra were normalized to an intensity of 1.0 at the observed λmax using Origin software prior to derivatization. The average of five scans was then subjected to smoothing using a 10-point smoothing average and a Savitzky-Golay algorithm. Finally, the second derivative of the smoothed spectrum was obtained using the same software. A smoothing step of the normalized data was required to reduce the noise in the second derivative.

The smoothing process was guided by the requirement that it maintains the original shape and intensity of the raw emission scan while also preserving the overall shape of the bands in the second derivative and eliminating excess noise.

1.4 Excited state lifetime measurements

Excited state lifetime fluorescence decay profiles of BHb and BHb-FA/CA/CGA complexes were recorded on Pico Master time correlated single photon counting (TCSPC) lifetime apparatus (PM-3) supplied by Photon Technology International (PTI), USA using an excitation wavelength of 295 nm from an LED source. The emission profiles were collected at a magic angle (54.7°) to remove any possible involvement from the anisotropy decay. Light scattered from a dilute colloidal solution consisting of dried non-dairy coffee whitener was used to determine the instrument response function (IRF). The following features were used to analyse the qualities of the graphical fit: (a) Durbin– Watson (DW) parameter (b) χ^2 values and (c) a visual scrutiny of the residuals of the fitted function to the data. The average lifetime (τ_{avg}) of the fluorophore is determined using the following equation (equation 2).[1]

$$\tau_{avg} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \qquad (2)$$

where α_i is the pre-exponential factor for the i^{th} decay time constant τ_i.

1.5 Energy transfer studies

Forster's resonance energy transfer (FRET) theory was used to determine the binding distance between the donor (Trp residue) and the acceptor (phenolic acids). The protein was excited at 295 nm and the fluorescence emission spectra were recorded for BHb (3 µM) and BHb-FA/CA/CGA complexes (1:1 molar ratio) in the wavelength region of 305-500 nm, keeping the slit width at 5/5 nm. The UV-vis spectra of the phenolic acids was also recorded in order to determine the energy transfer parameters.

1.6 Synchronous Fluorescence

Changes in and around the microenvironment of Trp and Tyr residues of the protein due to ligand binding can be determined by employing synchronous fluorescence technique. This study was also carried out in Fluoromax -4 Jobin Yvon (Horiba Scientific, Japan) fitted with a Newport temperature controller (Model 350 B, California, USA). The fluorescence emission spectra of 3 µM BHb and BHb in presence of increasing concentration of FA/CA/CGA (0-16.4 µM) was recorded at two offset wavelengths, $\Delta\lambda$=15 nm (specific for Tyr residue) and $\Delta\lambda$=60 nm (specific for Trp residue). The slit width was maintained at 5/5 nm.

1.7 Three-dimensional fluorescence studies

The instrument used to carry out three-dimensional (3D) fluorescence spectral measurements was Cary Eclipse Fluorescence Spectrophotometer (Agilent technologies, USA). The 3D spectra of 3 µM BHb and its complexes, BHb-FA/CA/CGA (molar ratio of 1:4) were carried out by recording the excitation and the emission spectra in the wavelength ranges of 200-380

nm (increment of 5 nm) and 250-500 nm (increment of 1 nm), respectively. The excitation and emission slit width were maintained at 10/5 nm, respectively.

1.8 Red edge excitation shift

Red edge excitation shift (REES) studies was carried out in Cary Eclipse Fluorescence Spectrophotometer (Agilent technologies, USA). The protein BHb (3μM) and its complexes with FA/CA/CGA (1:5 molar ratio) was excited at two different wavelength, 295 nm and 305 nm, keeping slit width fixed at 5/5 nm.

1.9 Fluorescence anisotropy measurements

The fluorescence anisotropy measurements were carried out using the following equation (equation 3).

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \qquad (3)$$

where I_{VV} and I_{VH} are the vertically and horizontally polarized components of probe emission with excitation by vertically polarized light at the respective wavelength and G is the sensitivity factor of the detection system.

Fluorescence anisotropy values of FA/CA/CGA (30 μM) alone as well as in the presence of BHb (15 μM and 30 μM) were obtained by recording emission scan for the ligands in all the four directions on a Cary Eclipse Fluorescence Spectrophotometer (Agilent technologies, USA). The measurement of temperature was done at room temperature and the excitation wavelength and emission wavelength were kept at 330 nm and 418 nm, respectively for FA, 330 nm and 422 nm, respectively, for CA, and 330 nm and 448 nm, respectively, for CGA.

1.10 Circular dichroism

The far UV circular dichroism (CD) spectra of 3 μM BHb and its 1:1 and 1:2 molar complexes with FA/CA/CGA were recorded on a Jasco 1500 CD spectrophotometer using a 0.1 cm quartz cuvette in the wavelength range of 190-240 nm. A scan rate of 100 nm/min and response time of 4 seconds was employed to collect the CD spectra. An online server called

DICHROWEB was used to calculate secondary structural alteration of native BHb and BHb-FA/CA/CGA complexes.

1.11 Fourier transform infrared (FTIR) measurements

The Fourier transform infrared (FTIR) spectra of BHb and BHb-FA/CA/CGA complexes were recorded on a PerkinElmer Spectrum Two spectrometer equipped with a single reflection diamond universal attenuated total reflectance (UATR) accessory. A stock solution of 10 mg/mL of BHb was prepared in 20 mM phosphate buffer (pH 7.4) and the concentration was determined spectrophotometrically. A 1:2 complex with FA/CA/CGA was then prepared and incubated for 30 mins prior to measurement. Each individual spectrum collected was corrected by subtracting the corresponding blank spectrum. The FTIR spectra of BHb and its complexes with the FA/CA/CGA was recorded using a 256 scan interferogram with a 4 cm^{-1} resolution

Using Byler and Susi method,[3] the secondary structural components of native BHb and BHb-FA/CA/CGA complexes were evaluated from amide I peak (1600-1700 cm^{-1}) due to its conformational sensitivity. The elimination of noises present in the corrected spectra has been done by smoothening at 15-point Savitzky-Golay smooth function.[4] With the help of Fourier self deconvolution and second derivative methodology the major peaks of the smoothed spectra were resolved. The Gaussian curve technique aided in determining the total area under the curve and the area corresponding to each secondary structural component in the region of 1600-1700 cm^{-1}.

1.12 Anti-oxidant activity of phenolic acids and BHb bound phenolic acids

The anti-oxidant properties of phenolic acids were determined in absence and presence of protein based on their DPPH radical scavenging activity. 30 μM BHb was incubated with 15 μM individual phenolic acids in dark for 10 minutes prior to DPPH assay. The absorbance of DPPH (0.1 mM) was measured at 517 nm in presence of FA, CA and CGA and BHb-

FA/CA/CGA complexes. The DPPH radical scavenging activity was calculated using the following equation (equation 4)

$$\%\ DPPH\ scavenging\ acitivity = \left[1 - \frac{A_{Test}}{A_{Control}}\right] \times 100 \qquad (4)$$

1.13 Anti-fibrillation studies

15 μM bovine hemoglobin was incubation in presence of 30 mM fructose in 50 mM phosphate buffer (pH 7.4). In order to test the anti-fibrillating properties of FA/CA/CGA, 150 μM concentration of these phenolic acids was added to the solution. Sodium azide (1 mM) was added to the solutions to prevent bacterial growth. The solutions were finally incubated at 37 ºC for 20 days. Control samples were prepared in a similar way in the absence of fructose. Sampling was carried out in appropriate time intervals upto 20 days of incubation.

1.13.1 Thioflavin T (ThT) fluorescence assay

ThT fluorescence was measured in a 1 cm path length quartz cell to monitor aggregation of BHb. The following parameters were adjusted for monitoring ThT fluorescence intensity during aggregation experiments: λ_{ex}=440 nm, λ_{em} =460–600 nm. Final concentration of protein in the sample was 1.5 μM whereas the concentration of ThT was 15 μM. Prior to ThT fluorescence assessment, all of the samples were pre-incubated for 10 minutes at room temperature in the dark.

1.13.2 8-Anilino-1-naphthalene-sulphonic acid (ANS) fluorescence measurements

ANS binding was measured by fluorescence emission spectra with excitation at 380 nm and emission was recorded from 400 to 600 nm. Typically, ANS concentration was 100 molar excess of the protein concentration and protein concentration was in the vicinity of 3 μM.

1.13.3 Turbidity Assay

The UV-vis absorption of unfibrillated BHb, fibrillated BHb and fibrillated BHb in presence of phenolic acids was recorded at 350 nm. The final concentration of the samples was kept 3 μM.

1.13.4 Congo red (CR) assay

Absorption spectroscopy was used to analyze the formation of aggregates with the help of CR dye. Here, 6 μM of the protein solution and protein solution in presence of phenolic acids were aliquot and mixed with 20 μM CR and 50 mM phosphate buffer, pH 7.4. The spectra were recorded from 400-600 nm.

1.13.5 Soret absorption spectroscopy

Soret absorption of the heme group of un-fibrillated BHb, fibrillated BHb and fibrillated BHb in presence of FA/CA/CGA was monitored using absorption spectroscopy. The final concentration of the protein in the solution was kept at 3 μM and the spectra was recorded from 200-600 nm.

1.13.6 Fluorescence microscopy

Fluorescence microscopy was carried out to visualize the fibril formation of BHb in absence and presence of phenolic acids. In this experiment, the ThT fluorescence of the fibrils was monitored in fluorescence microscope using an excitation filter of wavelength 365-460 nm.

1.13.7 Far-UV and Near-UV Circular dichroism (CD) studies

The far-UV CD measurements of 3 μM native BHb, fibrillated BHb and fibrillated BHb in the presence of phenolic acids was recorded the 190-240 nm wavelength range. Similarly, the near-UV CD spectra of 100 μM native BHb, fibrillated BHb and BHb in the presence of phenolic acids were recorded in the wavelength range of 240-320 nm. A Jasco 1500 CD spectrophotometer using a 0.1 cm quartz cuvette was used to carry out the above experiment. A scan rate of 100 nm/min and a response time of 4 seconds was employed to collect the CD spectra. An online server called DICHROWEB was used to calculate secondary structural alteration.[5]

Supplementary Tables

Table S1. K_{sv} and k_q values of BHb complexed with phenolic acids at three temperatures

Complexes	Temperature (K)	K_{SV} (M^{-1}), 10^4	k_q (M^{-1} s^{-1}), 10^{13}
BHb-FA	293	5.056±0.664	2.825±0.642
	300	3.943±0.185	2.203±0.179
	307	3.406±0.436	1.903±0.422
BHb-CA	293	5.313±0.378	2.968±0.210
	300	4.030±0.201	2.251±0.112
	307	2.876±0.351	1.607±0.196
BHb-CGA	293	4.530±0.259	2.530±0.144
	300	3.460±0.015	1.932±0.083
	307	2.983±0.047	1.666±0.026

Table S2. Comparison of binding constant values using two equations, double logarithmic equation and modified Stern-Volmer equation

Complexes	Temp (K)	Double logarithmic equation	Modified Stern-Vomer equation	
		K_b (10^4, M^{-1})	K_a (10^4, M^{-1})	f_a
BHb-FA	293	7.250±0.272	7.223±0.357	0.406±0.289
	300	6.576±0.222	6.104±0.211	0.449±0.242
	307	6.148±0.087	5.227±0.117	0.525±0.198
BHb-CA	293	7.512±0.212	7.987±0.362	0.392±0.331
	300	6.787±0.132	6.977±0.116	0.485±0.134
	307	6.154±0.110	5.584±0.267	0.588±0.219
BHb-CGA	293	6.411±0.296	6.809±0.232	0.394±0.123
	300	5.866±0.083	5.835±0.135	0.489±0.097
	307	5.420±0.049	5.384±0.108	0.432±0.125

Table S3. Comparison of thermodynamic parameters determined using the binding constant values obtained using double logarithmic equation and modified Stern-Volmer equation

Complexes	Temp (K)	Double logarithmic equation			Modified Stern-Vomer equation		
		ΔH^o (kJ mol^{-1})	ΔS^o (J mol^{-1} K^{-1})	ΔG^o (kJ mol^{-1})	ΔH^o (kJ mol^{-1})	ΔS^o (J mol^{-1} K^{-1})	ΔG^o (kJ mol^{-1})
BHb-FA	293	-(8.539±1.331)	+(63.862±1.218)	-(27.348±0.165)	-(7.185±1.215)	+(60.353±0.976)	-(25.031±0.287)
	300			-(27.703±0.072)			-(25.368±0.109)
	307			-(28.142±0.043)			-(25.873±0.098)
BHb-CA	293	-(10.620±1.028)	+(67.108±1.681)	-(27.353±0.073)	-(11.650±1.187)	+(69.543±1.432)	-(29.986±0.932)
	300			-(27.753±0.043)			-(30.411±0.162)
	307			-(28.153±0.040)			-(30.972±0.843)
BHb-CGA	293	-(8.637±0.232)	+(62.540±1.058)	-(26.961±0.103)	-(10.376±0.952)	+(58.450±1.138)	-(27.614±0.113)
	300			-(27.399±0.050)			-(27.921±0.168)
	307			-(27.837±0.054)			-(28.335±0.081)

Table S4. Energy transfer variables for the BHb-FA, BHb-CA, and BHb-CGA systems

Complex	E	J(λ) x10^{-15} M^{-1}cm^3	R_0 (nm)	r (nm)	k_T
BHb-FA	0.096	8.685	2.391	3.470	0.059
BHb-CA	0.074	4.868	2.171	3.302	0.046
BHb-CGA	0.096	11.123	2.491	3.620	0.060

Table S5. Synchronous fluorescence data of Stoke's shift and fluorescence intensities of (a) BHb-FA (b) BHb-CA (c) BHb-CGA complexes at offset wavelength Δλ=60 nm and Δλ=15 nm

System		Peak (nm)	Stokes shift Δλ (nm)	Fl. Intensity
BHb	**Offset Δλ =60 nm**	344	2	345380
BHb-FA		346		152906
BHb		344	3	348213
BHb-CA		347		147616
BHb		344	2	387186
BHb-CGA		346		212110
BHb	**Offset Δλ =15 nm**	308	0	132940
BHb-FA		308		58490
BHb		308	0	139363
BHb-CA		308		54910
BHb		308	1	142980
BHb-CGA		309		66743

Table S6. 3D spectral data of BHb and its 1:4 complexes with FA, CA, and CGA

System	Peak 1 (nm)		Stokes shift	Intensity	Peak 2 (nm)		Stokes shift	Intensity
	λ_{ex}	λ_{em}	$\Delta\lambda$ (nm)	(a.u.)	λ_{ex}	λ_{em}	$\Delta\lambda$ (nm)	(a.u.)
BHb	275	333	58	111.755	230	332	102	64.517
BHb-FA	275	338	63	83.351	230	339	109	49.690
BHb-CA	275	336	61	93.417	230	336	106	55.918
BHb-CGA	275	335	60	95.330	230	335	105	56.947

Table S7. Binding energies of different conformers of BHb complexed with phenolic acids

Complexes	Binding energies (kcal/mol)	Interacting residues
BHb-FA	-5.5	α_1-Arg41, α_1-Thr137, α_1-Ser138, α_1-Lys139, α_1-Tyr140, α_2-Lys127, α_2-Asp126, α_2-Ala130, β_2-Tyr35, β_2-Pro36, β_2-Trp37, and β_2-Leu105
	-5.4	
	-5.3	
	-4.6	
	-4.2	
	-4.2	
	-4.1	
	-4.1	
	-3.9	
BHb-CA	-6.2	α_1-Tyr140, α_1-Thr137, α_1-Ser138, α_1-Arg141, α_2-Ala123, α_2-Lys127, β_2-Val34, β_2-Val33, β_2-Tyr35, β_2-Trp3,7and β_2-Pro36
	-6.0	
	-5.3	
	-5.3	
	-4.8	
	-4.7	
	-4.5	
	-4.4	
	-4.4	
BHb-CGA	-8.0	α_1-Thr137, α_1-Tyr140, α_2-Lys99, α_2-Ser102, α_1-Pro95, α_2-His103, α_2-Leu106, α_2-Asp126, α_2-Lys127, β_2-Val34, β_2-Tyr35, β_2-Trp37, β_2-Asn108, β_2-Glu101, β_2-Lys104, and β_2-Leu105
	-7.2	
	-7.1	
	-7.0	
	-6.8	
	-6.8	
	-6.4	
	-6.4	
	-6.3	

Table S8. Free energy of binding of ligands with each chain of the protein in BHb-CGA, BHb-CA, and BHb-FA complexes (from MD Simulation studies)

Chains (BHb)	ΔG_{bind} (kJ mol^{-1})		
	BHb-CGA	BHb-CA	BHb-FA
1	-28.04	-9.48	-14.02
2	-20.36	-26.07	-33.84
3	-2.84	-8.90	-10.14
4	-35.25	-13.46	-35.43

Supplementary Figures

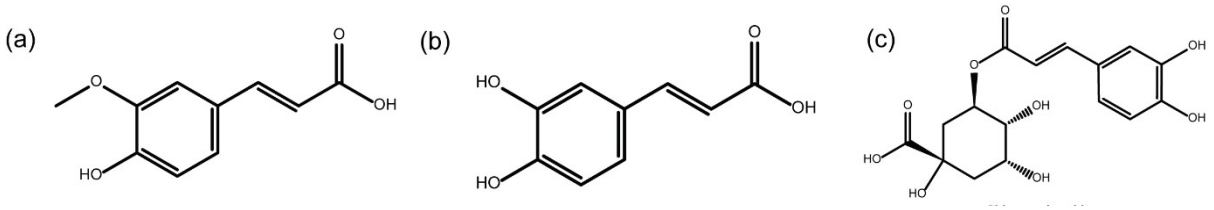

Fig. S1 2D illustrations of (a) FA, (b) CA, and (c) CGA.

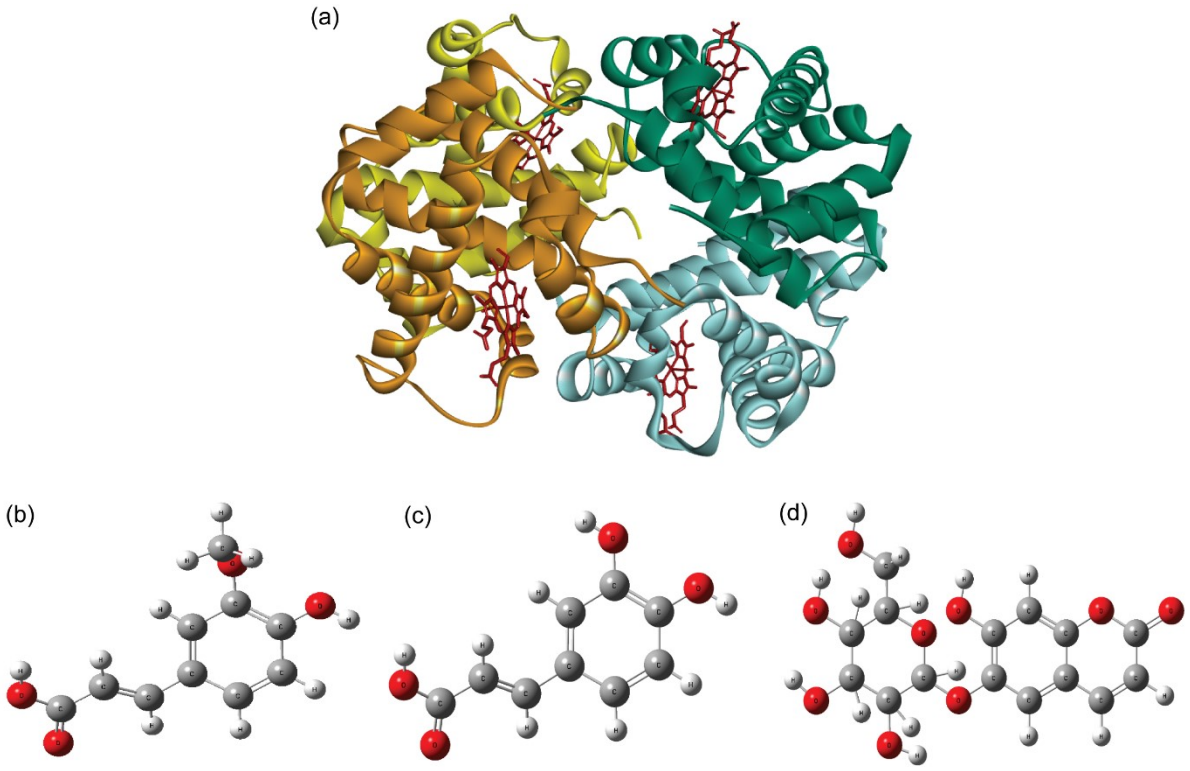

Fig. S2 (a) Crystal structure of bovine hemoglobin with PDB ID 1G09. (Chain α_1- orange, Chain α_2-green, Chain β_1- yellow and Chain β_2- grey, and heme moiety-red). Optimized structures of (b) FA, (c) CA, and (d) CGA (carbon- grey, hydrogen- white, and oxygen- red).

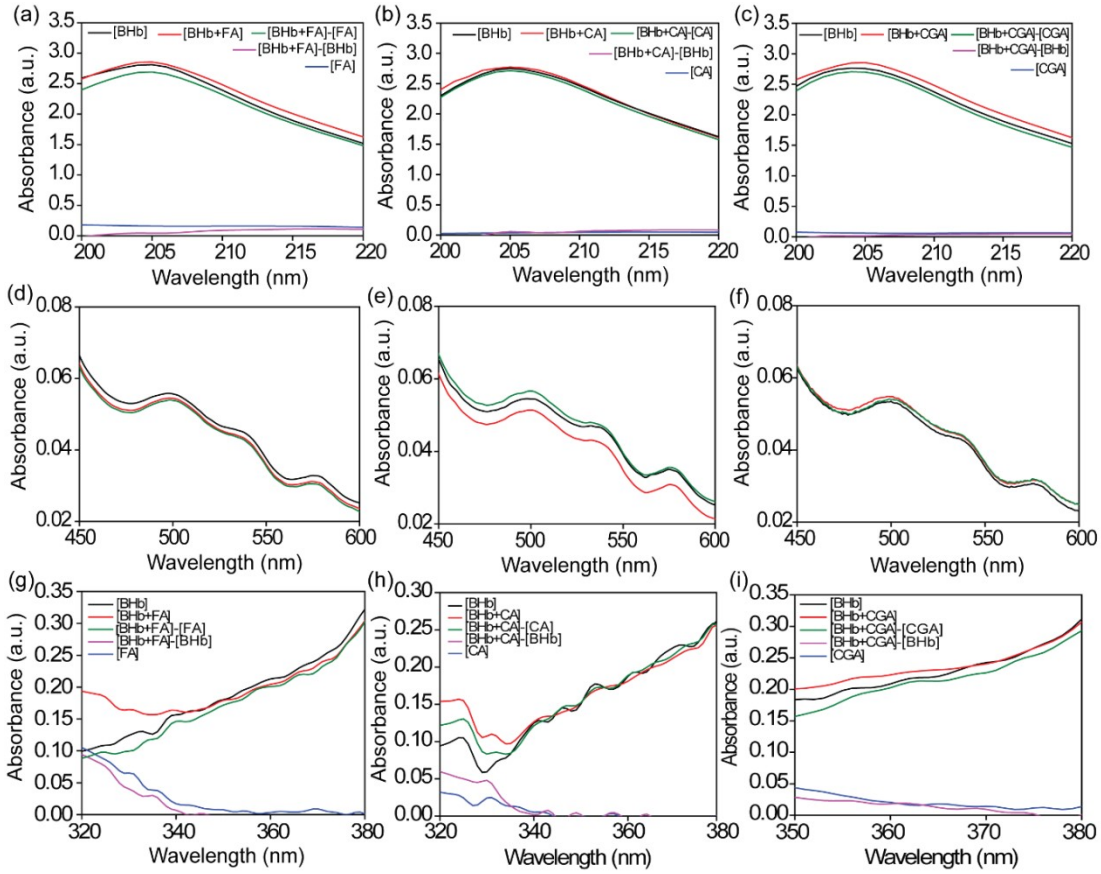

Fig. S3 UV-vis spectra of the wavelength region of 210 nm of BHb complexed with (a) FA, (b) CA, and (c) CGA. UV-vis spectra of oxy bands occurring in the wavelength region of 450-600 nm of BHb complexed with (d) FA, (e) CA, and (f) CGA. (g-i) UV-vis spectra displaying isosbestic points for BHb complexed with FA, CA, and CGA, respectively.

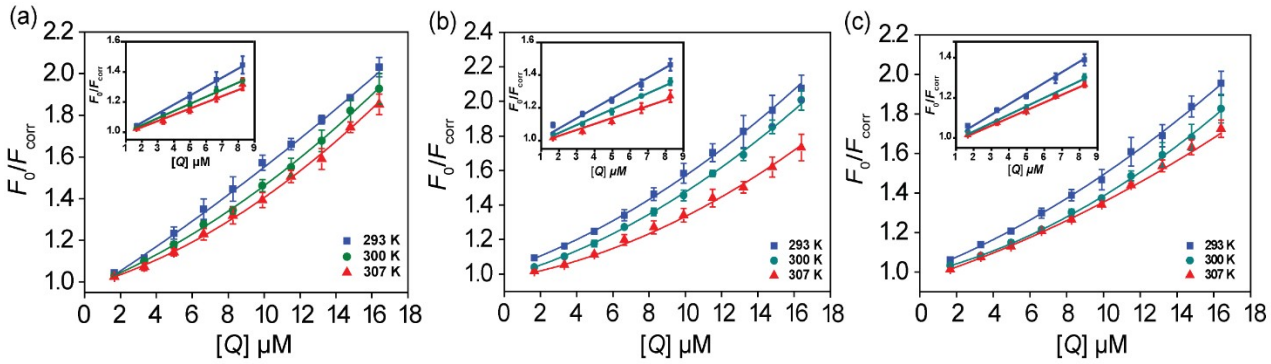

Fig. S4 Stern-Volmer plots of BHb complexed with (a) FA, (b) CA, and (c) CGA at three temperatures, 293, 300, and 307 K. Inset: Linear region on Stern-Volmer plot of BHb bound to the corresponding phenolic acids. [BHb]= 3μM. [FA]=[CA]=[CGA]= 0-16.4 μM.

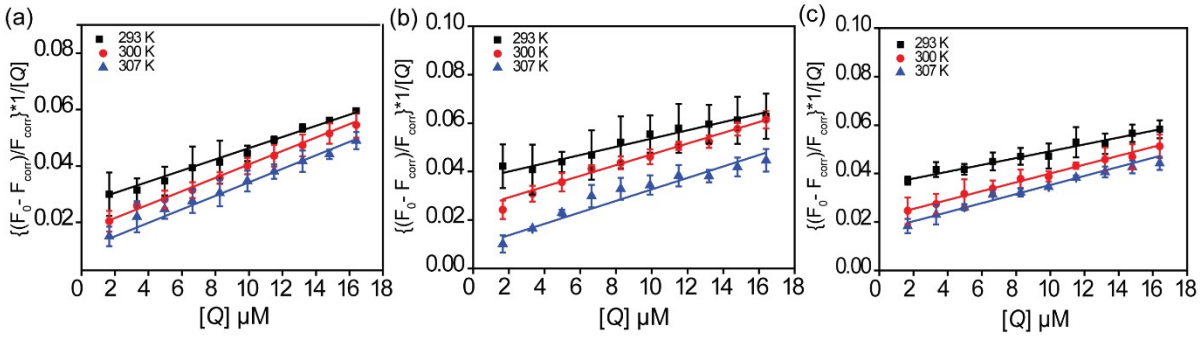

Fig. S5 Regression plots of BHb bound to (a) FA, (b) CA, and (c) CGA at three temperatures, 293, 300, and 307 K.

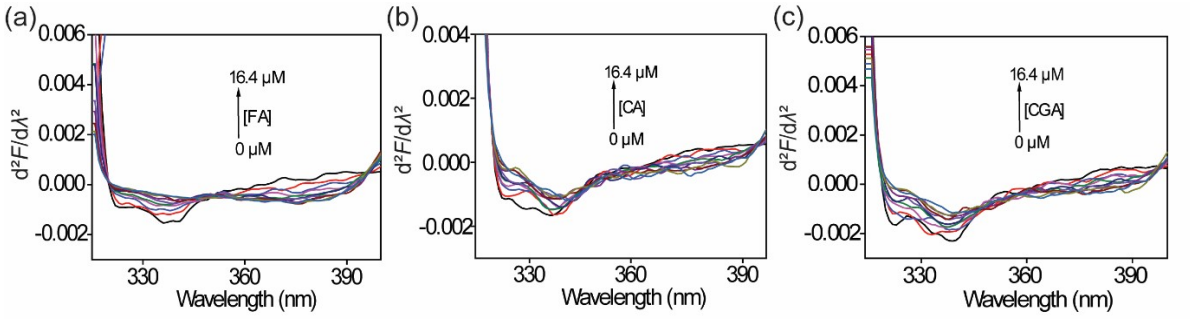

Fig. S6 Second derivative fluorescence spectra of BHb complexed with increasing concentrations of (a) FA, (b) CA, and (c) CGA.

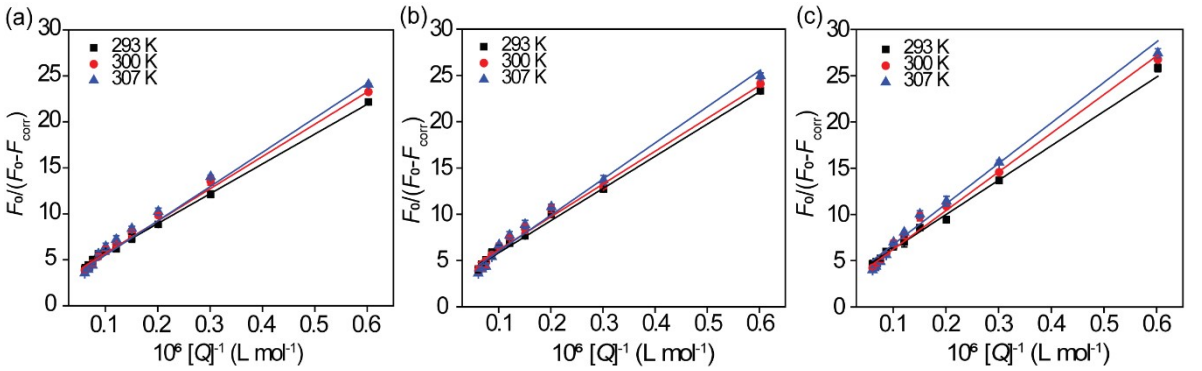

Fig. S7 Modified Stern-Volmer plots of BHb complexed with (a) FA, (b) CA, and (c) CGA at three different temperatures.

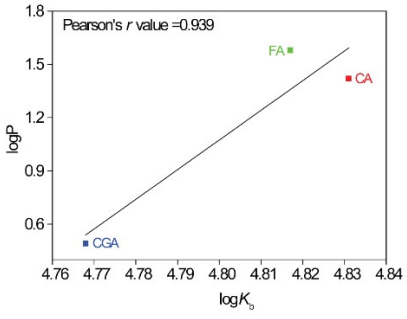

Fig. S8 Relationship between logK_b versus logP values of the phenolic acids and BHb at 300 K.

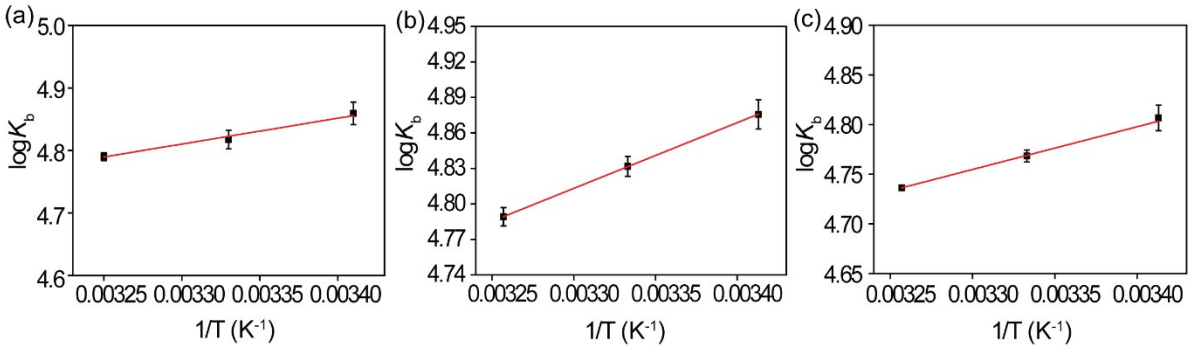

Fig. S9 van't Hoff plots of BHb in complexation with (a) FA, (b) CA, and (c) CGA at three temperatures, 293, 300, and 307 K.

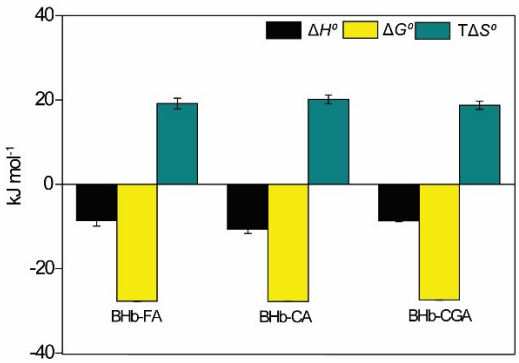

Fig. S10 Bar graph representation of thermodynamic parameters of BHb-FA, BHb-CA, and BHb-CGA complexes at 300 K.

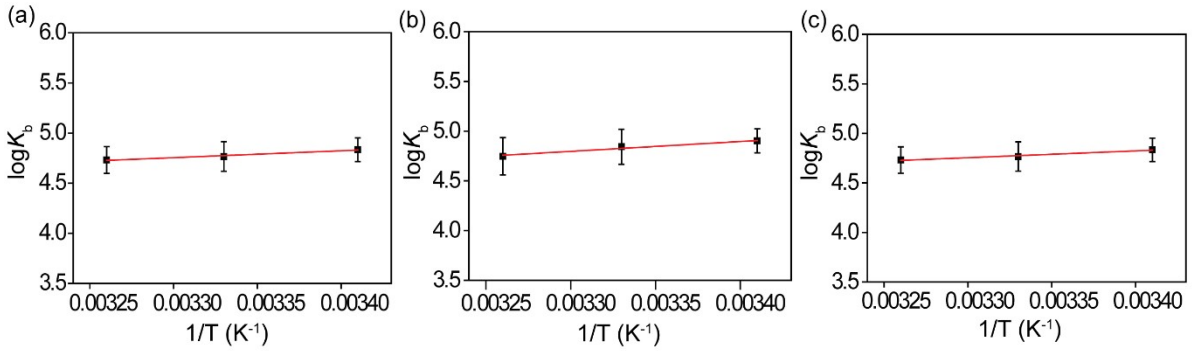

Fig. S11 van't Hoff plots of BHb complexed with (a) FA, (b) CA, and (c) CGA at three temperatures, 293, 300, and 307 K.

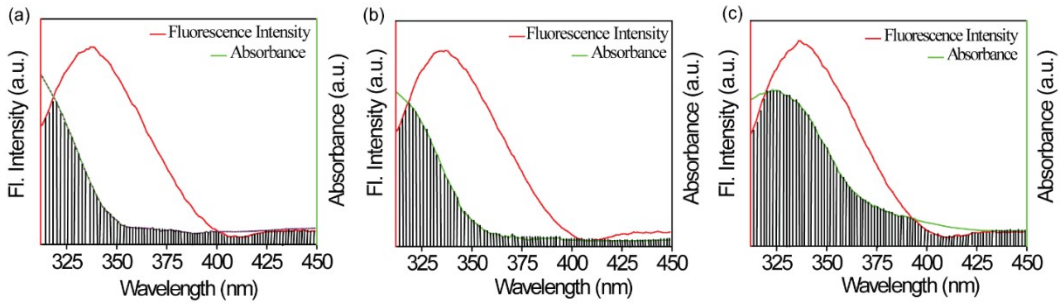

Fig. S12 Spectral overlap of fluorescence emission profile of BHb and absorbance spectra of (a) FA, (b) CA, and (c) CGA. [BHb] =3 µM.

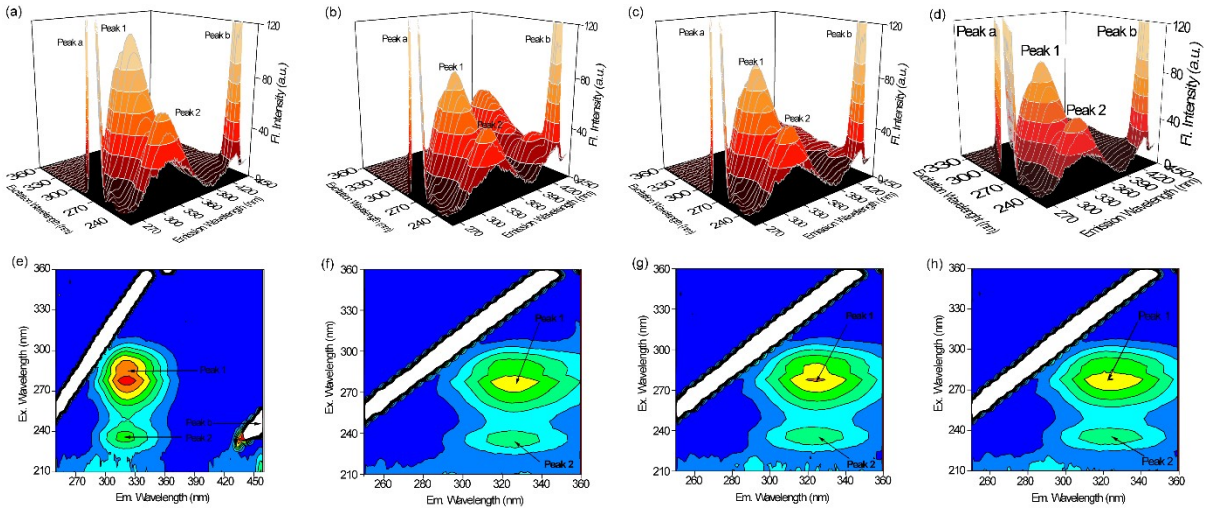

Fig. S13 3D spectral profiles of (a) native BHb and BHb in the presence of (b) FA, (c) CA, and (d) CGA along with their respective contour plots. [BHb]=3 µM.

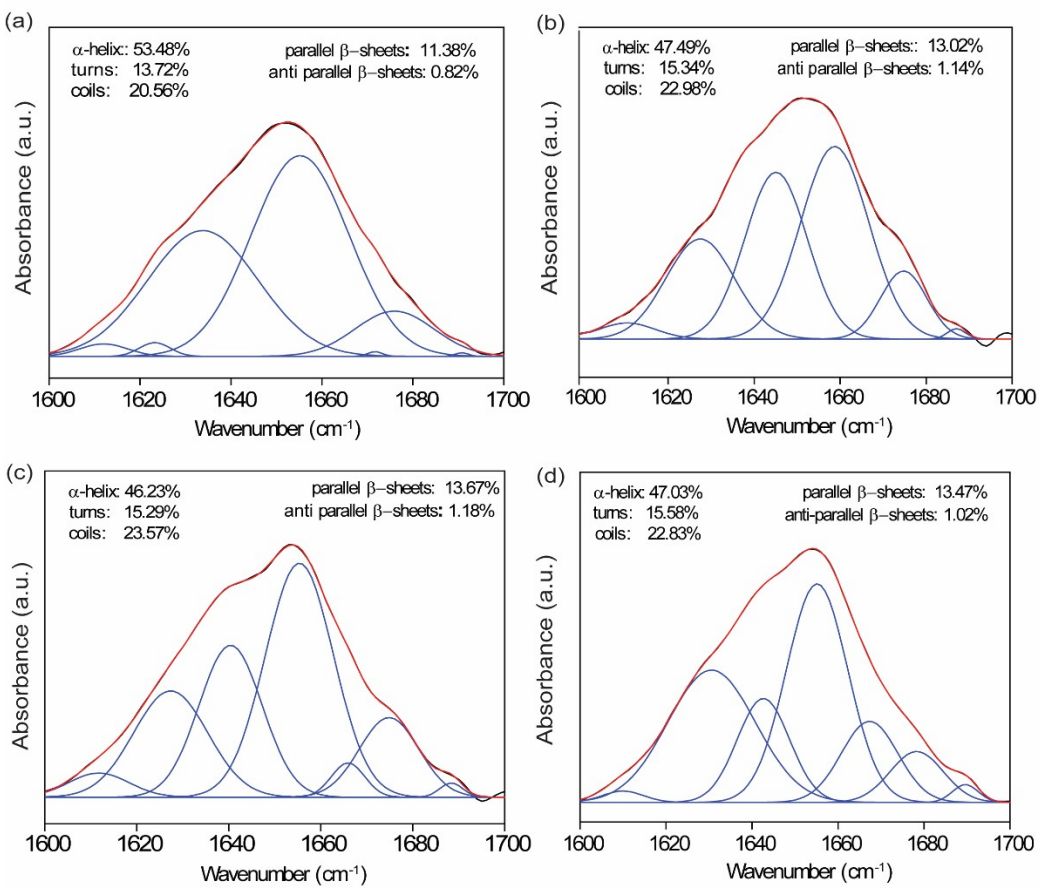

Fig. S14 Deconvolution plots of Amide I peak of BHb in complexation with (a) native BHb, (b) FA, (c) CA, and (d) CGA.

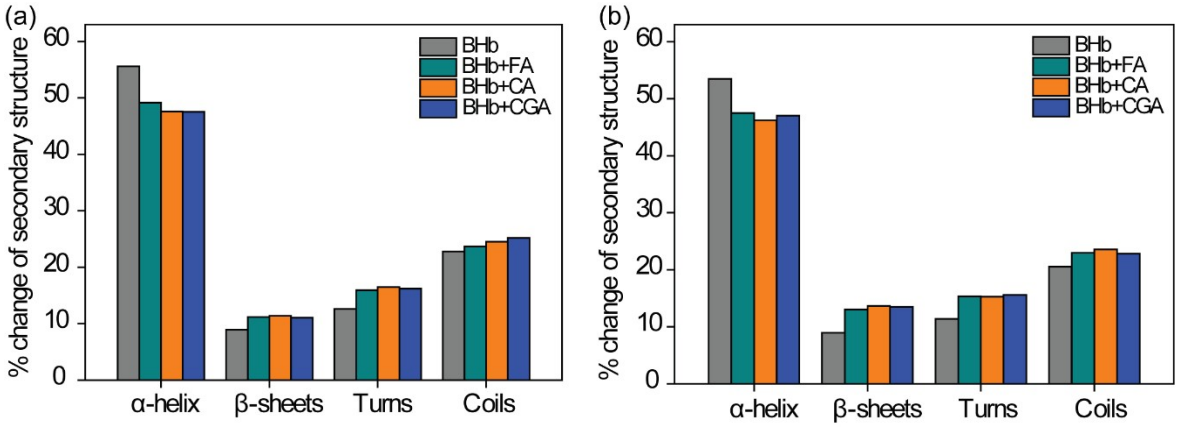

Fig. S15 Comparative bar graph representation of change in the secondary structure of BHb in the presence of FA/CA/CGA employing two methodologies: (a) Far-UV CD studies and (b) FT-IR measurements.

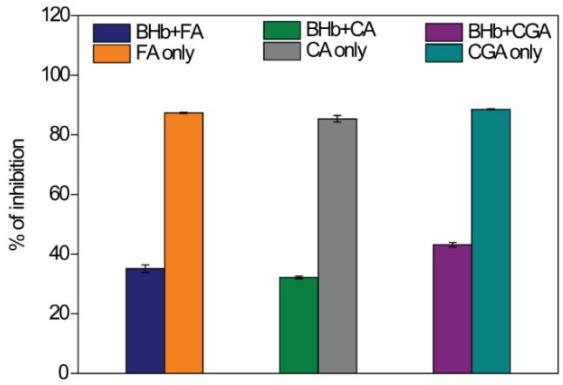

Fig. S16 Bar graph representation of the percentage of radical scavenging activity of phenolic acids and phenolic acids bound to BHb.

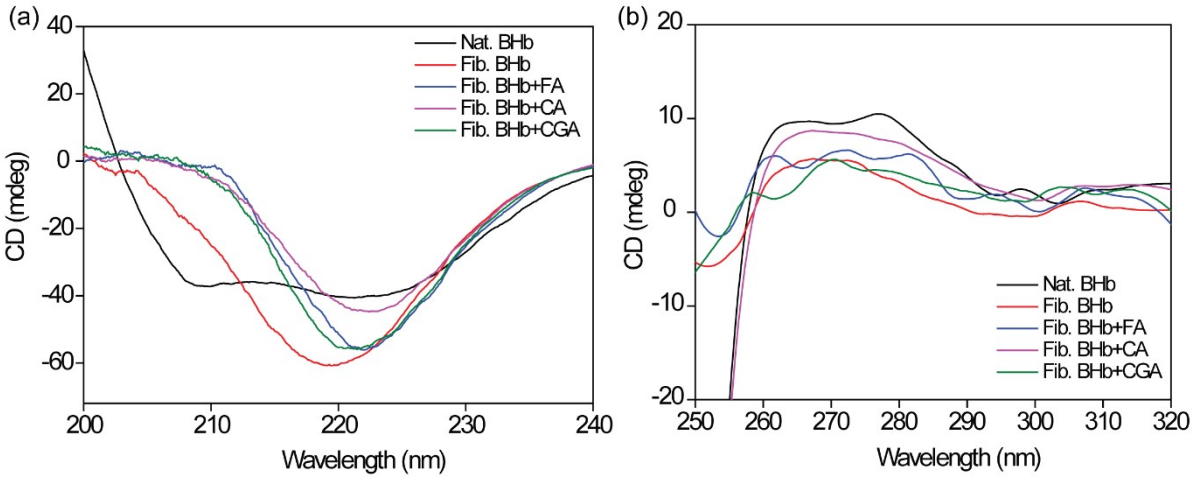

Fig. S17 (a) Far-UV CD spectra and (b) Near-UV CD spectra of native BHb and fibrillated BHb in the absence and presence of FA, CA, and CGA.

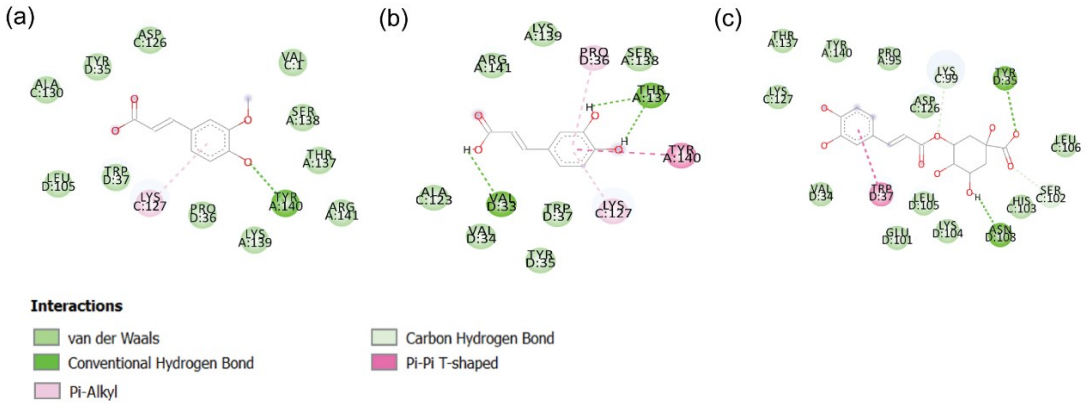

Fig. S18 2D plots of BHb docked with (a) FA, (b) CA, and (c) CGA showing the interacting amino acid residues and type of interacting forces existing.

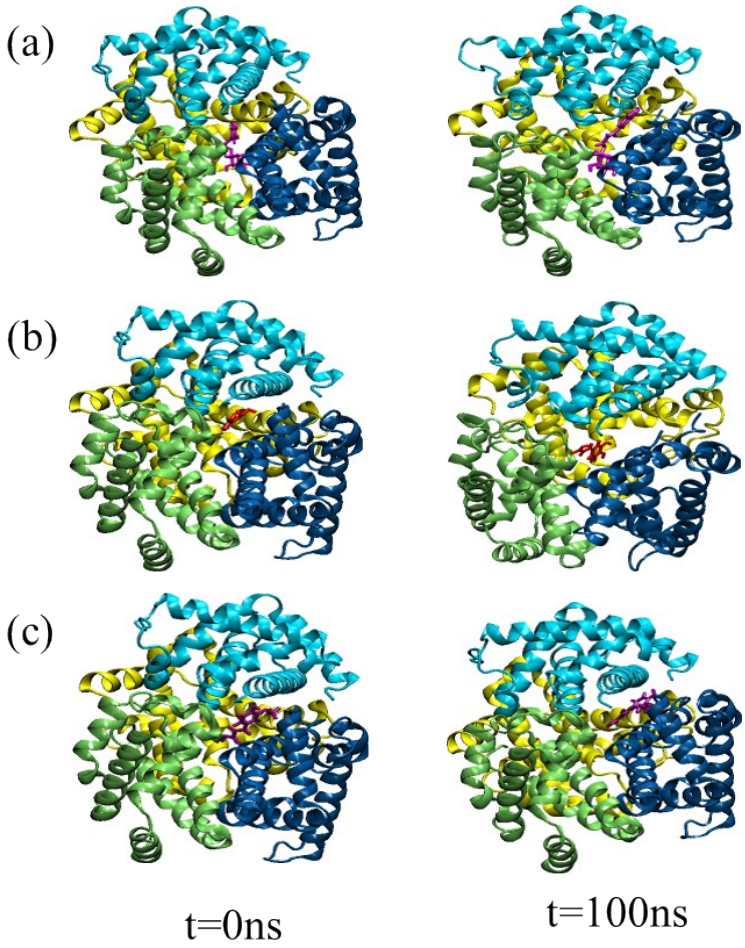

Fig. S19 (a) Represents BHb-CGA complex, (b) Represents BHb-CA complex, and (c) Represents BHb-FA complex at (i) t=0 ns and (ii) t=100 ns. Here, the color codes cyan, yellow, blue, and lime represent Chain-1, Chain-2, Chain-3, and Chain-4 of BHb protein, and Ligand CGA, CA, and FA are represented by magenta, red, and orange, respectively.

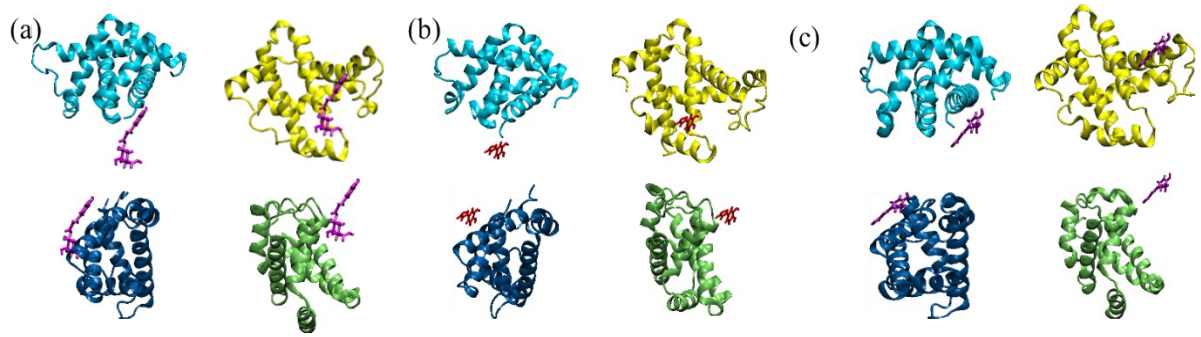

Fig. S20 A representative snapshot of the position of the ligands with 4 chains of BHb protein in (a) BHb-CGA complex, (b) BHb-CA complex, and (c) BHb-FA complex at t =100 ns.

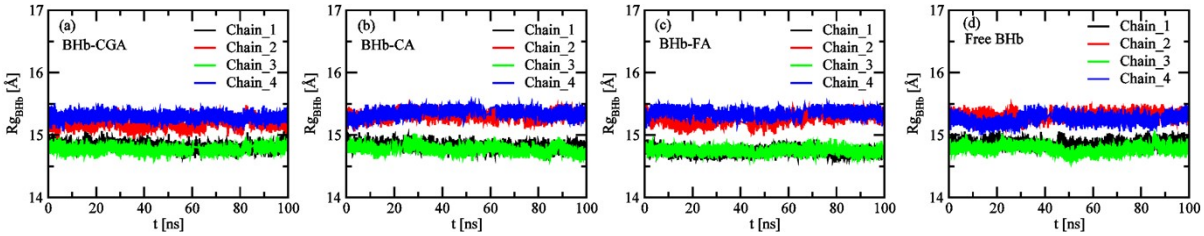

Fig. S21 Time-dependent R_g plots of four protein chains in their complexed states in (a) BHb-CGA, (b) BHb-CA, (c) BHb-FA, and (d) Free BHb, respectively.

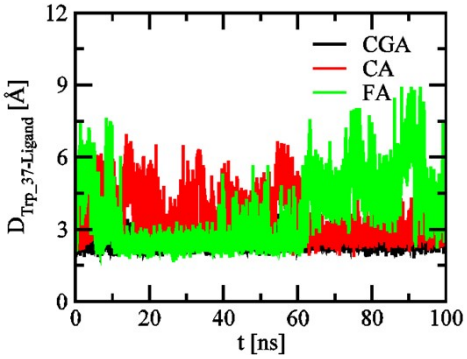

Fig. S22 Distance of β_2-Trp-37 residue with ligands (CGA, CA, and FA) throughout the simulation trajectory.

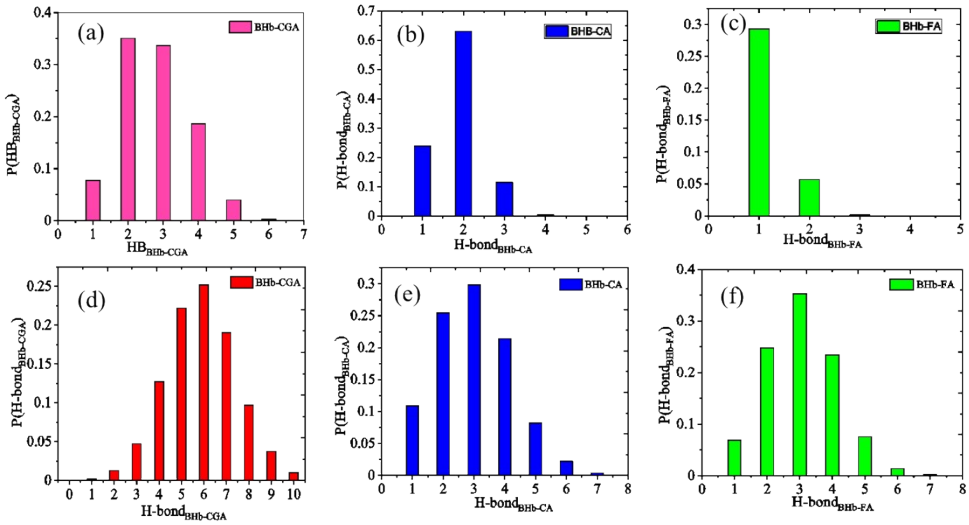

Fig. S23 Probability of the number of direct hydration bonds formed between BHb protein and the three different ligands CGA, CA, FA (a-c) as well as the hydrogen bonds formed between ligands and water (d-f).

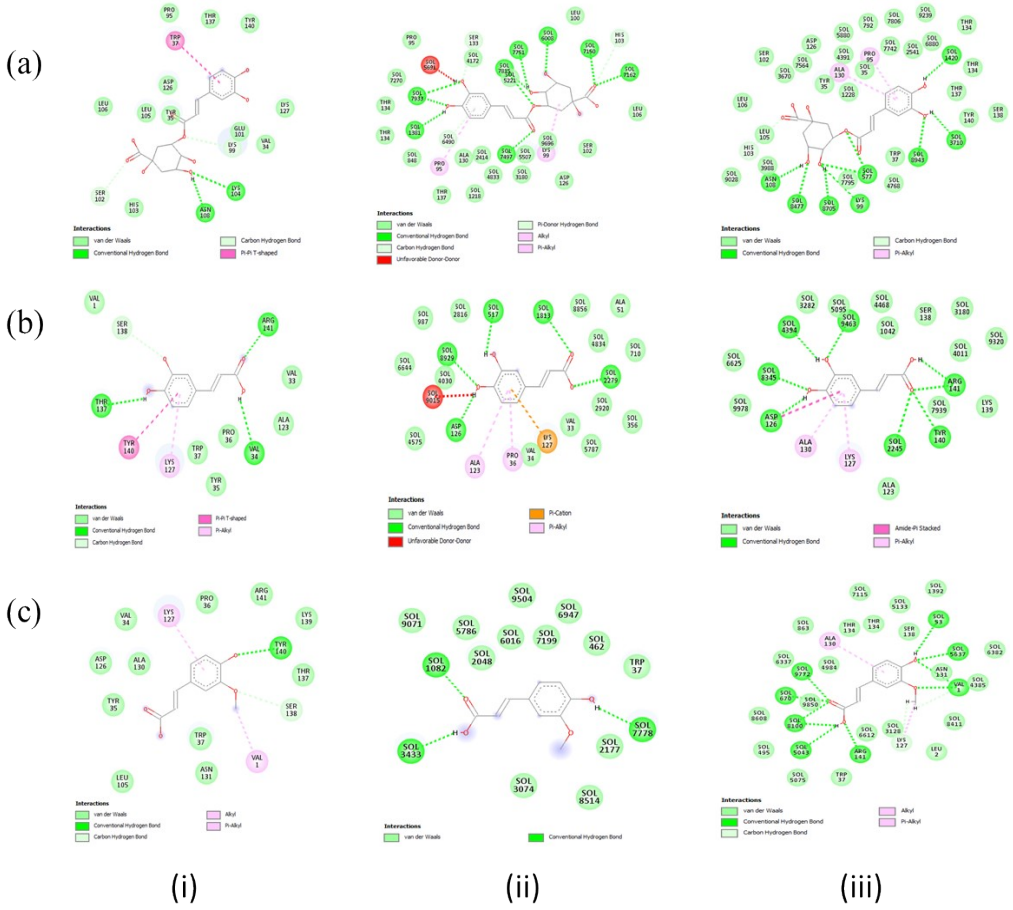

Fig. S24 2D interaction plot (a) BHb-CGA, (b) BHb-CA and (c) BHb-FA complexes at (i) t= 0 ns, (ii) t =50 ns and (iii) t =100 ns.

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