Discover Electrochemistry

Research

An insight for the interaction of Aceclofenac with hemoglobin using spectroscopic, electrochemical and in silico approaches

Sandeep Yadav[1,2] · Madhur Babu Singh[1,2] · Shubham Sewariya[3,4] · Shyam Bharat Yadav[3] · Prashant Singh[1] · Ramesh Chandra[3] · Shrikant Kukreti[3] · Sandeep Kumar Singh[5] · Pallavi Jain[2] · Kamlesh Kumari[6]

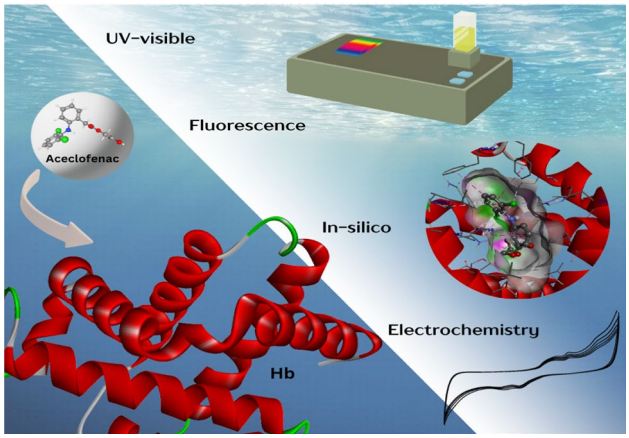
Received: 8 July 2024 / Accepted: 1 October 2024
Published online: 15 October 2024

Abstract
The interactions between protein and ligand are crucial to practically every biological function in the body, and investigations of these interactions are critical from the perspective of logical medication development. In this work, several biophysical methods like UV–Visible spectroscopy, fluorescence spectroscopy, and *in-silico* studies have been explored to examine the interaction between aceclofenac and haemoglobin. Spectrofluorimetric data confirmed the static nature of the protein quenching mechanism caused by the medication. ΔG for the formation of the complex via the interaction was found to be − 12.65 kJ/mol. Molecular docking showed a strong interaction between aceclofenac and hemoglobin with a binding energy of − 31.79 kJ/mol. Further, molecular dynamics simulations using Gromacs were run to investigate the stability of the protein with and without aceclofenac, and the ΔG for the formation of the complex (hemoglobin-aceclofenac) is − 105.09 kJ/mol with an error of 13.51 kJ/mol. Spectroscopic and *in-silico* calculations indicate the feasible binding of the aceclofenac with hemoglobin. Further, the interaction of haemoglobin with aceclofenac was studied by cyclic voltammetry.

Sandeep Yadav, Madhur Babu Singh and Shubham Sewariya have equal authorship.

✉ Prashant Singh, psingh@arsd.du.ac.in; ✉ Kamlesh Kumari, kkumari@zoology.du.ac.in; Sandeep Yadav, 96y.sandeep@gmail.com; Madhur Babu Singh, 19madhursingh@gmail.com; Shubham Sewariya, shubhamsewariya94@gmail.com; Shyam Bharat Yadav, sy62407@gmail.com; Ramesh Chandra, acbrdu@hotmail.com; Shrikant Kukreti, skukreti@chemistry.du.ac.in; Sandeep Kumar Singh, sandeepk.singh@jgu.edu.in; Pallavi Jain, palli24@gmail.com | [1]Department of Chemistry, Atma Ram Sanatan Dharma College, University of Delhi, New Delhi, India. [2]Department of Chemistry, SRM Institute of Science and Technology, Delhi-NCR Campus, Modinagar, India. [3]Department of Chemistry, University of Delhi, Delhi, India. [4]School of Pharmacy and Biomedical Sciences, University of Central Lancashire, Preston, UK. [5]O.P. Jindal Global Business School, OP Jindal Global University, Sonipat, India. [6]Department of Zoology, University of Delhi, Delhi, India.

Graphical Abstract



Keywords Haemoglobin · Aceclofenac · Spectroscopic techniques · In-silico studies · Cyclic voltammetry

1 Introduction

Aceclofenac, a nonsteroidal anti-inflammatory drug (NSAID), is widely utilized for its potent analgesic, anti-inflammatory, and antipyretic properties. It serves as a cornerstone in managing conditions such as rheumatic disorders, musculoskeletal pain, and acute inflammatory episodes like postoperative discomfort. Aceclofenac effectively suppresses prostaglandin synthesis by inhibiting cyclooxygenase enzymes, particularly Cox-2, Aceclofenac effectively suppresses prostaglandin synthesis, mitigating inflammation and alleviates pain. Its multifaceted pharmacological actions, including antioxidant effects and modulation of inflammatory cytokines, underscore its significance in clinical practice for providing symptomatic relief and improving patient outcomes.

Understanding the interactions between aceclofenac and biomolecules, particularly hemoglobin, holds paramount importance in pharmacological and toxicological contexts. Hemoglobin, a crucial protein responsible for oxygen transport in the body, is a vital biomarker for assessing drug interactions and potential toxicity. Investigating aceclofenac's binding affinity and effects on hemoglobin can shed light on its pharmacokinetic and pharmacodynamic properties, aiding in optimising dosing regimens and minimising adverse effects. Furthermore, elucidating the impact of aceclofenac on hemoglobin stability and function is essential for ensuring drug safety and efficacy, particularly in patients with underlying hemoglobinopathies or other haematological disorders. Therefore, comprehensive monitoring of Aceclofenac's interactions with hemoglobin offers valuable insights into its therapeutic potential and safety profile, contributing to informed clinical decision-making and enhanced patient care.

There are several studies in which the interactions of small molecules and Hb are reported, mainly using spectroscopic techniques to study the interactions. Liu et al. studied the interactions of cefpiramide sodium (CPMS) with bovine hemoglobin (BHb) at various temperatures. Electrostatic forces contributed significantly to the conjugation process between BHb and CPMS. [1] In another study by Tunc et al. studied changes in human serum albumin (HSA) and human hemoglobin (HMG) due to chloroquine diphosphate (CQP) and phenelzine sulfate (PS). CQP showed static quenching. Also, electrostatic interactions were confirmed with an adverse change in enthalpy and increased entropy after complex formation [2]. Several spectroscopic methods have revealed the interactions between BHb and phosmet. Cyclic voltammetry and UV–Vis spectroscopy were used to evaluate the binding affinity between ferrocenylmethylnitroaniline derivatives (Fc2N, Fc3N, and Fc4N) and DNA, revealing that the interaction is driven primarily by electrostatic attraction [3]. Another study by Kamaljeet et al. used UV–Vis absorbance, fluorescence spectroscopy, and molecular modeling to reveal that four dyes strongly interact with hemoglobin, potentially forming toxic interactions, with all dyes binding within hemoglobin's central cavity [4].

The structural changes in BHb due to phosmet were examined by employing Fourier transform infrared spectroscopy (FTIR) and circular dichroism (CD) [5]. Biophysical and in silico investigations gave insight into the interaction between BHb and substituted coumarins [6]. The binding interaction among 9-bromo-noscapine-based ionic liquid and human hemoglobin (Hb) was investigated using computational calculations and spectroscopic investigations and concluded that ionic liquid forms complex with Hb in a stoichiometric ratio of 1:1 [7]. Interaction of ionic liquids (ILs) of biological potential molecules with Hb has been studied, and static quenching was observed with both the ILs [MeNOS]NTf_2 and [MeBrNOS]NTf_2 [8]. Recently, our group have explored the interactions between dinotefuran (a pesticide) and ofloxacin (a drug) with Hb using electrochemical, spectroscopic and computational methods and observed significant structural changes [9, 10]. Herein, we aim to examine the interaction between aceclofenac and Hb through different spectroscopic, electrochemical and in silico studies.

2 Methodology

2.1 Materials

The PBS buffer capsules (Product ID-31390E) were procured from MOLYCHEM. Lyophilised powder of haemoglobin human (Product ID-H7379) was obtained from MERCK. Aceclofenac was provided by Agilent Technologies, Inc.

2.2 Spectroscopic studies

2.2.1 UV–Visible spectroscopy

UV–Visible spectrophotometry was employed to investigate the interaction between aceclofenac and hemoglobin (Hb) using a Thermo Scientific Evolution 300 spectrophotometer. UV–visible spectroscopy, a fundamental technique in analytical chemistry, was utilised to assess electronic structure and chemical environment. Initially, the UV–visible spectrum of pure Hb at 2.5 µM served as a baseline. Subsequently, aceclofenac was incrementally added to the Hb solution (0–50 µM), with spectra recorded at each step. Changes in absorbance indicated alterations in the electronic environment of Hb upon interaction with aceclofenac. This allowed for quantitative analysis of molecular interactions and determination of binding constants and stoichiometry.

2.2.2 Fluorescence spectroscopy

The Hitachi F-7000 Fluorescence Spectrophotometer was utilised for steady-state fluorescence spectroscopy analysis. Excitation spectra for haemoglobin (Hb) in the presence and absence of aceclofenac were recorded at a fixed wavelength of 280 nm. We obtained emission spectra in the 300–500 nm region. The interaction between aceclofenac and Hb was studied at 298, 303, and 308 K. This allowed for the characterisation of changes in fluorescence intensity and spectral shifts in Hb after interaction with aceclofenac.

2.3 Computational analysis

2.3.1 Molecular docking

Molecular docking is an efficient tool for predicting the best binding pose of a receptor/protein–ligand complex. Various search algorithms such as fast shape matching, incremental construction, Monte Carlo, and genetic algorithms are employed to run the docking simulations to generate the docking scores. Parameters like van der Waals radius, structure flexibility and interatomic interactions are considered while predicting the conformations of ligand binding at the target site of the protein. iGEMDOCK, Schrodinger, AutoDock, etc., are the computation tools that are available to perform molecular docking between the compound and protein. Herein, AutoDock Vina was used to perform molecular

docking between aceclofenac and Hb (PDB ID: 2DN1) [11, 12]. The structure of aceclofenac was drawn using ChemDraw [13] and the structure of the protein was taken from the RCSB database (https://www.rcsb.org/) and prepared using Chimera 1.8 [14].

2.3.2 Molecular dynamics simulations

Molecular dynamics (MD) simulations are used to investigate the binding of ligands with the proteins in a dynamic state. Movement and trajectories of atoms over a fixed period are determined using Newton's laws of motion. MD simulations can reveal biomolecular processes like protein folding, conformational changes, ligand binding and movement of atoms from their mean positions. AMBER, GROMACS, CHARMM, NAMD, and LAMMPS are some tools generally used to perform MD simulations. In this work, GROMACS was used to perform MD simulations, and the CHARMM27 force field model was used to calculate the forces involved in interatomic interactions [15, 16]. The topology of the ligand (aceclofenac) was created using the SwissParam webserver (https://www.swissparam.ch/). SPC216 water model and cubic box (6.625 nm $\times$ 6.625 nm $\times$ 6.625 nm) type were used to perform the simulations [17]. The box volume came out to be 290.81 nm^3 with 1001.04 g/L density. 8816 water molecules were added to solvate the MD simulation box. We have used the complex obtained from molecular docking to run the MD simulations using GROMACS [18]. The trajectories produced from MD simulations were used to get root mean square deviation (RMSD), root mean square fluctuation (RMSF), and radius of gyration (Rg) of Hb with and without aceclofenac [19, 20].

2.3.3 Density functional theory calculations

Density functional theory (DFT) calculations were used to investigate the electronic structure of aceclofenac and understand its structure and reactivity. Quantum mechanical models are used in DFT calculations to predict the electronic structure and energies of the molecule. Optimised geometry, total energy, energy of frontier orbitals and dipole moment are obtained from the DFT calculations. VASP, Quantum ESPRESSO, Gaussian, ORCA, NWChem and Abinit are some of the software used to perform DFT calculations. In this work, Gaussian was employed to execute the calculations [21]. The B3LYP method, along with the 6311-G basis set, was used to compute the optimised geometry and various energies of Aceclofenac [22, 23].

2.4 Electrochemical studies

Electrochemical analysis of the interactions occurring between aceclofenac and Hb was done by the 604E series electrochemical analyser of CH Instruments Inc. A three-electrode assembly was used to investigate the interactions between aceclofenac and Hb in aqueous medium. The experiment was conducted in an aqueous medium to closely replicate the natural environment of hemoglobin. 0.1 mM aceclofenac solution and 10 µM Hb solution were prepared in PBS buffer of 7.4 pH. 1 mL of aceclofenac solution was added to 100 mL of Hb, and various electrochemical measurements were carried out using the mixture. Concentrations of aceclofenac and Hb were kept almost similar in the final solution. The potential range for the cyclic voltammetry was kept from − 1.8 to 2 V with a sensitivity of 1×10^{-4}. A time-dependent study (3 h) of the solution containing aceclofenac and Hb was done to observe the changes occurring in the electrochemical response over a period of time. Multi-scan cyclic voltammograms of aceclofenac solution were recorded to characterise the reaction occurring at the electrode [9, 10]. The GCE was cleaned with 0.3-micron alumina paste, followed by ultrasonication before each measurement, to prevent any inconsistencies caused by residual hemoglobin or aceclofenac on the electrode surface from prior scans. Cyclic voltammetry was chosen for this study due to its sensitivity in detecting redox-active species and its ability to provide real-time insights into electron transfer processes. This technique is particularly advantageous for studying protein–drug interactions as it allows for monitoring subtle changes in electrochemical behavior, offering valuable information about binding dynamics and the conformational changes of proteins upon drug interaction.

3 Results and discussion

3.1 Spectroscopic studies

3.1.1 UV–visible spectroscopy

Analyzing protein–ligand interactions using UV–visible spectroscopy helps understand the conformational changes in Hb [24]. The three distinctive absorption peaks of Hb in the 200–500 nm range are (i) $\lambda_{max} = 212$ nm (n to pi* transitions responsible for hemoglobin's helical structure; (ii) $\lambda_{max} = 274$ nm, which is associated with the pi to pi* transitions of the aryl groups of amino-acids; and $\lambda_{max} = 406$ nm for Soret band of porphyrin [25–28]. When interacting Hb with increasing concentrations of aceclofenac, authors found that the absorption intensity increases at 278 nm, and a minor change in absorption intensity at 406 nm is observed, as shown in Fig. 1. The findings suggest a drug-protein complex forms within the haemoglobin protein segment upon gradually adding aceclofenac. As the concentration of aceclofenac increases, there is a corresponding rise in the aromatic interactions between the ligand and the protein, leading to an increase in the intensity of the pi to pi* peak. Thus, it is conceivable that aceclofenac exhibits significant binding affinity to hemoglobin.

3.1.2 Fluorescence spectroscopy

Fluorescence spectroscopy is a well-established technique that can be used in addition to UV–visible spectroscopy to investigate how a drug molecule and protein interact. The tryptophan (Trp37) is particularly susceptible to structural modifications and is responsible for the deviation in fluorescence intensities upon aceclofenac-Hb binding. [29, 30] As shown in Fig. 2, authors noticed a reduction in the emission intensity of Hb at 336 nm with steadily increasing aceclofenac doses. As a result, the research supported our hypothesis that aceclofenac and Hb may interact around the Trp37 residue, as seen in absorption spectral analyses.

Then, to determine the quenching process and quantify the corresponding thermodynamic parameters, the temperature has varied (Fig. 3) [31–33]. Aceclofenac-Hb interaction causes dynamic quenching, while ground-state complex formation indicates static quenching. Equations (1) and (2) are for the dynamic and static quenching, respectively [29, 33]. For mixed type quenching, the dependence of F_0/F on [Q] is non-linear (Fig. 3a). Types of quenching are differentiated through the value of the Stern–Volmer quenching constant, K_{sv} and bimolecular quenching constant, K_q (Table 1). The relationship between F_0/F and [Q] can be understood by Eq. (3) [33].

$$\frac{F_o}{F} = 1 + K_{sv}[Q] = K_q \tau_o [Q], \tag{1}$$

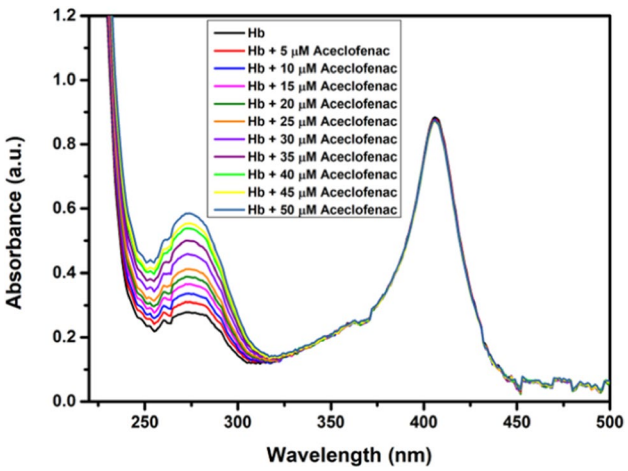


Fig. 1 UV–visible spectra of Hb against varying concentrations of (**a**) aceclofenac at 298 K

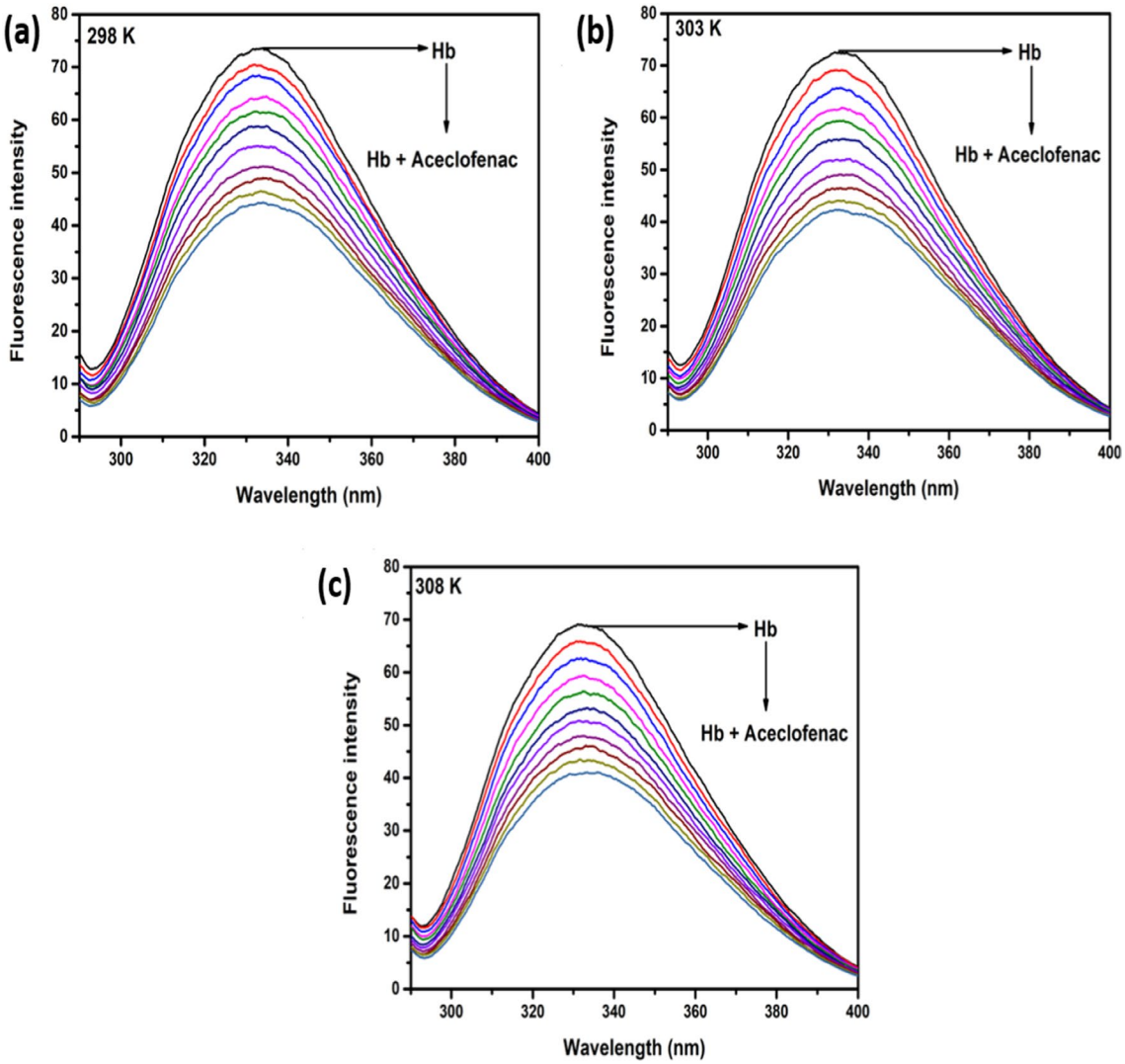


Fig. 2 Emission spectra of Hb with changing the concentrations (0–50 µM) of aceclofenac at (**a**) 298 K (**b**) 303 K and (**c**) 308 K

$$\frac{F_o}{F} = 1 + K_s[Q], \tag{2}$$

$$\frac{F_o}{F} = (1 + K_D[Q])(1 + K_S[Q]), \tag{3}$$

Authors have drawn a plot of F_o/F against *[Q]* shown in Fig. 3a**,** and for the concentrations of drug between 0 to 45 µM, it is non-linear. For concentrations of aceclofenac ranging from 0 to 20 µM, we found that the plot was linear (Fig. 3b) It was used to determine K_q and K_{sv} (Table 1). We observed an increase in the value of the Stern–Volmer quenching constant with an increase in the temperature of the system. This observation indicates dynamic quenching Hb using aceclofenac. However, we have also observed the value of bimolecular quenching constants in the order of 10^{12} M^{-1} s^{-1} [26, 34–36]. It corresponds to static quenchin,g and therefore, it is mixed quenching.

Literature reported several binding sites are present in the Hb, and herein, the authors have explored the modified Stern–Volmer equation (Eq. 4) and made a plot (Fig. 4a) to get the binding constant as well a number of interacting sites [32].

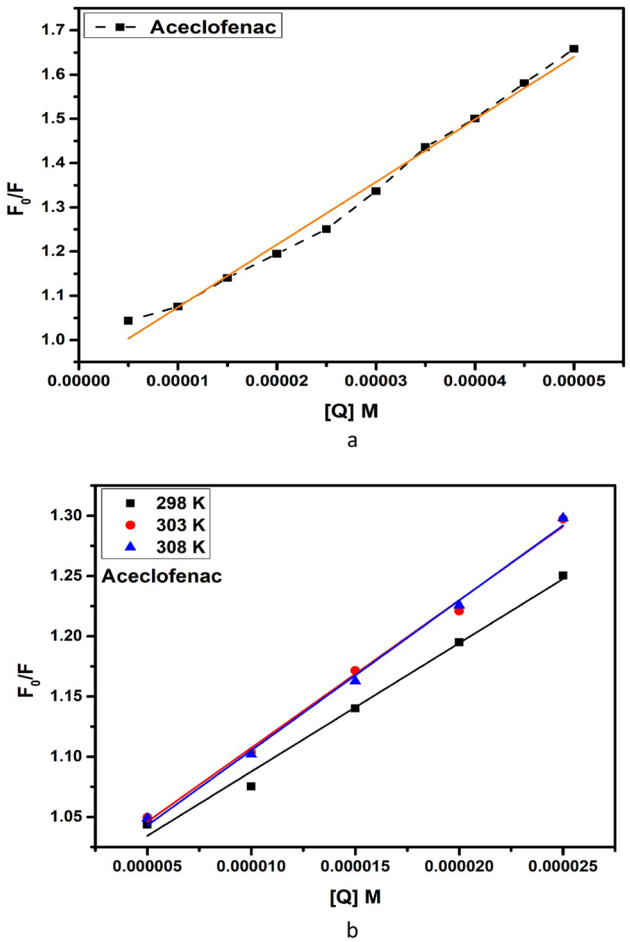


Fig. 3 Stern Volmer plot of Hb (**a**) different concentrations of aceclofenac at 298 K (**b**) aceclofenac at different temperatures

Table 1 Stern Volmer constant and bimolecular quenching constants for the formation of complex at different temperature

Temperature (K)	K_{sv} $(M^{-1}) \times 10^4$	K_q $(M^{-1}\ s^{-1}) \times 10^{12}$
298	1.07	1.07
303	1.23	1.23
308	1.24	1.24

$$log\left(\frac{F_o - F}{F}\right) = \log K_b + n\log[Q]. \tag{4}$$

In Eq. (6), K_b and n corresponds to the binding constant as well as available binding sites. At 298 K, the value of the binding constant is $1.26 \times 10^5\ M^{-1}$ (Table 2) for the aceclofenac-Hb complex. Further, n was found to be close to 1 and it means aceclofenac and Hb bind in 1:1 ratio. Later, authors have determined the thermodynamic parameters for aceclofenac-Hb complex from the Van't Hoff's plot shown in Fig. 4b using Eqs. (5) and (6).

$$\ln K_b = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}, \tag{5}$$

$$\Delta G = \Delta H - T\Delta S. \tag{6}$$

Here, R is the universal gas constant, ΔG, $\Delta H and \Delta S$ are the change in Gibb's free energy, enthalpy and entropy, respectively.

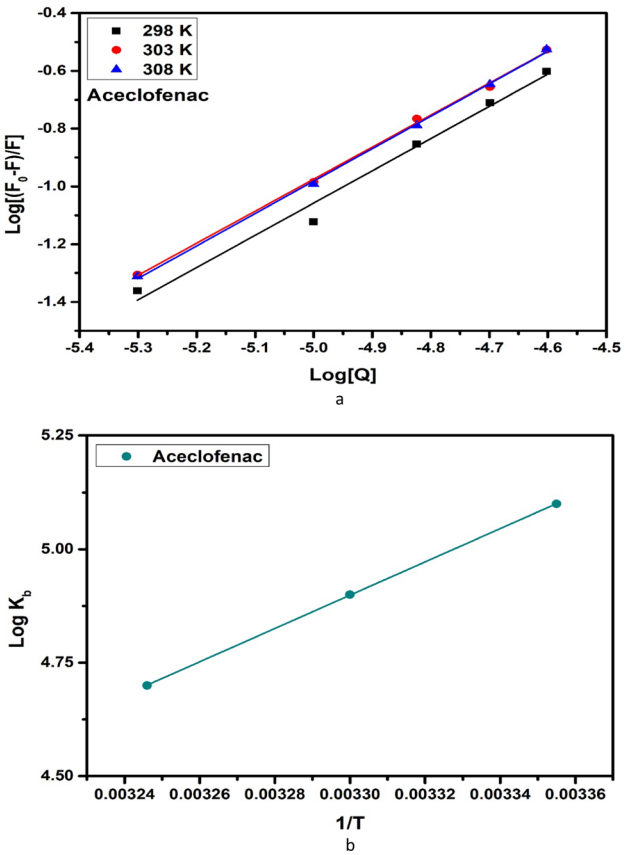


Fig. 4 **a** Modified Stern–Volmer plot of Hb at different concentrations of aceclofenac. **b** Van't Hoff's plot for aceclofenac-Hb

Table 2 Thermodynamic parameters of Hb-aceclofenac complex

Temperature (Kelvin)	K_b (M^{-1})	n	ΔG ($kJmol^{-1}$)	ΔH ($kJmol^{-1}$)	ΔS ($Jmol^{-1}K^{-1}$)
298	1.26×10^5	1.23	− 12.64	− 30.51	− 17.86
303	79,433	1.18			
308	50,119	1.14			

The nature for the interaction of aceclofenac with Hb could be investigated by values of enthalpy and entropy [37]. From Table 2**,** the value of Δ*G* is found to − 12.64 $kJmol^{-1}$ and indicates the spontaneity. The negative values of change in enthalpy (Δ*H*) and change in entropy (Δ*S*) indicate that the binding was mainly driven by hydrogen bonding and Vander Waals interactions. Further, the negative value of Δ*H* disclose that the complex formation process was exothermic in nature and negative value of Δ*S* correspond to formation of a more compact resultant complex compared to free hemoglobin. These observations are in line with literature reports for other such studies [7, 38, 39].

3.2 Computational studies

3.2.1 Molecular docking

Molecular docking was performed by AutoDock. The molecule of aceclofenac was docked at an active site of 2DN1. A triplicate study of the docking has been done to validate its accuracy. Figure 5 contains aceclofenac's two-dimensional and three-dimensional docked poses within 2DN1 obtained from a triplicate docking study. Various types of interactions like hydrogen bonding, pi-alkyl and electrostatic attractions were reported in all three runs. The 3D pose indicates the fitting of the aceclofenac in the binding site, and the 2D pose suggests the interaction of different amino acids in the protein cavity. In all three runs, aceclofenac binds mainly with the PHE, VAL, HIS, LEU and TYR at different positions of the protein. A few variations in the bonding was observed in Run 3, resulting in a slight variation in the binding energy.

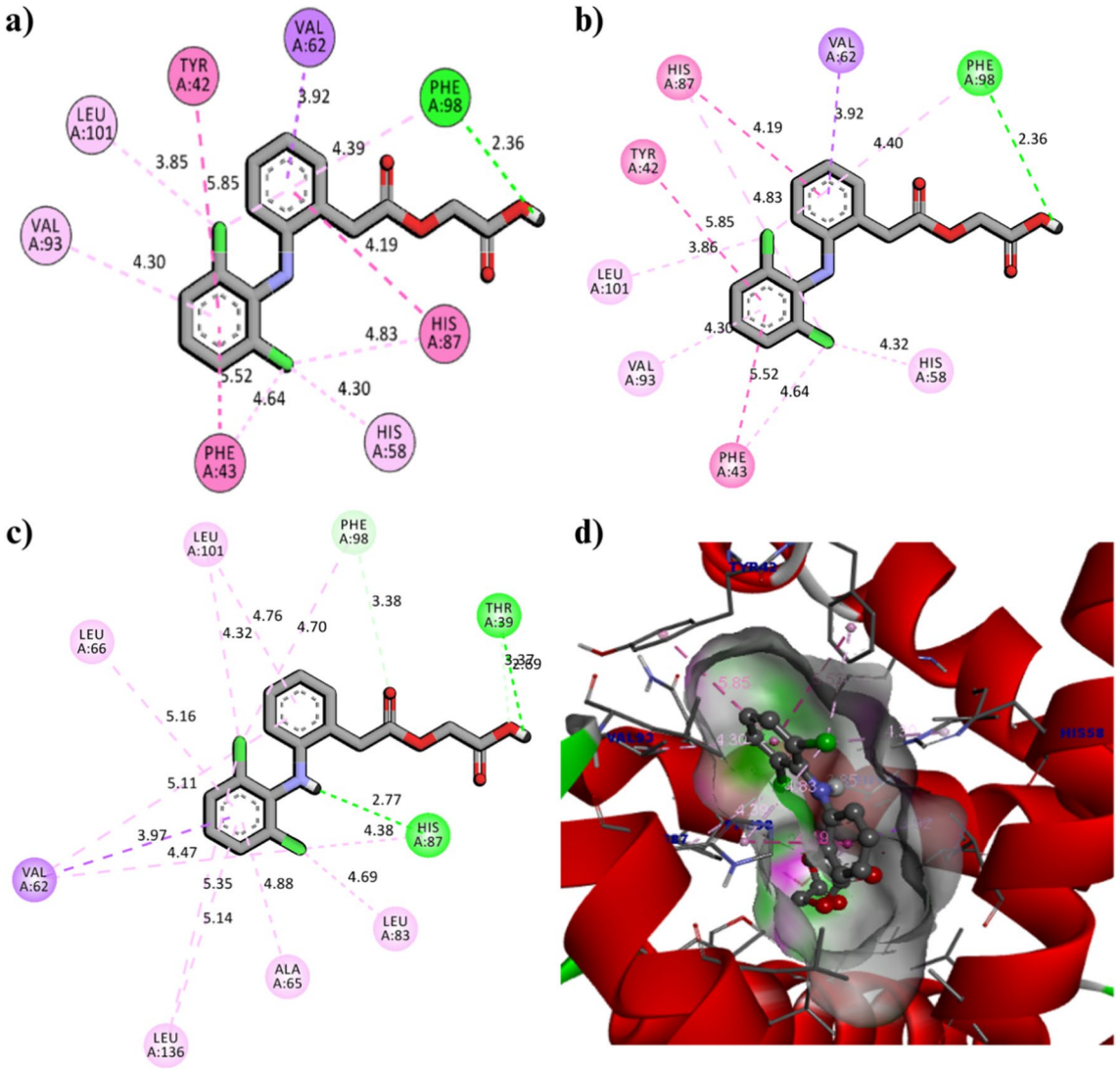


Fig. 5 Two-dimensional poses for the docked pose of aceclofenac within the binding pocket of Hb in (**a**) Run 1, (**b**) Run 2, (**c**) Run 3 and (**d**) three-dimensional view of docked pose in Run 1

In Run 1 aceclofenac showed hydrophobic interactions with HIS A:58 (4.30 Å), VAL A:93 (4.30 Å), LEU A:101 (3.85 Å), HIS A:87 (4.19 Å, 4.83 Å), TYR A:42 (5.85 Å), PHE A:43 (5.52 Å, 4.64 Å). Electrostatic interactions were shown with VAL A:62 (3.92 Å). Hydrogen bonding was also observed between the carboxylic group of aceclofenac and PHE A:98 amino acid with a bond length of 2.36 Å.

Binding energies and their RMSD obtained from the triplicate docking of aceclofenac within 2DN1 are reported in Table 3. The binding energy for the formation of the complex was found to be -31.79 kJ/mol in Run 1. The relative RMSD of the binding energies was found to be 0.635%, which is in the acceptable range, indicating the study's accuracy. Molecular docking is primarily used to find the binding pose of a small molecule in a macro-molecule. The energy or binding affinity

Table 3 Binding energies obtained from the triplicate docking of aceclofenac within 2DN1

Run	Binding energy (kJ/mol)	RMSD (kJ/mol)	Relative RMSD
Run 1	− 31.79	0.203	0.635%
Run 2	− 31.79		
Run 3	− 32.22		

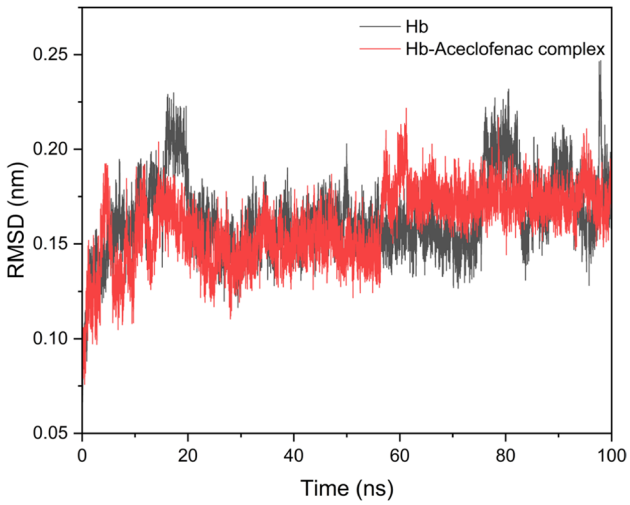


Fig. 6 RMSD for the Hb and Hb-aceclofenac complex obtained from 100 ns MD simulations

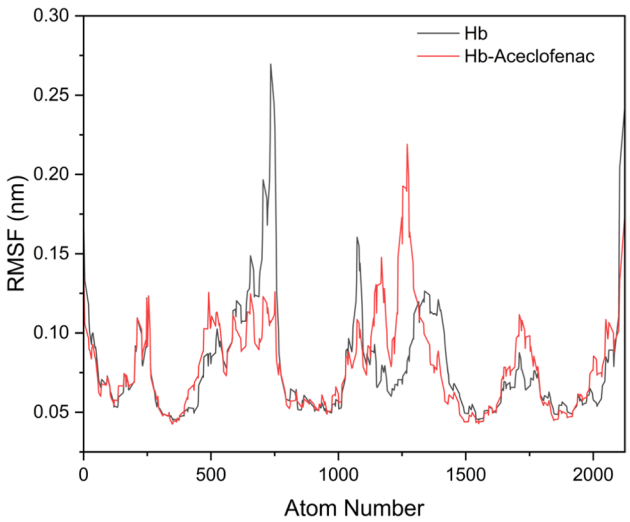


Fig. 7 Root mean square fluctuation for the Hb and Hb-aceclofenac complex obtained from 100 ns MD simulations

values obtained from docking results are not considered at par with experimental values. Rather, it helps to do further studies like MD simulations [40, 41].

3.2.2 Molecular dynamics simulations

To further understand the formation of complex, authors have performed molecular dynamics simulations using GROMACS 2022, and the thermodynamics parameters have been calculated by the MM-PBSA method. Herein, authors have investigated the the complex (Hb-aceclofenac) and protein (Hb) using trajectories like root mean square deviation (RMSD) and root mean square fluctuation (RMSF). The data points of RMSD associated with the Hb protein and Hb-aceclofenac complex have been plotted (Fig. 6). It can be seen that deviations in the complex are less than that for the Hb alone. The significant deviations in Hb in the range of 17–19 ns and 77–81 ns are stabilised upon the addition of aceclofenac. Additional minor deviations at 60 ns were observed in the case of Hb-aceclofenac complex. The MD simulations were performed for 100 ns, and the RMSD for the Hb and Hb-aceclofenac was below 0.25 nm and comes to an

Table 4 Various energies for forming the complex (Hb-aceclofenac) using the MM-PBSA calculations

E_{vdw} (kJ/mol)	E_{elec} (kJ/mol)	E_{PS} (kJ/mol)	E_{SASA} (kJ/mol)	$E_{Binding}$ (kJ/mol)
− 166.47 (±) 11.41	− 40.06 (±) 7.68	121.10 (±) 8.16	− 19.66 (±) 0.80	− 105.09 (±) 13.51

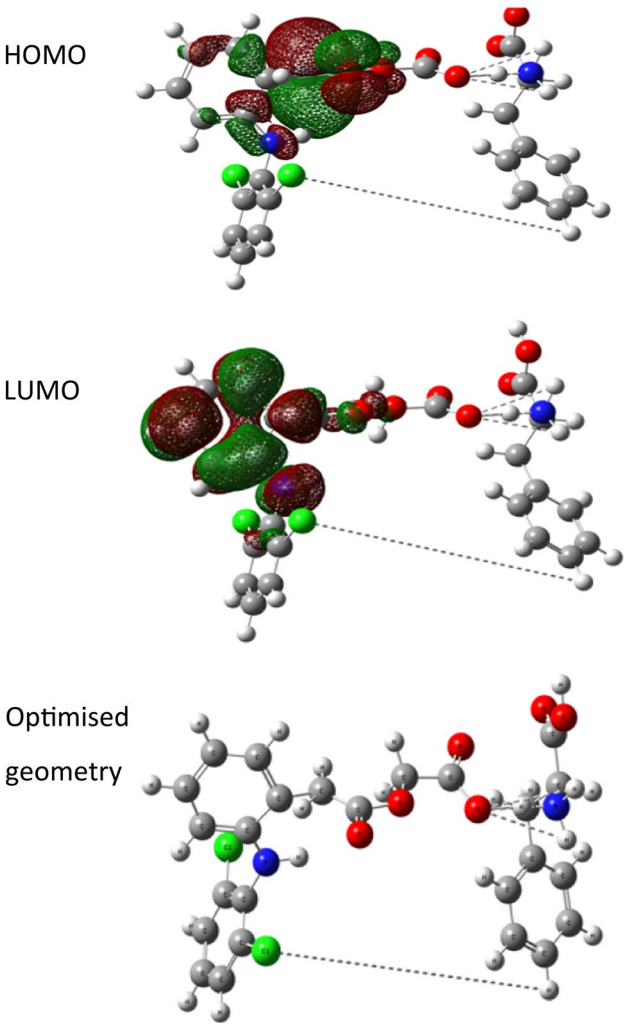


Fig. 8 Frontier orbital contours and optimised geometry of aceclofenac-PHE obtained by DFT calculations

acceptable value. This clearly shows the formation of the stable complex, and it is concluded that the drug (aceclofenac) binds with Hb [42–44].

The RMSF assessment in MD simulations evaluates the degree of protein flexibility. It quantifies the deviations of atoms from their mean locations. Studying the RMSF of both the protein and the protein–ligand complex facilitates the identification of areas of elasticity and the impact of ligand attachment on the behaviour of the protein. RMSF for the Hb and Hb-aceclofenac is given in Fig. 7. Major fluctuations of Hb protein from 580 to 760 atom numbers, were reduced significantly upon the addition of aceclofenac, implying increased rigidity of these amino acids due to binding of aceclofenac. Fluctuations around atom numbers 1073, 1340 and 1390 were also damped. The reduction in the fluctuation can be related to the complex formation leading to the reduction in the movement of residues. Increased fluctuations in the atoms ranging from 1140 to 1300 atom numbers might correspond to the opening of the protein to accommodate the incoming ligand, owing to the flexibility of the protein. So, it can be concluded that the complex (Hb-aceclofenac) is stable and that the aceclofenac binds strongly with the Hb.

Further, MM-PBSA calculations have been performed for the formation of the complex (Hb-aceclofenac) as in Table 4. The MM-PBSA approach is utilised to calculate interaction-free energy in biomolecular research and is progressively employed in *Insilico* drug development. The computation of interaction energies involves combining three factors: the alteration in potential energy in a vacuum, the energy required for desolvation, and the entropy associated with the configuration. MM-PBSA is commonly used to research biomolecular complexes because it incorporates conformational variations, and entropy contributes to binding energy when paired with molecular dynamics simulations. Various energies, including van der Waal energies (E_{vdw}), electrostatic energies (E_{elec}), polar solvation energies (E_{PS}) and solvent-accessible surface area energies (E_{SASA}) of the protein–ligand complex, were obtained from MM-PBSA calculations [45, 46].

Table 5 Thermodynamic parameters of aceclofenac-Phe complex

System	Optimisation energy (Hartree)	Zero-point energies ± corrections (Hartree)	Thermal energies ± corrections (Hartree)	Thermal enthalpies ± corrections (Hartree)	Thermal enthalpies ± corrections (Hartree)	Dipole moment (Debye)
Aceclofenac-PHE	− 2448.30	− 2447.16 ± 0.42	− 2447.13 ± 0.45	− 2447.13 ± 0.37	− 2447.22 ± 0.42	9.08

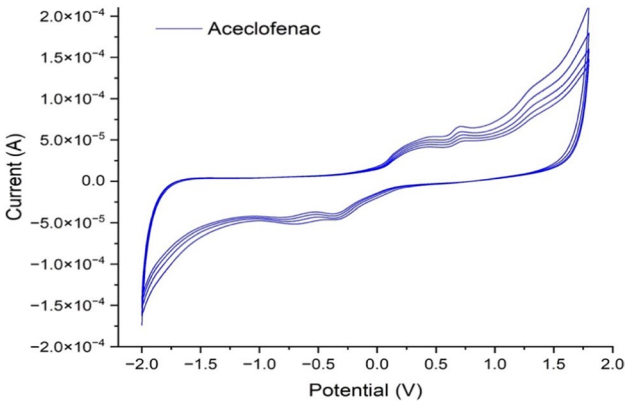


Fig. 9 Consecutive cyclic voltammograms of 0.1 mM aceclofenac in PBS (pH = 7) with a scan rate of 500 mV/s

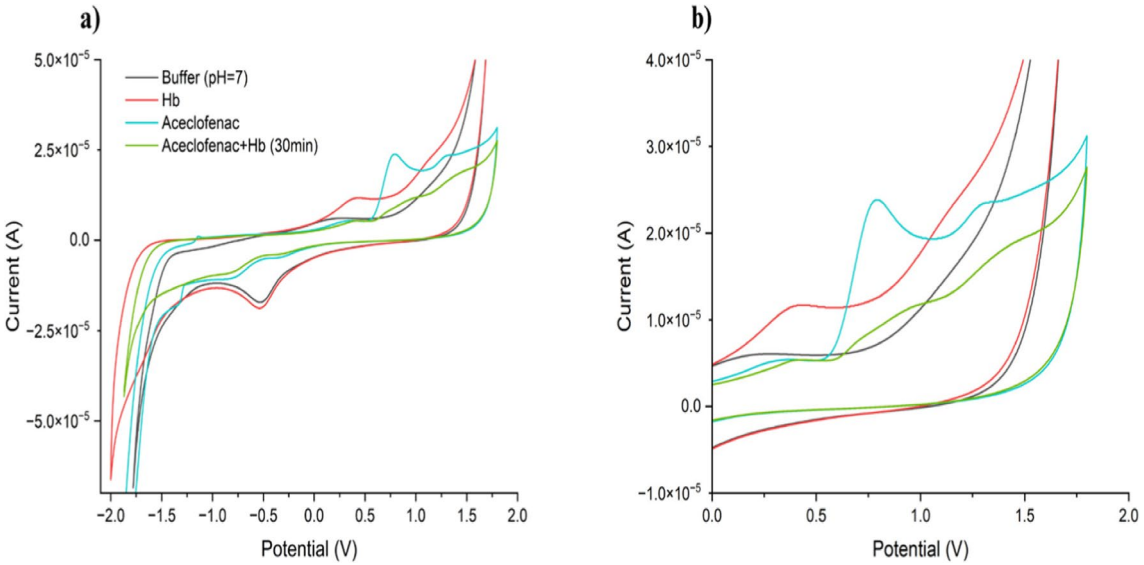


Fig. 10 Cyclic voltammograms of (**a**) PBS, Hb (μM), aceclofenac (0.1 mM) and Hb-aceclofenac (30 min) solutions at 100 mV/s scan rate and (**b**) zoom in view of a The time-dependent study of the interactions occurring between aceclofenac and Hb revealed that most of the binding occurred within 30 min of mixing aceclofenac and Hb solution. Figure 11 contains the cyclic voltammograms of aceclofenac + Hb solution at 0 min, 5 min, 15 min, 30 min, 60 min and 180 min of mixing. The voltammograms were taken at 100 mV/s scan rate. There was a significant decrease in the oxidative peak of aceclofenac at 30 min. There was no significant change in the voltammograms after 30 min of mixing

3.2.3 Density functional theory calculations

Based on molecular docking, the aceclofenac showed hydrogen bonding with the phenylalanine (PHE). Therefore, the authors have tried to investigate the interaction of the formation aceclofenac-Phe using DFT calculations, and it can be seen clearly that chlorine of aceclofenac showed interaction with the aromatic hydrogen atom of PHE as well as the oxygen of aceclofenac showed interaction with hydrogen of methyl of PHE as well as hydrogen of amine of PHE. (Fig. 8).

Thermodynamic parameters like optimisation energy, zero-point energy, thermal energies and enthalpies, along with the corrections, were obtained from the DFT results (Table 5) [47, 48]. The DFT calculations were employed to investigate the interaction between aceclofenac and the PHE residue of Hb, as it showed significant interactions in molecular docking. The study highlighted specific interactions, including hydrophobic interactions between the non-polar regions of aceclofenac and PHE, which play a crucial role in stabilising the complex. Additionally, the significant dipole moment of 9.08 Debye indicates strong electrostatic interactions, suggesting a substantial attraction between the charged regions of the molecules. The

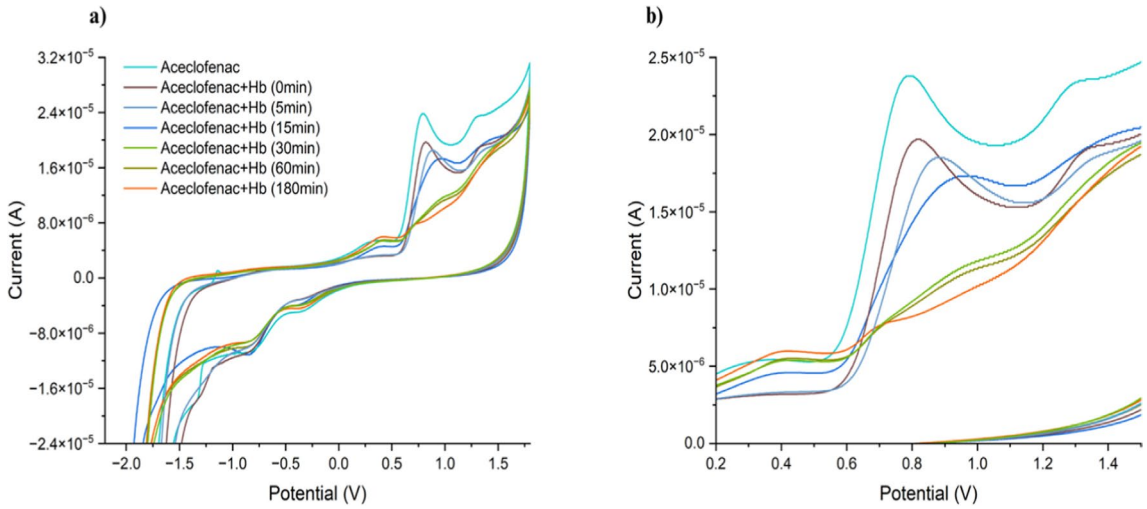


Fig. 11 Cyclic voltammograms of (**a**) aceclofenac + Hb solution at 0 min, 5 min, 15 min, 30 min, 60 min and 180 min of mixing and (**b**) zoom in view of a UV–Visible spectra exhibited that aceclofenac bind with Hb at the $\alpha_1\beta_2$ interface of the protein i.e., in the vicinity of aromatic amino acid residues (hyperchromic shift at 274 nm in Fig. 1). This observation was in accordance with fluorescence spectroscopy results as quenching was observed at 336 nm (Fig. 2) in the presence of the drug attributed to fluorescence quenching in the aromatic amino acid residues. The same observations were confirmed from molecular docking displaying the interactions between aceclofenac and aromatic amino acid residues in Hb (namely Tyrosine, Phenylalanine and Histidine) in 2D-docked pose (Fig. 5). Moreover, the decreased deviations in RMSD plot of aceclofenac-Hb complex (Fig. 6) and net negative $E_{binding}$ from MM-PBSA calculations (Table 4) insinuated the formation of a stable complex which was consistent with the obtained negative value of ΔG (negative ΔG means the process is feasible) in Table 2 and stabilised fluctuations in RMSF plot (Fig. 7) implied the formation of a more compact complex structure and reduced protein flexibility which was coherent with obtained negative value of ΔS in Table 2. In electrochemical studies, reduced peak current of aceclofenac implied binding of drug molecule with Hb. All these studies collectively imply the binding of aceclofenac with Hb, highlighting the stability and feasibility of the drug–protein complex

negative optimisation energy value of − 2447.16 Hartree signifies a stable and favorable binding between aceclofenac and PHE, as lower (more negative) optimisation energy values generally indicate greater stability. The thermodynamic parameters, including zero-point and thermal energies, further support the stability of the complex, providing a comprehensive understanding of the binding mechanism. This detailed analysis enhances the understanding of how aceclofenac interacts with hemoglobin.

3.3 Electrochemical analysis

The electrochemical behaviour of aceclofenac was studied using GCE (3 mm diameter) in PBS buffer (pH = 7). Figure 9 shows the successive cyclic voltammograms of 0.1 mM aceclofenac solution with a scan rate of 500 mV/s. Two oxidative peaks at + 0.7 V and + 1.3 V and two reductive peaks at − 0.3 V and − 0.7 V were reported. A remarkable decrease in the oxidative and reductive peak current with each successive scan was observed, which can be attributed to the blocking of the electrochemically active surface area of the electrode with each consecutive scan. Thus, in further studies, the first cyclic voltammogram was reported and the GCE was thoroughly cleaned with alumina paste after each scan.

The interactions of aceclofenac and Hb were studied by comparing the cyclic voltammograms of pure protein and pure drug with the voltammogram of the solution containing both. Figure 10 consists of cyclic voltammograms of PBS buffer, Hb, aceclofenac and aceclofenac + Hb solution which was kept for 30 min. 10 µM Hb solution in PBS gave a small oxidative peak at + 0.4 V. Another reductive peak at − 0.52 V was observed which can be attributed to the PBS as it was evident in the voltammogram of PBS. It is observed that upon mixing both the drug and the protein, there is a significant reduction in the peaks of both drug and protein. No new peaks are observed upon mixing of drug and protn. As aceclofenac binds with Hb protein, the number of free aceclofenac molecules in the solution was decreased resulting in the reduction of peak current of the drug [9, 10, 49]. Due to the binding of aceclofenac, there could be structural changes in Hb which caused the disappearance of Hb oxidative peak at + 0.4 V.

4 Conclusion

Spectroscopic, electrochemical and in silico studies are explored to understand the interaction of aceclofenac with Hb. UV–Visible and fluorescence spectroscopic data revealed the stable complex formation. Value of the bimolecular quenching constant is in the order of 10^{12} M^{-1} s^{-1}, suggests static quenching. Therefore, authors inferred that the quenching is mixed quenching during the binding of aceclofenac with Hb. At 298 K, the binding constant in order of 10^5 M^{-1} and the *value of n* was found to be approximately 1. Further, molecular docking revealed significant interaction between aceclofenac and haemoglobin at molecular level. Aceclofenac mainly binds with the PHE, VAL, HIS, LEU and TYR of the Hb and the binding energy was found out be – 31.79 kJ/mol. Then, the molecular dynamics simulations validated binding of aceclofenac with Hb. Various trajectories like RMSD and RMSF confirmed the formation of stable complex (aceclofenac-Hb) and the change in free energy for the formation of complex at molecular level was found to be – 105.09 kJ/mol with an error of 13.51 kJ/mol. Electrochemical studies also, confirmed the interactions between aceclofenac and Hb as the oxidative peaks of Hb (+ 0.4 V) and aceclofenac (+ 0.7 V and + 1.3 V) almost disappeared within 30 min.

Acknowledgements Authors convey sincere thanks to Prof. B. Jayaram, Incharge, SCFBio, Indian Institute of Technology, Delhi for allowing to use the computational facilities. M.B. Singh convey thanks to Council of Scientific and Industrial Research (CSIR), New Delhi for financial support. Authors convey thanks to Dr Vikas Bansal for Agilent Technology Inc. India for providing the aceclofenac. K. Kumari convey thanks to IoE, University of Delhi for the financial support.

Author contributions Sandeep Yadav: Conceptualization, Methodology, Investigation, Data curation, Writing—original draft, Visualization. Madhur Babu Singh: Conceptualization, Methodology, Investigation, Data curation, Writing—original draft, Visualization. Shubham Sewariya: Conceptualization, Methodology, Investigation, Data curation, Writing—original draft, Visualization. Shyam Bharat Yadav: Conceptualization, Methodology, Investigation, Data curation, Writing—original draft, Visualization. Shrikant Kukreti: Resources, review, editing, data curation, supervision. Prashant Singh: Resources, review, editing, data curation, supervision. Ramesh Chandra: Resources, review, editing, data curation, supervision. Sandeep Kumar Singh: Review, validation, editing, data curation, supervision. Pallavi Jain: Methodology, Investigation, Data curation, Writing—original draft, Visualization. Kamlesh Kumari: Conceptualization, validation, supervision, funding acquisition.

Funding Not applicable.

Data availability Data will be made available from the corresponding author on reasonable request of reader.

Declarations

Ethics approval and consent to participate Not applicable.

Competing interests The authors declare no competing interests.

References:

1. Yan X, Liu B, Chong B, Cao S. Interaction of Cefpiramide sodium with bovine hemoglobin and effect of the coexistent metal ion on the protein–drug association. J Lumin. 2013;142:155–62. https://doi.org/10.1016/J.JLUMIN.2013.04.009.
2. Tunç S, Duman O, Bozoğlan BK. Studies on the interactions of chloroquine diphosphate and phenelzine sulfate drugs with human serum albumin and human hemoglobin proteins by spectroscopic techniques. J Lumin. 2013;140:87–94. https://doi.org/10.1016/J.JLUMIN.2013.03.015.
3. Lanez T, Feizi-Dehnayebi M, Lanez E. Assessment of the electrostatic binding of ferrocenylmethyl-nitroaniline derivatives to DNA: a combined experimental and theoretical study. J Mol Struct. 2024;1308: 138386. https://doi.org/10.1016/J.MOLSTRUC.2024.138386.
4. Kamaljeet, Bansal S, Gupta Sen U Sen. A study of the interaction of bovine hemoglobin with synthetic dyes using spectroscopic techniques and molecular docking. Front Chem. 2017;5: 223050. https://doi.org/10.3389/FCHEM.2016.00050/BIBTEX.
5. Kaur L, Singh A, Datta A, Ojha H. Multispectroscopic studies of binding interaction of phosmet with bovine hemoglobin. Spectrochim Acta A Mol Biomol Spectrosc. 2023;296: 122630. https://doi.org/10.1016/J.SAA.2023.122630.

6. Lyndem S, Hazarika U, Athul P, Bhatta A, Prakash V, Jha AN, et al. A comprehensive in vitro exploration into the interaction mechanism of coumarin derivatives with bovine hemoglobin: spectroscopic and computational methods. J Photochem Photobiol A Chem. 2023;436: 114425. https://doi.org/10.1016/J.JPHOTOCHEM.2022.114425.
7. Sewariya S, Sehrawat H, Mishra N, Singh MB, Singh P, Kukreti S, et al. Comparative assessment of 9-bromo noscapine ionic liquid and noscapine: synthesis, in-vitro studies plus computational & biophysical evaluation with human hemoglobin. Int J Biol Macromol. 2023;247: 125791. https://doi.org/10.1016/J.IJBIOMAC.2023.125791.
8. 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: 124227. https://doi.org/10.1016/J.IJBIOMAC.2023.124227.
9. Yadav S, Sewariya S, Babu Singh M, Sachdeva B, Singh P, Chandra R, et al. Employing spectroscopic, electrochemical, and in silico research to investigate the interaction of ofloxacin. Antibacterial Drug Haemoglobin Chem Select. 2024;9: e20240077. https://doi.org/10.1002/slct.202400770.
10. Yadav S, Sewariya S, Singh P, Chandra R, Jain P, Kumari K. Analytic and in silico methods to understand the interactions between dinotefuran and haemoglobin. Chem Biodivers. 2024;21: e202400495. https://doi.org/10.1002/cbdv.202400495.
11. Morris GM, Ruth H, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, et al. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. J Comput Chem. 2009;30:2785. https://doi.org/10.1002/JCC.21256.
12. Forli S, Huey R, Pique ME, Sanner MF, Goodsell DS, Olson AJ. Computational protein-ligand docking and virtual drug screening with the AutoDock suite. Nat Protoc. 2016;11:905–19. https://doi.org/10.1038/NPROT.2016.051.
13. Cousins RK. ChemDraw Ultra 9.0 CambridgeSoft, 100 CambridgePark Drive, Cambridge, MA 02140. www.cambridgesoft.com. See Web site for pricing options. J Am Chem Soc. 2005;127: 4115–4116. https://doi.org/10.1021/ja0410237.
14. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera—a visualization system for exploratory research and analysis. J Comput Chem. 2004;25:1605–12. https://doi.org/10.1002/JCC.20084.
15. Bjelkmar P, Larsson P, Cuendet AM, Hess B, Lindahl E. Implementation of the CHARMM force field in GROMACS: analysis of protein stability effects from correction maps, virtual interaction sites, and water models. J Chem Theory Comput. 2010;6:459–66. https://doi.org/10.1021/ct900549r.
16. Abraham MJ, Murtola T, Schulz R, Páll S, Smith JC, Hess B, et al. GROMACS: high performance molecular simulations through multi-level parallelism from laptops to supercomputers. SoftwareX. 2015;1–2:19–25. https://doi.org/10.1016/J.SOFTX.2015.06.001.
17. Mark P, Nilsson L. Structure and dynamics of the TIP3P, SPC, and SPC/E water models at 298 K. J Phys Chem A. 2001;105:9954–60. https://doi.org/10.1021/JP003020W/ASSET/IMAGES/LARGE/JP003020WF00004.JPEG.
18. Babu Singh M, Himani, Yadav S, Singh P. A theoretical study to understand the impact of Mpro of nCoV on the hormones. ChemistrySelect. 2024;9:e202304767. https://doi.org/10.1002/SLCT.202304767.
19. Raman APS, Singh MB, Vishvakarma VK, Jain P, Kumar A, Sachdeva S, et al. An investigation for the interaction of gamma oryzanol with the Mpro of SARS-CoV-2 to combat COVID-19: DFT, molecular docking, ADME and molecular dynamics simulations. J Biomol Struct Dyn. 2023;41:1919–29. https://doi.org/10.1080/07391102.2022.2029770.
20. Singh MB, Sharma R, Kumar D, Khanna P, Mansi, Khanna L, et al. An understanding of coronavirus and exploring the molecular dynamics simulations to find promising candidates against the Mpro of nCoV to combat the COVID-19: a systematic review. J Infect Public Health. 2022;15:1326–1349. https://doi.org/10.1016/J.JIPH.2022.10.013.
21. Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, et al. Gaussian 16, Rev. C.01. Gaussian 16, Rev. C. 01. 2016.
22. Tirado-Rives J, Jorgensen WL. Performance of B3LYP density functional methods for a large set of organic molecules. J Chem Theory Comput. 2008;4:297–306. https://doi.org/10.1021/CT700248K/SUPPL_FILE/CT700248K-FILE002.PDF.
23. Singh MB, Prajapat A, Jain P, Kumar A, Singh P, Bahadur I, et al. Investigate the significance of DES to enhance the solubility of noscapine: DFT calculations, MD simulations, and experimental approach. Ionics (Kiel). 2024;30:1795–813. https://doi.org/10.1007/S11581-023-05359-3/FIGURES/11.
24. Nichols WL, Zimm BH, Ten Eyck LF. Conformation-invariant structures of the α1β1 human hemoglobin dimer. J Mol Biol. 1997;270:598–615. https://doi.org/10.1006/JMBI.1997.1087.
25. Shi JH, Lou YY, Zhou KL, Pan DQ. Elucidation of intermolecular interaction of bovine serum albumin with Fenhexamid: a biophysical prospect. J Photochem Photobiol B. 2018;180:125–33. https://doi.org/10.1016/J.JPHOTOBIOL.2018.01.025.
26. Wang Q, Huang CR, Jiang M, Zhu YY, Wang J, Chen J, et al. Binding interaction of atorvastatin with bovine serum albumin: spectroscopic methods and molecular docking. Spectrochim Acta A Mol Biomol Spectrosc. 2016;156:155–63. https://doi.org/10.1016/J.SAA.2015.12.003.
27. Shi JH, Wang Q, Pan DQ, Liu TT, Jiang M. Characterization of interactions of simvastatin, pravastatin, fluvastatin, and pitavastatin with bovine serum albumin: multiple spectroscopic and molecular docking. J Biomol Struct Dyn. 2017;35:1529–46. https://doi.org/10.1080/07391102.2016.1188416.
28. Kritika N, Pant M, Yadav M, Verma AK, Mahapatro AK, Roy I. Comparative analysis of cobalt ferrite and iron oxide nanoparticles using bimodal hyperthermia, along with physical and in silico interaction with human hemoglobin. J Mater Chem B. 2023;11:4785–98. https://doi.org/10.1039/D2TB02447K.
29. Wang BL, Pan DQ, Zhou KL, Lou YY, Shi JH. Multi-spectroscopic approaches and molecular simulation research of the intermolecular interaction between the angiotensin-converting enzyme inhibitor (ACE inhibitor) benazepril and bovine serum albumin (BSA). Spectrochim Acta A Mol Biomol Spectrosc. 2019;212:15–24. https://doi.org/10.1016/J.SAA.2018.12.040.
30. Wang BL, Kou SB, Lin ZY, Shi JH, Liu YX. Insights on the interaction mechanism of brigatinib to human α-1-acid glycoprotein: experimental and computational approaches. Int J Biol Macromol. 2020;157:340–9. https://doi.org/10.1016/J.IJBIOMAC.2020.04.151.
31. Wang BL, Kou SB, Lin ZY, Shi JH. Insight into the binding behavior of ceritinib on human α-1 acid glycoprotein: multi-spectroscopic and molecular modeling approaches. Spectrochim Acta A Mol Biomol Spectrosc. 2020;232: 118160. https://doi.org/10.1016/J.SAA.2020.118160.
32. Hazra S, Suresh KG. Structural and thermodynamic studies on the interaction of iminium and alkanolamine forms of sanguinarine with hemoglobin. J Phys Chem B. 2014;118:3771–84. https://doi.org/10.1021/JP409764Z/SUPPL_FILE/JP409764Z_SI_001.PDF.

33. Alpert B, Jameson DM, Weber G. Tryptophan emission from human Hemoglobin and its isolated subunits. Photochem Photobiol. 1980;31:1–4. https://doi.org/10.1111/J.1751-1097.1980.TB03674.X.
34. Corchnoy SB, Swartz TE, Lewis JW, Szundi I, Briggs WR, Bogomolni RA. Intramolecular proton transfers and structural changes during the photocycle of the LOV2 domain of phototropin 1. J Biol Chem. 2003;278:724–31. https://doi.org/10.1074/jbc.M209119200.
35. Lakowicz JR, Weber G. Quenching of fluorescence by oxygen. A probe for structural fluctuations in macromoleculest. Biochemistry. 1973;12:4161–70.
36. Manna A, Chakravorti S. Role of block copolymer-micelle nanocomposites in dye–bovine serum albumin binding: a combined experimental and molecular docking study. Mol Biosyst. 2012;9:246–57. https://doi.org/10.1039/C2MB25368B.
37. Tong JQ, Tian FF, Li Q, Li LL, Xiang C, Liu Y, et al. Probing the adverse temperature dependence in the static fluorescence quenching of BSA induced by a novel anticancer hydrazone. Photochem Photobiol Sci. 2012;11:1868–79. https://doi.org/10.1039/C2PP25162K/METRICS.
38. Khan AY, Kumar GS. Probing the binding of anticancer drug topotecan with human hemoglobin: Structural and thermodynamic studies. J Photochem Photobiol B. 2016;163:185–93. https://doi.org/10.1016/J.JPHOTOBIOL.2016.08.031.
39. Ovung A, Luikham S, Bhattacharyya J. Interaction of hemoglobin and sulfamethazine: A spectrofluorimetric characterization of the binding thermodynamics. J Indian Chem Soc. 2020; 2667–2672.
40. Pantsar T, Poso A. Binding affinity via docking: fact and fiction. Molecules. 2018;23:1899. https://doi.org/10.3390/MOLECULES23081899.
41. Babu Singh M, Jain P, Tomar J, Kumar V, Bahadur I, Arya DK, et al. An in silico investigation for acyclovir and its derivatives to fight the COVID-19: molecular docking, DFT calculations, ADME and td-molecular dynamics simulations. J Indian Chem Soc. 2022;99: 100433. https://doi.org/10.1016/J.JICS.2022.100433.
42. Rani A, Aslam M, Khan J, Pandey G, Singh P, Maharia RS, et al. computational insights into chromene/pyran derivatives: molecular docking, ADMET studies, DFT calculations, and MD simulations as promising candidates for Parkinson's disease. Chem Biodivers. 2024;21: e202400920. https://doi.org/10.1002/CBDV.202400920.
43. Raman APS, Kumari K, Jain P, Vishvakarma VK, Kumar A, Kaushik N, et al. In silico evaluation of binding of 2-deoxy-D-glucose with Mpro of nCoV to combat COVID-19. Pharmaceutics. 2022;14:135. https://doi.org/10.3390/PHARMACEUTICS14010135.
44. Massey I, Yadav S, Kumar D, Maharia RS, Kumari K, Singh P. An insight for the inhibition of anxiolytic and anti-convulsant effects in zebrafish using the curcumins via exploring molecular docking and molecular dynamics simulations. Mol Divers. 2024; 1–17. https://doi.org/10.1007/S11030-024-10865-1/TABLES/9.
45. Kumari R, Kumar R, Lynn A. G-mmpbsa—a GROMACS tool for high-throughput MM-PBSA calculations. J Chem Inf Model. 2014;54:1951–62. https://doi.org/10.1021/CI500020M/SUPPL_FILE/CI500020M_SI_001.PDF.
46. Rastelli G, Del Rio A, Degliesposti G, Sgobba M. Fast and accurate predictions of binding free energies using MM-PBSA and MM-GBSA. J Comput Chem. 2010;31:797. https://doi.org/10.1002/jcc.21372.
47. Aslam M, Pandey G, Deshwal N, Kumar A, Kumari K, Bahadur I, et al. Study the solubility of pharmaceutical ingredients and their eutectic mixtures: an in-depth density functional theory and molecular dynamics simulations approaches. J Mol Liq. 2024;397:124070. https://doi.org/10.1016/J.MOLLIQ.2024.124070.
48. Kumar A, Kumari K, Raman APS, Jain P, Kumar D, Singh P. An insight for the interaction of drugs (acyclovir/ganciclovir) with various ionic liquids: DFT calculations and molecular docking. J Phys Org Chem. 2022;35:e4287. https://doi.org/10.1002/POC.4287.
49. Sandford C, Edwards MA, Klunder KJ, Hickey DP, Li M, Barman K, et al. A synthetic chemist's guide to electroanalytical tools for studying reaction mechanisms. Chem Sci. 2019;10:6404–22. https://doi.org/10.1039/C9SC01545K.