# Supplementary Information

# In-vitro Interactions of Esculin and Esculetin with Bovine Hemoglobin alter its Structure and Inhibit Aggregation: Insights from Spectroscopic and Computational Studies

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## 1. Experimental Methodologies

### 1.1 Inner filter effect correction

As esculin and esculetin possesses certain amount of absorbance at the excited and emission wavelength of BHb (295 nm and 337 nm, respectively), it became a necessity to correct the emission profiles for inner filter effect. The following equation (eqn. 1) was used in order to do so.[1,2]

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

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 esculin/esculetin.

### 1.2Steady state and excited state fluorescence

Temperature studies of BHb in presence of increasing concentration of esculin and esculetin, in a 1 cm path length quartz cuvette, was carried out in a Fluoromax -4 Jobin Yvon (Horiba Scientific, Japan) fitted with a Newport temperature controller (Model 350 B, California, USA). 3 μM BHb was titrated, separately, with successive additions of esculin and esculetin (0-16.4 μM) and the fluorescence intensity was recorded at three temperatures, 288, 298 and 308 K, on exciting the Trp residue at 295 nm. The emission spectrum was recorded in the wavelength region of 305-500 nm using a slit width of 5/5 nm.

The binding distance between the donor (Trp fluorophore) and the acceptor (coumarin derivatives) can be calculated based on Forster's theory.[3]The protein was excited at 295 nm and the fluorescence emission spectra were recorded for BHb (3 μM) and BHb-Esculin/Esculetin 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 coumarin derivatives was also collected in order to determine the energy transfer parameters.

Synchronous fluorescence was carried out in the previously mentioned instrument for BHb (3 μM) and BHb in presence of increasing concentration of esculin/ esculetin (0-16.4 μM) at two offset wavelengths, i.e., Δλ=15 nm (specific for Tyr residue) and Δλ=60 nm (specific for Trp residue) maintaining the slit width at 5/5 nm.[4]

Investigation into microenvironmental changes around the Trp residue, induced due to ligand binding, was carried out through 3-D fluorescence measurements. The 3-D spectra of BHb (3 μM) and its 1:4 complexes with BHb-Esculin/Esculetin was recorded in a Cary Eclipse Fluorescence Spectrophotometer (Agilent technologies, USA) using an excitation range of 200-380 nm with 5 increments and emission range was set at 250-500 nm with a 1 nm interval. The excitation and emission slit width were maintained at 10/5 nm, respectively.

Red edge excitation shift (REES) studies were executed of a Fluoromax-4 instrument by exciting 3μM BHb and its 1:5 complexes (BHb-Esculin/Esculetin) at two wavelength, 295 nm and 305 nm, keeping the slit width at 5/5 nm.

Fluorescence anisotropy readings were recorded by exciting the samples and recording the emission at appropriate wavelengths. The anisotropy values ($r$) were calculated using the following expression (eqn. 2).[5]

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

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 esculin and esculetin (30 μM) alone as well as in the presence of BHb (15 μM and 30 μM) were obtained

by recording emission scan for the coumarin derivatives in allthe four directions on a Cary Eclipse Fluorescence Spectrophotometer (Agilent technologies, USA). The measurement was carried out at room temperature and the excitation and emission wavelength were kept at 336 nm and 409 nm, respectively, for esculin, and 350 nm and 467 nm, respectively, for esculetin.

Excited state lifetime fluorescence decay profiles of BHb and BHb-Esculin/Esculetin 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) $\chi^2$ values and (c) a visual scrutiny of the residuals of the fitted function to the data.The average lifetime ($\tau_{avg}$) of the fluorophore is determined using the following equation (eqn. 3).[5]

$$\tau_{avg} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \qquad (3)$$

where $\alpha_i$ is the pre-exponential factor for the $i^{th}$ decay time constant $\tau_i$.

**1.3 UV/vis absorption studies**

UV/vis absorption measurements were executed on a PerkinElmer Lambda 365 spectrophotometer using quartz cuvette in the range of 200-700 nm. The absorption spectra of 1.5 μM native BHb dissolved in 20 mM phosphate buffer (pH 7.4) as well as its 1:3 complex with esculin and esculetin was recorded in this wavelength region of 200-700 nm. The absorption spectra of only the coumarin derivatives was also recorded in this range.

### 1.4 Circular dichroism

The far UV CD spectra of BHb and BHb-Esculin/Esculetin complexeswere recordedon a Jasco 1500 CD spectrophotometer using a 0.1 cm quartz cuvettein 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. DICHROWEB was used to calculate secondary structural alteration of the native protein and protein in the presence of the coumarin derivatives.[6] For this experiment, a solution of 3 μM BHb and its complexes with coumarin derivatives, at a molar ratio of 1:1 and 1:2, were scanned in the region of 190-240 nm.

### 1.5 Fourier transform infrared measurements

A PerkinElmer Spectrum Two spectrometer equipped with a single reflection diamond universal attenuated total reflectance (UATR) accessory was used to record the Fourier transform infrared (FTIR) spectra of BHb and its BHb-Esculin/Esculetin complexes. 20 mM phosphate buffer (pH 7.4) was used to dissolve 10 mg/mL of BHb after which its concentration was determined spectrophotometrically. A 1: 2 molar ratio of the complexes were prepared from the stock solution and incubated 30 minutes prior to recording the spectra. A 256 scan interferogram employing a 4 $cm^{-1}$ resolution was employed to record the FTIR spectra. The blank spectrum was subtracted from each corresponding spectra to obtain the corrected spectra.

Using Byler and Susi method,[7]the secondary structural components of native BHb and BHb-Esculin/Esculetin 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.[8]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 $cm^{-1}$.

### 1.6 Fibrillation inhibition studies

15 μM bovine haemoglobin was incubation in presence of 30 mM fructose in 50 mM phosphate buffer (pH 7.4).[9] In order to test the anti-fibrillating properties of esculin and esculetin, 150 μM concentration of these coumarin derivatives was added to the solution. Sodium azide (1 mM) was added to the solutions to prevent bacterial growth.[10] 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.

#### 1.6.1 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: $\lambda_{ex}$= 440 nm, $\lambda_{em}$ = 460–600 nm.[11]Final concentration of protein in the sample was 1.5μM whereas the concentration of ThT was 15 μM. Prior to ThT fluorescence assessment, all of the samples were pre-incubated for 10 minutes in room temperature in the dark.

#### 1.6.2 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.[12]Typically, ANS concentration was 100 molar excess of the protein concentration and protein concentration was in the vicinity of 3 μM.[13]

### 1.6.3 Soret absorption spectroscopy

Soret absorption of the heme group of un-fibrillated BHb, fibrillated BHb and fibrillated BHb in presence of esculin and esculetin 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.

### 1.6.4 Congo red (CR) assay

Absorption spectroscopy was used to analyze the formation of aggregates with the help of CR dye. Here,6 μMof the protein solutionand protein solution in presence of coumarin derivatives were aliquot and mixed with 20 μM CR and 50 mM phosphate buffer, pH 7.4.[14] The spectra was recorded from 400-600 nm.

### 1.6.5 Fluorescence microscopy

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

## 2. Supplementary Tables

**Table S1.** $K_{SV}$ and $k_q$ values of BHb-Esculin and BHb-Esculetin at three temperatures

| Complex | Temp (K) | $K_{SV}$ ($M^{-1}$) × $10^4$ | $k_q$ ($M^{-1}$ $s^{-1}$) × $10^{13}$ |
|---|---|---|---|
| **BHb-Esculin** | **288** | 4.083±0.207 | 2.077±0.120 |
| | **298** | 3.353±0.212 | 1.935±0.112 |
| | **308** | 2.936±0.179 | 1.867±0.076 |
| **BHb-Esculetin** | **288** | 7.626±0.131 | 2.885±0.049 |
| | **298** | 6.053±0.026 | 2.707±0.219 |
| | **308** | 5.073±0.023 | 2.584±0.341 |

**Table S2.** Data obtained from synchronous fluorescence of BHb in presence of increasing concentration of esculin and esculetin at two offset wavelengths

| System | | Peak (nm) | Stoke's shift Δλ (nm) | Fl. Intensity |
|---|---|---|---|---|
| **BHb** | **Offset Δλ=60 nm** | 344 | 2 | 418573 |
| **BHb-Esculin** | | 342 | | 222573 |
| **BHb** | | 344 | 1 | 366760 |
| **BHb-Esculetin** | | 343 | | 275210 |
| **BHb** | **Offset Δλ=15 nm** | 309 | 0 | 112023 |
| **BHb-Esculin** | | 309 | | 60046 |
| **BHb** | | 309 | 0 | 113450 |
| **BHb-Esculetin** | | 309 | | 64626 |

**Table S3.** 3D fluorescence data for BHb, BHb-Esculin and BHb-Esculetin complexes

| System | Peak 1 (nm) | | Stokes shift Δλ (nm) | Intensity (a.u.) | Peak 2 (nm) | | Stokes shift Δλ (nm) | Intensity (a.u.) |
|---|---|---|---|---|---|---|---|---|
| | $\lambda_{ex}$ | $\lambda_{em}$ | | | $\lambda_{ex}$ | $\lambda_{em}$ | | |
| **BHb** | 275 | 334 | 59 | 109.875 | 230 | 332 | 102 | 64.942 |
| **BHb-Esculin** | 275 | 330 | 55 | 98.234 | 230 | 329 | 99 | 55.834 |
| **BHb-Esculetin** | 275 | 330 | 55 | 83.244 | 230 | 330 | 100 | 53.294 |

**Table S4.** REES data for BHb and BHb in presence of esculin and esculetin obtained at two excitation wavelengths.

| System | $\lambda_{ex}$ = 295 nn | $\lambda_{ex}$ = 305 nm | Δλ(nm) |
|---|---|---|---|
| **BHb** | 337 | 346 | 9 |
| **BHb-Esculin** | 336 | 347 | 11 |
| **BHb-Esculetin** | 335 | 348 | 13 |

**Table S5.** Binding energy of docked conformers for BHb-Esculin and BHb-Esculetin complexes

| Complex | Binding energy (kcal $mol^{-1}$) |
|---|---|
| **BHb-Esculin** | -7.8 |
| | -7.8 |
| | -7.7 |
| | -7.6 |
| | -7.6 |
| | -7.4 |
| | -7.4 |
| | -7.4 |
| | 7.3 |
| **BHb-Esculetin** | -6.8 |
| | -6.4 |
| | -6.3 |
| | -6.1 |
| | -6.0 |
| | -6.0 |
| | -5.9 |
| | -5.9 |
| | -5.9 |

**Table S6.** RMSF value for binding site residues in comparison with BHb for the two systems

| Sl No. | Residues name | RMSF value forbinding site residue | | |
|---|---|---|---|---|
| | | **BHb** | **BHb-Esculin** | **BHb-Esculetin** |
| 1 | $\alpha_1$-Lys139 | 0.2179 | 0.1320 | 0.1658 |
| 2 | $\alpha_1$-Tyr140 | 0.0934 | 0.0879 | 0.1000 |
| 3 | $\alpha_1$-Arg141 | 0.2569 | 0.2418 | 0.2551 |
| 4 | $\alpha_2$-Lys99 | 0.1391 | 0.1155 | 0.1146 |
| 5 | $\alpha_2$-Asp126 | 0.0843 | 0.0706 | 0.0848 |
| 6 | $\alpha_2$-Lys127 | 0.0954 | 0.0802 | 0.0921 |
| 7 | $\alpha_2$-Ala130 | 0.0590 | 0.0580 | 0.0648 |
| 8 | $\beta_2$-Trp37 | 0.0921 | 0.0949 | 0.0903 |

**Table S7.** Distance value for binding site residues in comparison with BHb for the two systems BHb-Esculin and BHb-Esculetin

| Sl No. | Residues name | Time steps (ns) | Distance value for binding site residue | |
|---|---|---|---|---|
| | | | **BHb-Esculin** | **BHb-Esculetin** |
| 1 | $\alpha_1$-Pro95 | 0 | 0.967 | 1.126 |
| | | 50 | 0.964 | 1.040 |
| | | 100 | 0.911 | 1.007 |
| 2 | $\alpha_1$-Thr137 | 0 | 0.980 | 0.816 |
| | | 50 | 0.822 | 0.662 |
| | | 100 | 0.741 | 0.828 |
| 3 | $\alpha_1$-Ser138 | 0 | 0.466 | 0.882 |
| | | 50 | 0.460 | 0.826 |
| | | 100 | 0.459 | 0.746 |
| 4 | $\alpha_1$-Tyr140 | 0 | 0.723 | 0.745 |
| | | 50 | 0.601 | 0.686 |
| | | 100 | 0.598 | 0.529 |
| 5 | $\alpha_2$-Lys127 | 0 | 0.641 | 0.536 |
| | | 50 | 0.497 | 0.524 |

| | | | | |
|---|---|---|---|---|
| | | 100 | 0.489 | 0.518 |
| 6 | $\beta_2$-Val34 | 0 | 1.065 | 0.757 |
| | | 50 | 0.943 | 0.772 |
| | | 100 | 0.866 | 0.743 |
| 7 | $\beta_2$-Pro36 | 0 | 1.092 | 0.740 |
| | | 50 | 0.924 | 0.694 |
| | | 100 | 0.891 | 0.635 |
| 8 | $\beta_2$-Trp37 | 0 | 0.977 | 0.778 |
| | | 50 | 0.976 | 0.772 |
| | | 100 | 0.912 | 0.768 |

**Table S8.** The distributed non-bonded interactions numbers between the BHb and the ligand were counted throughout the entire simulation period

| Protein-ligand | Time (ns) | Non Bonded Interactions | | | | | | | Total number of interaction |
|---|---|---|---|---|---|---|---|---|---|
| | | Conventional H-bond | van der Waals | Carbon Hydrogen Bond | Pi-Pi T-shaped | Pi-Sigma | Pi-alkyl | Unfavourable Donor-Donor | |
| BHb-Esculin | 0 | 4 | 8 | 0 | 1 | 0 | 2 | 0 | 15 |
| | 50 | 5 | 9 | 1 | 1 | 0 | 1 | 0 | 17 |
| | 100 | 5 | 9 | 1 | 1 | 0 | 1 | 0 | 17 |
| BHb-Esculetin | 0 | 1 | 7 | 0 | 0 | 1 | 3 | 0 | 12 |
| | 50 | 2 | 7 | 0 | 1 | 0 | 3 | 1 | 14 |
| | 100 | 4 | 6 | 0 | 0 | 0 | 2 | 0 | 12 |

**Table S9.** Average ligand distances from the $\beta_2$-Trp37 residues of the BHb (1G09)

| BHb | Ligand | Average Distance (nm) | Standard Deviation (nm) |
|---|---|---|---|
| $\beta_2$-Trp37 | Esculin | 0.950 | 0.054 |
| | Esculetin | 0.756 | 0.047 |

## 3. Supplementary Figures

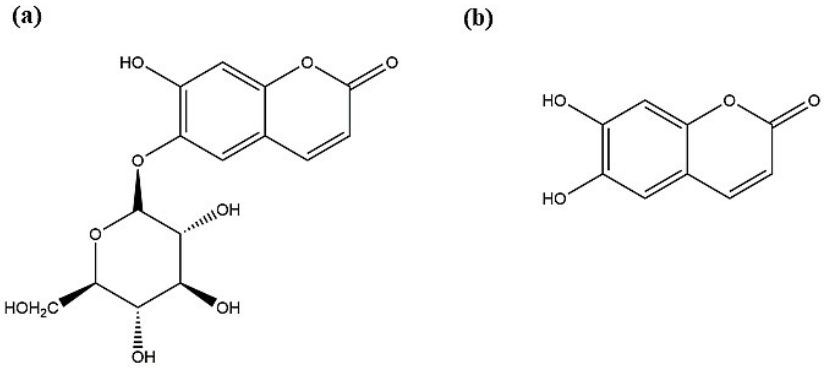


**Fig. S1.** Illustration of the 2D chemical structure of (a) Esculin and (b) Esculetin.

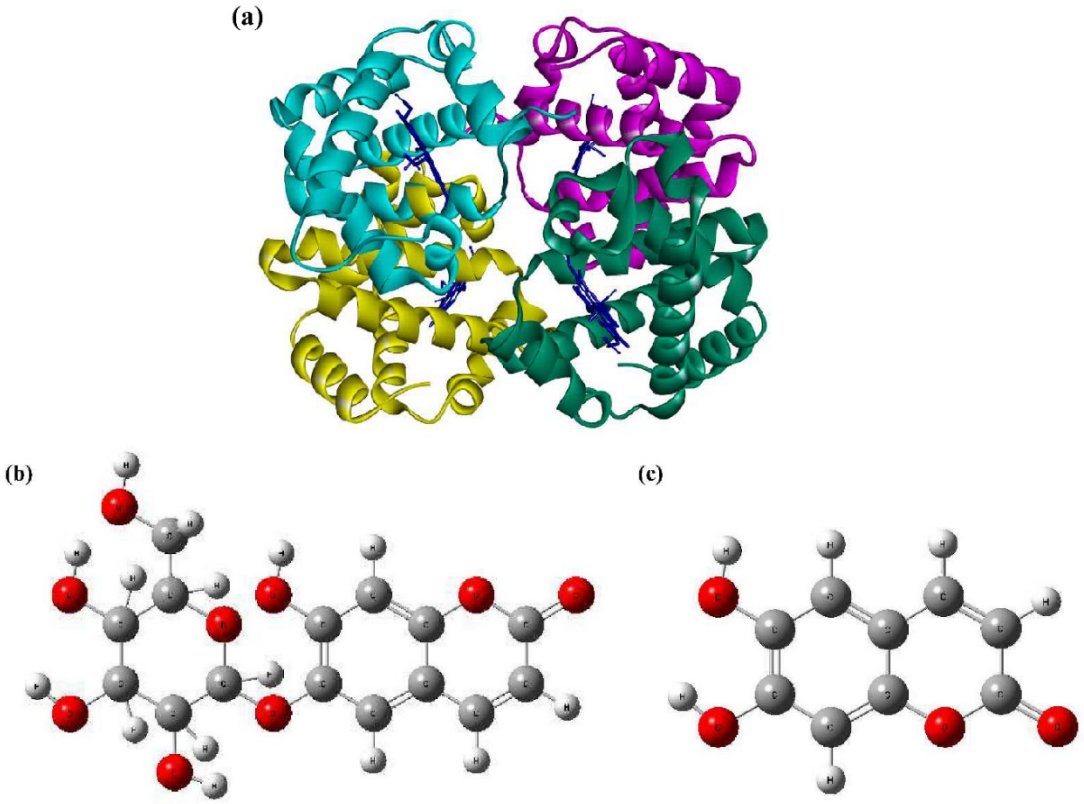


**Fig. S2.** Schematic representation of (a) The crystal structure of bovine hemoglobin (PDB ID: 1G09) ($\alpha_1$: cyan, $\beta_1$: yellow; $\alpha_2$: purple; $\beta_2$: dark green;heme: dark blue).(b) Optimised

structure of esculin and (c) Optimised structure of esculetin. (gray: carbon; red: oxygen; white: hydrogen).

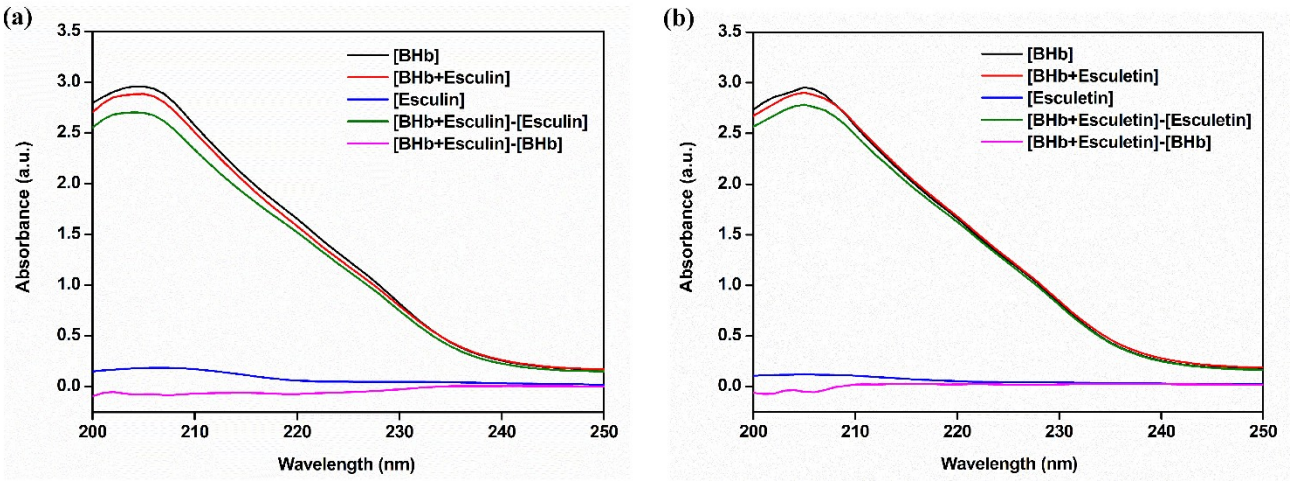


**Fig. S3.** The UV/vis absorption spectra of (a) BHb-Esculin and (b) BHb-Esculetin in the wavelength range of 200-250 nm. [BHb]=1.5 μM. [Esculin]=[Esculetin]=4.5 μM.

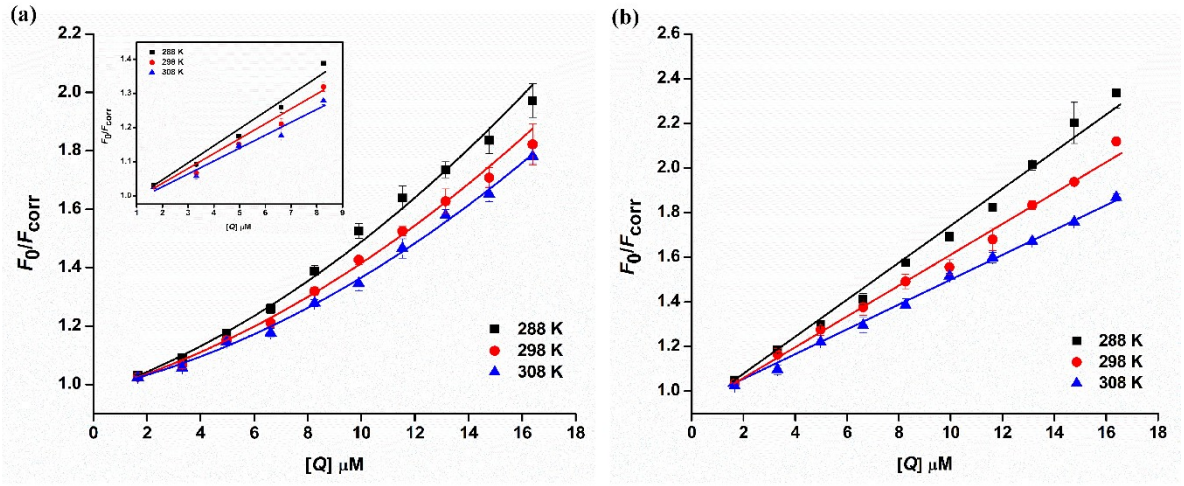


**Fig. S4.** The Stern-Volmer plot of (a) BHb-Esculin (Inset: Linear portion of Stern-Volmer plot) and (b) BHb-Esculetin complexes at three temperatures carried out in 20 mM phosphate buffer (pH 7.4). [BHb]=3μM.

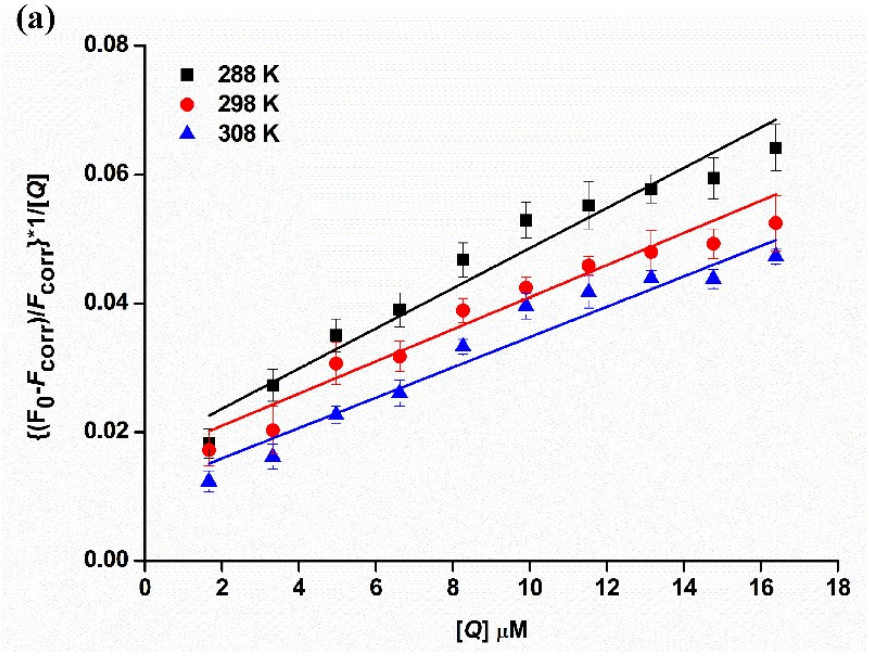


**Fig. S5.** Regression plot of BHb in presence of esculin at three different temperatures. [BHb]=3 µM.

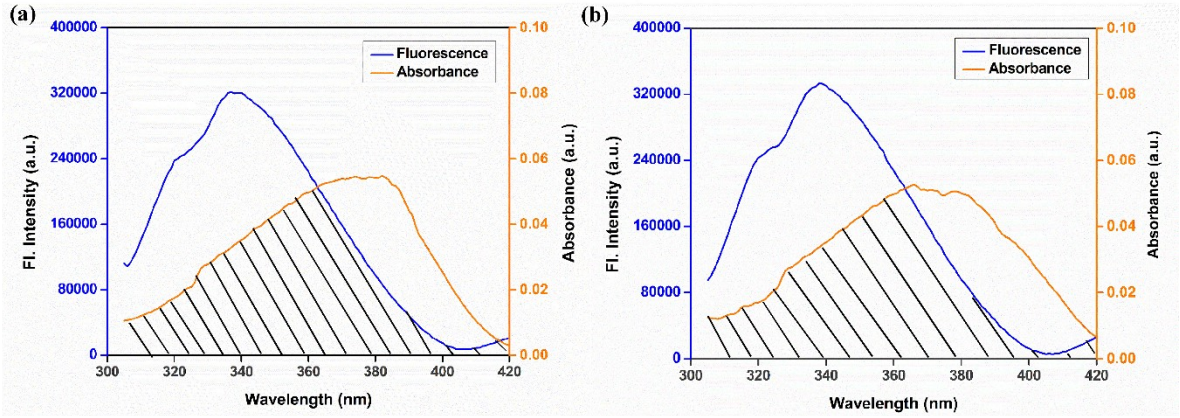


**Fig.S6.** Spectral overlap of emission spectra of BHb with the absorption spectra of (a) Esculin and (b) Esculetin in 20 mM phosphate buffer. [BHb]=[Esculin]=[Esculetin]= 3µM.

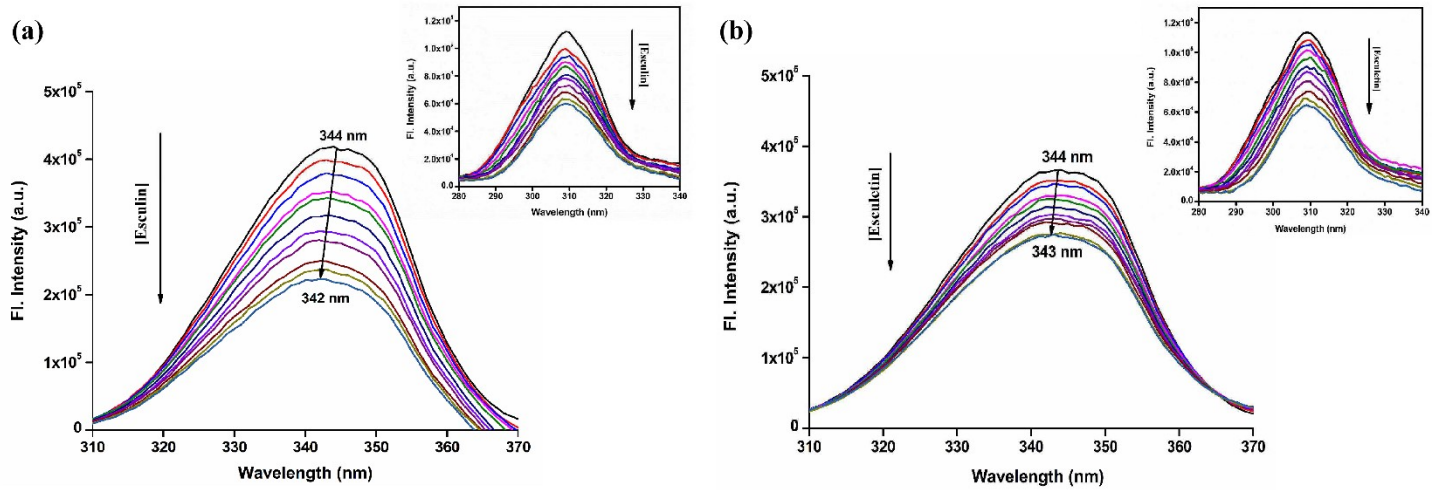


**Fig.S7.** Synchronous spectra at offset wavelength of 60 nm of BHb in absence and presence of increasing concentration of (a) Esculin and (b) Esculetin in 20 mM phosphate buffer pH 7.4. [Inset: Synchronous spectra at offset wavelength 15 nm of BHb and the respective coumarin derivative]. [BHb]=3 µM.

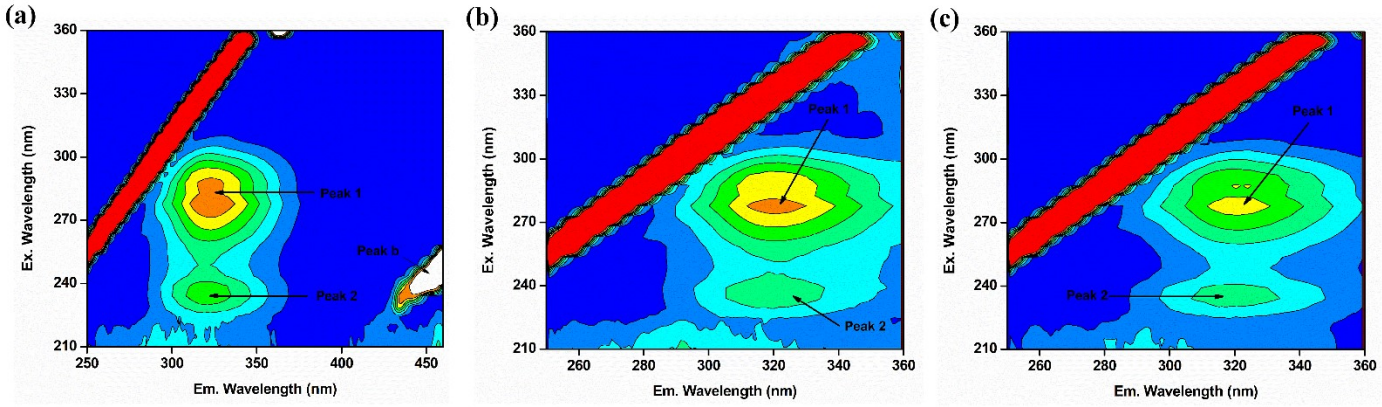


**Fig. S8.** The contour plots obtained from 3D fluorescence for (a) BHb and its 1:4 molar complexes (b) BHb-Esculin and (c) BHb-Esculetin in 20 mM phosphate buffer (pH 7.4).

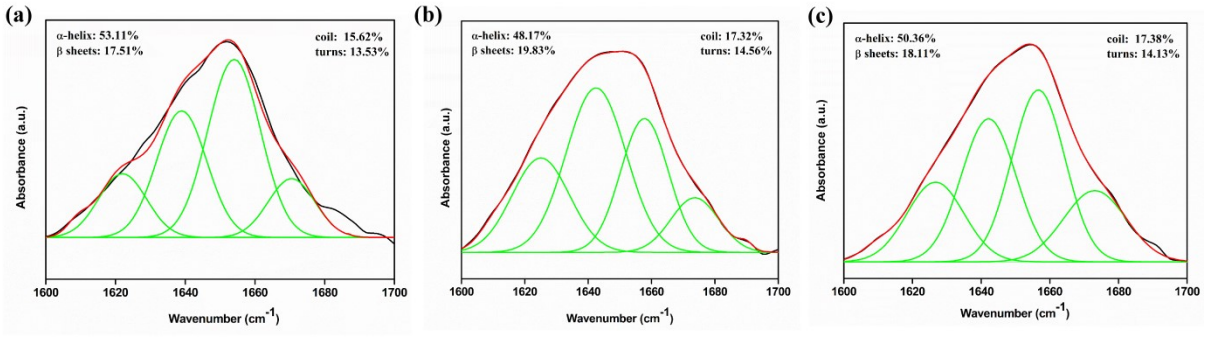


**Fig. S9.** FTIR amide I peak (1600-1700 $cm^{-1}$) after deconvolution of (a) Native BHb (b) BHb-Esculin complex and (c) BHb-Esculetin complex.

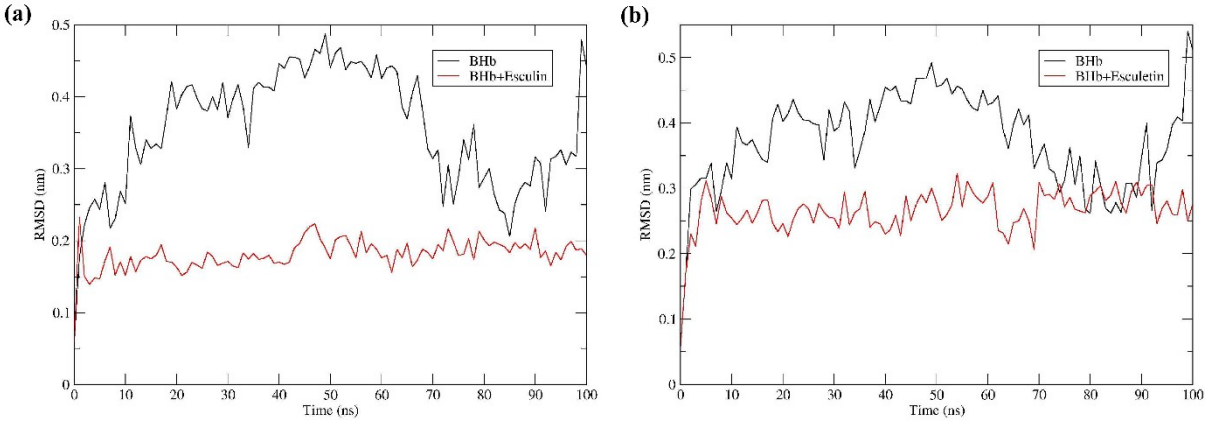


**Fig. S10.** RMSD plot for (a) BHb and BHb+Esculin system (b) BHb and BHb+Esculetin system specifically for binding site residues only.

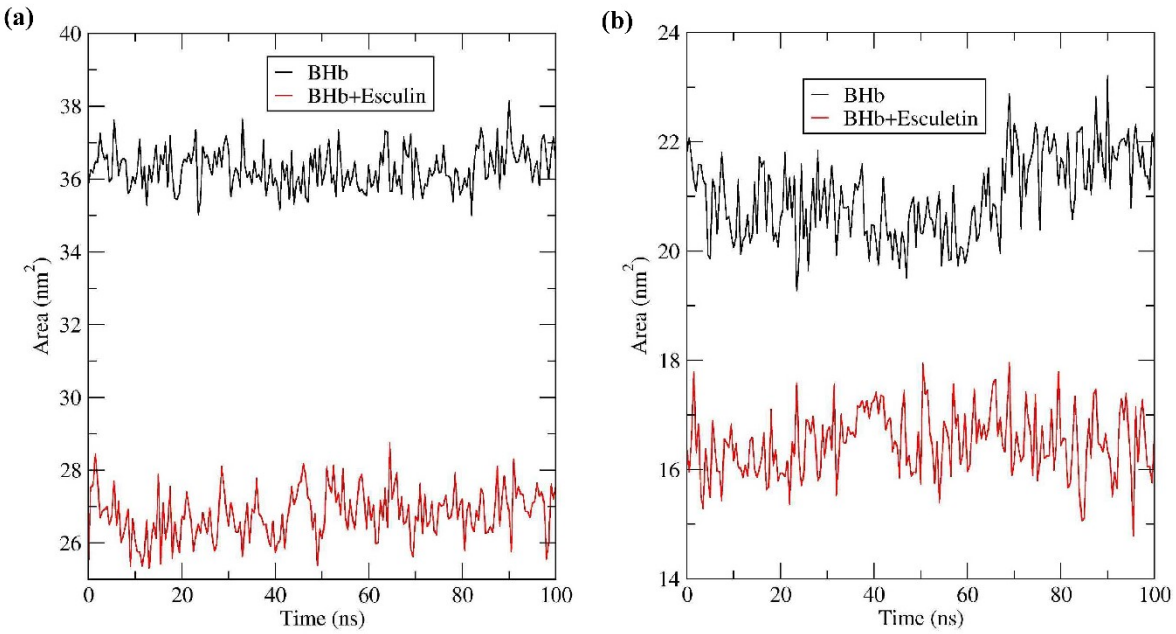


**Fig. S11.** SASA Plot for binding site residue of (a) BHb+Esculin and (b) BHb+Esculetin system.

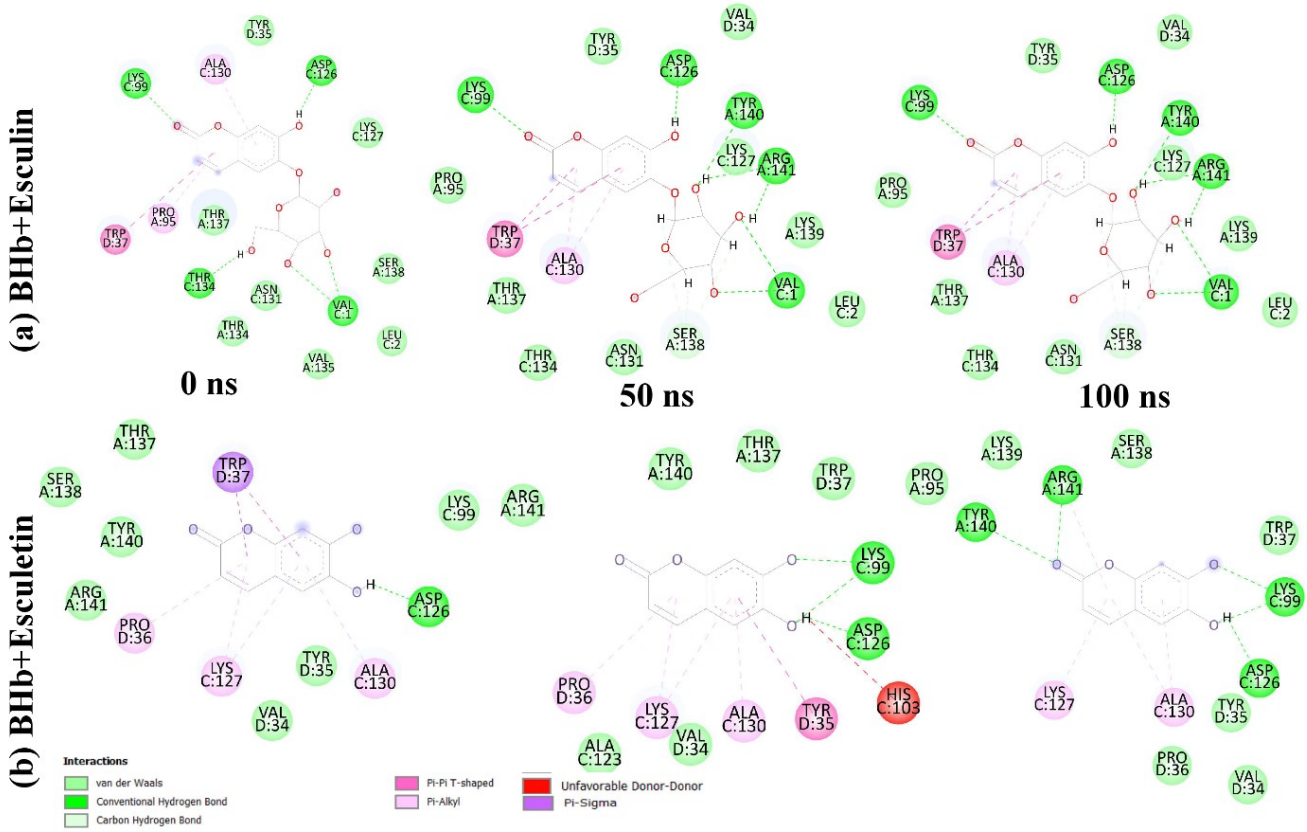


**Fig. S12.** The 2D interaction map displays how BHb interacts with ligands at intervals of 0, 50, and 100 ns for the BHb-Esculin and BHb-Esculetin complexes, respectively.

## References


1 Z. Chi and R. Liu, *Biomacromolecules*, 2011, **12**, 203–209.
2 F. Macii and T. Biver, *J. Inorg. Biochem.*, 2021, **216**, 111305.
3 T. Förster, *Ann. Phys.*, 1948, **437**, 55–75.
4 T. Vo-Dinh, *Anal. Chem.*, 1978, **50**, 396–401.
5 J. Lakowicz, *Lakowicz JR (2006) Principles of fluorescence spectroscopy, 3rd edn. Springer, New York, 2006*, 2006.
6 L. Whitmore and B. A. Wallace, *Nucleic Acids Res.*, 2004, **32**, W668–W673.
7 D. M. Byler and H. Susi, *Biopolymers*, 1986, **25**, 469–487.
8 H. Susi, D. M. Byler and J. M. Purcell, *J. Biochem. Biophys. Methods*, 1985, **11**, 235–240.
9 S. Ghazanfari-Sarabi, M. Habibi-Rezaei, R. Eshraghi-Naeeni and A. A. Moosavi-Movahedi, *PLoS One*, 2019, **14**, e0214725.
10 J. O. Alben and L. Y. Fager, *Biochemistry*, 1972, **11**, 842–847.
11 S. A. Hudson, H. Ecroyd, T. W. Kee and J. A. Carver, *FEBS J.*, 2009, **276**, 5960–5972.
12 A. Hawe, M. Sutter and W. Jiskoot, *Pharm. Res.*, 2008, **25**, 1487–1499.
13 A. Iram, T. Alam, J. M. Khan, T. A. Khan, R. H. Khan and A. Naeem, *PLoS One*, 2013, **8**, e72075.
14 F. Chiti, N. Taddei, F. Baroni, C. Capanni, M. Stefani, G. Ramponi and C. M. Dobson, *Nat. Struct. Biol.*, 2002, **9**, 137–143.