Investigating binding dynamics of trans resveratrol to HSA for an efficient displacement of aflatoxin B$_1$ using spectroscopy and molecular simulation

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Resveratrol is a polyphenol belonging to the class stilbenes. The active and stable form of resveratrol is trans-resveratrol. This polyphenol is bestowed with numerous biological properties. Aflatoxin B$_1$ is a hepato-carcinogen and mutagen that is produced by Aspergillus species. In this study, the interaction of trans-resveratrol with HSA followed by competitive dislodging of AFB$_1$ from HSA by trans-resveratrol has been investigated using spectroscopic studies. The UV-absorption studies revealed ground state complex formation between HSA and trans-resveratrol. Trans-resveratrol binds strongly to HSA with the binding constant of ~ $10^7$ M$^{-1}$ to a single binding site ($n = 1.58$), at 298.15 K. The Stern–Volmer quenching constant was calculated as $7.83 \times 10^4$ M$^{-1}$ at 298.15 K, suggesting strong fluorescence quenching ability of trans-resveratrol. Site markers displacement assay projected subdomain IIA as the binding site of trans-resveratrol to HSA. The molecular docking approach envisages the amino acid residues involved in the formation of the binding pocket. As confirmed from the site marker displacement assays, both trans-resveratrol and AFB$_1$ binds to HSA in the same binding site, subdomain IIA. The study explores the ability of trans-resveratrol to displace AFB$_1$ from the HSA-AFB$_1$ complex, thereby affecting the toxicokinetic behavior of AFB$_1$ associated with AFB$_1$ exposure.

Resveratrol belongs to a class of polyphenols called stilbenes having C$_6$-C$_2$-C$_6$ skeleton and is a natural stilbene present in ample amounts in grapes. In nature, Cis and trans form of resveratrol are found predominantly where the trans form is biologically more stable and active. Trans-resveratrol is chemically 3, 5, 4-trihydro-trans-stilbene, first obtained in 1939 from Veratrum album. It is a phytoalexin produced in response to stress or mechanical injury to a plant. It is identified in approximately 70 species of plants and is predominantly found in the skin and seeds of red grapes. About 50–100 μg/g concentration of trans-resveratrol is found in the skin of grapes. Trans-resveratrol is known to possess numerous properties, including anti-cancerous, anti-oxidant, and anti-inflammatory. Its neuroprotective, antimicrobial and antifungal property has also been reported. The effect of dietary resveratrol in AFB$_1$ induced changes in broiler chicken has also been evaluated earlier. The chemical structure of trans-resveratrol molecule is shown in Fig. 1a.

Aflatoxin B$_1$ is a hazardous material and is regarded as a potent hepato-carcinogen and mutagen. It is obtained from fungus, Aspergillus flavus, Aspergillus parasiticus and Aspergillus nomius. However, AFB$_1$ production from other species of Emericella has also been reported. The crops like cereals, peanuts, and corns are exposed to these mycotoxin-producing fungi, leading to their contamination with AFB$_1$. AFB$_1$ contamination is a common problem in developing countries due to the lack of proper infrastructure and facilities. It contaminates agricultural feed and poses a severe risk to the health of animals and humans. Aflatoxicosis becomes a serious concern when linked with hepatitis B (HBV) and hepatitis C (HCV) virus. Generally, ≤ 20% of the population in developing countries is affected by HBV and HCV infection. However, when HBV or HCV infection and aflatoxicosis are combined, the probability of developing the cancer increases. The binding dynamics of AFB$_1$ with HSA have already been documented, and it binds moderately with a binding constant of ~ $10^4$ M$^{-1}$. The molecular structure of AFB$_1$ is depicted in Fig. 1b.
HSA is a major transport protein in humans, having a molecular weight of 66.5 kDa comprising 585 amino acid residues, with single tryptophan viz. Trp-214. The presence of aromatic amino acid residues like Trp, Tyr, and Phe imparts HSA its fluorescent property. Nevertheless, the significant contribution is due to tryptophan residues. It is a model protein that has been explored to investigate ligand–protein interactions.

Pharmacokinetics and pharmacodynamics studies of molecules using biophysical tools are prerequisites in determining its efficacy, toxicity, and elimination process from the body. There are a number of studies exploring the hepatocellular toxicity of AFB1 using in vitro and in vivo approaches. Though, studies aiming at the fate of this hepatocarcinogen in the presence of serum albumin and vice versa are fewer. Recently few studies focused on the binding behavior of AFB1, with BSA, HSA, and chicken egg albumin, provided information about its binding constant, site of binding, and thermodynamic parameters. Still, these studies have gaps that need to be filled by an approach that could unload the mycotoxin from the precise binding location in serum albumin, leading to its biotransformation followed by its elimination from the body. This study focuses on the very same approach using a polyphenol trans-resveratrol that could effectively dislodge the AFB1 from serum albumin using fluorescence spectroscopic tools. In the present study, UV–absorption spectroscopy is used to investigate the structural alterations and ground-state complex formation between HSA and trans-resveratrol, the fluorescence spectroscopy was used to calculate the binding constant of the polyphenol and HSA, thermodynamic parameters (ΔG, ΔH, and ΔS), followed by site marker displacement assay to examine the binding site of trans-resveratrol on HSA. The Circular dichroism study was performed to investigate secondary structure changes induced by trans-resveratrol in HSA, followed by the calculation of melting temperature (Tm). Temperature–dependent heat denaturation and unfolding profile of HSA in the presence of trans-resveratrol were also determined. Molecular docking analysis visualized the amino acid residues involved in the binding of trans-resveratrol with HSA along with major binding forces stabilizing the interacting entities. In silico amino acid substitution study was performed to ascertain the role of Trp-214 in the binding process of the ligand to the protein molecule. After confirming the binding location of the polyphenol, a comparative analysis of the fluorescence quenching strength of trans-resveratrol and AFB1 was established for HSA. Later on, the displacement assays were performed to analyze the dislodging potential of trans-resveratrol to displace AFB1 from the HSA and vice versa. The outcome of this study will help the researchers to understand the kinetics and dynamics of the binding process of trans-resveratrol and AFB1.

Results and discussion

Spectroscopic studies. The binding and interactive mode of flavonoid trans-resveratrol with HSA was investigated before its use as a dislodging agent for HSA bound AFB1. The binding behavior was studied using spectroscopic tools like fluorescence spectroscopy and UV–visible spectroscopy. UV-absorption spectroscopy was used to ascertain the structural changes induced by trans-resveratrol upon binding with HSA. The UV-absorption property of HSA is by virtue of its aromatic amino acids (Trp, Tyr, and Phe) that impart a strong UV absorption signal at 280 nm. Nevertheless, trans-resveratrol shows the absorption signal at 319 nm, as shown in Fig. 2a. From the Fig. 2a, it is clear that in the presence of an increasing concentration of trans-resveratrol, the hyperchromic effect was observed at absorption maxima (λmax) of HSA, coupled with bathochromic shift, suggesting structural alterations in the native structure of HSA and a ground state complex formation between trans-resveratrol and HSA. The absorption spectrum also gives a clue about the existence of static quenching between trans-resveratrol and HSA, since in static type of quenching, the absorption spectra of native protein changes in the presence of a ligand molecule, however in dynamic quenching, it remains unaffected. Fluorescence spectroscopy was performed to get insight into the binding and thermodynamics parameters associated with the interaction of trans-resveratrol with HSA. When excited at 280 nm, a strong fluorescence quenching was observed in the fluorescence emission spectrum of HSA in the presence of an increasing concentration of trans-resveratrol (0–14 µM) as shown in Fig. 2b. HSA consists of fluorophores, critical for the fluorescent property of the protein viz, Trp, Tyr, and Phe, where the major contribution is from Trp-214 residue. Quenching is accompanied by redshift suggesting structural and conformational alterations in the native structure of HSA in the presence of trans-resveratrol. Bathochromic shift is the result of the increase in the polarity around fluorophores in HSA in the presence of trans-resveratrol.

Stern–Volmer quenching constant (Ksv) for binding trans-resveratrol with HSA was calculated according to Eq. (1). There is linearity between the concentration of T-res (Q) and F0/F, as shown in Fig. 3a, at 298.15, 303.15, and 308.15 K. With the rise in temperature, a decrease in the slope of the Stern–Volmer plot is observed, the binding between HSA and trans-resveratrol also destabilizes with the increase in the temperature, suggesting a static mode of fluorescence quenching and quashing the existence of dynamic quenching mechanism operating between HSA and trans-resveratrol. Nevertheless, from Fig. 3a, if we look at the F0/F vs Q plot at 298.15 K, at a higher concentration of trans-resveratrol, the F0/F vs Q plot is shifted towards y axis, envisaging a mixed
Figure 2. (a) UV-absorption spectrum of HSA (5 µM), and (b) fluorescence emission spectrum of HSA (5 µM) in the presence of an increasing concentration of trans-resveratrol (0–14 µM) at 298.15 K and pH 7.4. λ_ex of HSA = 280 nm. The concentration of trans-resveratrol alone is 2 µM.

Figure 3. (a) Plot of F_0/F versus Q for the calculation of Stern–Volmer quenching constant (K_SV), (b) plot of log (F_0/F)/F versus log (Q) for the calculation of binding constant (K_b), (c) Van’t Hoff plot of lnK versus 1/T (K) for the calculation of thermodynamic parameters for HSA-T-res system, at 298.15, 303.15 and 308.15 K and pH 7.4.
HSA. All the thermodynamic parameters are reported in Table 2.

That was found to be negative, suggesting a favorable and spontaneous process of binding.

Figure 3c shows the lnK versus 1/T (K) plot, and the value of Kb was calculated to be 6.36 ± 0.32 × 107 M⁻¹ at 298.15 K. Such a high binding constant is an indication of strong binding affinity between HSA and trans-resveratrol. The values of Kb and number of binding sites (n) at three different temperatures are reported in Table 2.

The Gibbs free energy (ΔG), enthalpy change (ΔH), and entropy change (ΔS) are the essential thermodynamic parameters that portray the spontaneity and favorability of a chemical reaction. These thermodynamic values are calculated using Eqs. (6) and (7) of the “Methods” section. Figure 3c shows the lnK versus 1/T (K) plot, the intercept and slope of the plot were used in the calculation of ΔS and ΔH, which was calculated to be −257.67 cal mol⁻¹ K⁻¹ and −87.60 kcal mol⁻¹, respectively. The magnitude of both ΔS and ΔH is negative, signifying hydrogen bonding and van der Waals interaction as the major forces acting in HSA and trans-resveratrol complex stabilization. The principal forces acting between the protein and ligand molecule are hydrogen bonding and van der Waals interaction when ΔS < 0 > ΔH.

Location of the binding site of trans-resveratrol in HSA using site markers displacement assay.

Our molecule of interest, trans-resveratrol, binds to HSA with a much higher affinity as compared to AFB₁, hence possessing the potential to compete with AFB₁. We further explored the binding site of trans-resveratrol on HSA using site markers, warfarin, and ibuprofen. These two site markers are routinely used probes to locate the binding site of a small molecule on protein. Most of the ligand binds to the protein at Sudlow's site I (subdomain IIA) and Sudlow's site 2 (subdomain IIIA). Warfarin binds to subdomain IIA, and ibuprofen binds to subdomain IIIA. The binding location of AFB₁ on HSA has already been studied by Tan et al. and Poor et al., confirming Sudlow's site 1 as the binding pocket of AFB₁ on HSA. If trans-resveratrol and AFB₁ share the same binding site on the protein molecule, trans-resveratrol, by virtue of its higher binding constant (Kₐ) for HSA than AFB₁, could easily displace the mycotoxin from the HSA and increase its availability in the body in free form rather than bound form. The percentage displacement was calculated from the plot of F₂/F1 × 100 versus probe/HSA, obtained using Eq. (7) of the “Methods” section.

Table 1. Tabular representation of Stern–Volmer quenching constant (Ksv), bimolecular quenching constant (Kq) for the HSA-T-res system at 298.15, 303.15, and 308.15 K and pH 7.4.

| Temp (K) | Ksv (M⁻¹) | R² | n | ΔG (kcal mol⁻¹) | ΔH (kcal mol⁻¹) | ΔS (cal mol⁻¹ K⁻¹) |
|----------|-----------|----|---|----------------|----------------|------------------|
| 298.15   | 6.36 ± 0.32 × 10⁷ | 0.990 | 1.58 ± 0.006 | −10.63 | −87.60 | −257.67 |
| 303.15   | 5.29 ± 0.33 × 10⁷ | 0.987 | 1.42 ± 0.006 | −9.32 | −87.60 | −257.67 |
| 308.15   | 4.99 ± 0.23 × 10⁷ | 0.992 | 1.27 ± 0.004 | −8.03 | −87.60 | −257.67 |

Table 2. Tabular representation of binding constant (Kb) and thermodynamic parameters like ΔH, ΔG, and ΔS for HSA-T-res system at three different temperatures, 298.15, 303.15, and 308.15 K.

| Temperature (K) | Kb (M⁻¹) | R² | n | ΔG (kcal mol⁻¹) | ΔH (kcal mol⁻¹) | ΔS(cal mol⁻¹ K⁻¹) |
|----------------|----------|----|---|----------------|----------------|------------------|
| 298.15         | 7.83 × 10⁷ | 0.993 | 2.06 × 10⁷ | 3.95 × 10¹³ | 0.005 | 0.001 |
| 303.15         | 4.35 × 10⁷ | 0.998 | 2.06 × 10⁷ | 3.95 × 10¹³ | 0.001 | 0.001 |
| 308.15         | 2.06 × 10⁷ | 0.999 | 2.06 × 10⁷ | 3.95 × 10¹³ | 0.001 | 0.001 |
the percentage displacement of trans-resveratrol by warfarin is more prominent than ibuprofen, which provides a clue that trans-resveratrol binds at the site in HSA where warfarin binds, suggesting subdomain IIA or Sudlow’s site 1 as the binding site of trans-resveratrol in HSA. Various studies on ligand and protein interactions have used site markers displacement assays as a reliable method for locating the binding site of the ligand of interest in protein molecules.

Circular dichroism measurement. Circular dichroism is a valuable tool for deciphering the conformational or secondary structure change in the protein induced by a ligand. It is a routinely used technique involved in ligand and protein interaction to investigate the nature of the binding between protein and molecules. Interaction of chromophores in the protein molecule, in an asymmetric milieu, with the polarized light results in CD signals. Peptide bonds absorb polarized light in the far UV–region. The far UV-CD signal of HSA with predominant alpha helix exhibits two negative ellipticity at 208 and 222 nm as a consequence of n→π* and π→π* transition. From Fig. 5a, it is observed that the native HSA exhibited two peaks at 208 and 222 nm, suggesting the predominance of the alpha helix. The MRE value at 208 nm and percentage alpha helix was calculated using Eqs. (8) and (9) of the “Methods” section.

Figure 4. Graphical representation of site marker displacement assay for the location of binding site of trans-resveratrol on HSA at 298.15 K and pH 7.4, warfarin, and ibuprofen site markers were used for subdomain IIA and subdomain IIIA specific binding, respectively. At the level of 0.05 (p < 0.05), the data is significant. Error bar represents the standard deviation value (mean ± SD).

Figure 5. (a) Far UV-CD spectra of HSA (5 µM) in the presence of a different concentration of trans-resveratrol with HSA (10 µM and 20 µM) at 298.15 K and pH 7.4. (b) Bar graph showing the CD values in mdeg, at 280 and 222 nm, for HSA alone and HSA in the presence of different molar ratio of trans-resveratrol (1:2 and 1:4).
Native HSA showed 61.39% alpha-helical content. In the presence of trans-resveratrol (10 and 20 µM), the alpha-helical content was changed to 65.07 and 68.24%, respectively. Figure 5a,b indicate trans-resveratrol induced conformational changes and increase in alpha-helix in HSA, suggesting stabilization of native structure of HSA in the presence of trans-resveratrol. Lower concentrations of trans-resveratrol (2–8 µM) induced insignificant changes in the secondary structure of HSA. Figure 5b shows the CD values at 208 and 222 nm for HSA in the absence and presence of trans-resveratrol, which depicts a clear picture of the increase in CD (mdeg) values corresponding to increase in an alpha helix at 10 and 20 µM trans-resveratrol. Some phytochemicals and drug molecules, on interaction with albumins, have shown to increase the alpha helix and thereby its stability, and it is due to an increase in the extent of hydrogen bonding in the protein molecules.

Thermal stability of HSA in the presence of trans-resveratrol was also investigated using CD spectroscopy, by measuring changes in CD signal at 222 nm by sigmoidal fitting, as a function of temperature (20–90 °C). Hydrophobic interactions are the major contributors to the folding mechanism of protein. However, other factors like hydrogen bonding and electrostatic interactions also play significant roles in stabilizing protein structure. The thermal stability of the protein is directly proportional to its T_m value. In other words higher the T_m value more is the thermal stability of the protein. The native HSA exhibited T_m values of 63.75 °C. However, in the presence of trans-resveratrol, it was increased to 66.25 °C. The increase in the T_m value from 63.75 °C to 66.25 °C confirms the trans-resveratrol-assisted folding of HSA. The thermal unfolding experiment further explores that the thermal stability of HSA is increased in the presence of trans-resveratrol. Certain drugs which bind to subdomain IIA, like warfarin and virstatin have been known to increase the T_m of HSA, as reported in earlier studies. Figure 6 shows the melting profile of HSA in the presence of trans-resveratrol.

Far UV- three dimensional CD spectra of HSA in the absence and presence of trans-resveratrol as a function of temperature were also plotted to envisage the ligand induced structural and conformational perturbation of HSA at each temperature ranging from 20 to 90 °C. From Fig. 7, it is clear that with each rise in temperature from 20 to 90 °C, the negative ellipticity at 208 and 222 nm decreases, suggesting the unfolding of HSA as a function of temperature. In the presence of 20 µM trans-resveratrol, the alpha-helical content of HSA is protected at each rise in temperature, suggesting the trans-resveratrol mediated stabilization of the secondary structure of native HSA. From Table 3, it is evident that at 20 °C, native HSA showed 61.30% alpha-helix, and at 90 °C, it was reduced to 27.69% as a result of unfolding and temperature-induced denaturation. However, trans-resveratrol bound HSA at 20 °C exhibited 67.58%, higher than the HSA alone, at each increment in the temperature; HSA bound trans-resveratrol showed higher alpha-helix as compared to HSA alone at the same temperature. The drastic decrease in the alpha helix in HSA as a function of temperature is attributed to the reduction in the hydrogen bonding in the amino acids. Nevertheless, trans-resveratrol proved to be effective in protecting the unfolding of HSA, thereby restoring hydrogen bonding in the amino acid residues at a given temperature range. Figure 7 reflects the far UV-CD spectra of HSA in the presence of trans-resveratrol from 20 to 90 °C.

Displacement of AFB_1 from HSA by trans-resveratrol. After confirming that AFB_1 and trans-resveratrol shares the same binding site, subdomain IIA in HSA, displacement assay was followed to check the dislodging potential of trans-resveratrol against AFB_1 competing for the same binding pocket. In Fig. 8a, it is shown that each increasing concentration of trans-resveratrol (0–20 µM), displaces the AFB_1 bound to HSA from the HSA-AFB_1 system. However, when the displacing potential of AFB_1 against trans-resveratrol was studied for the HSA-Tres system, as shown in Fig. 8b, AFB_1 failed to dislodge trans-resveratrol from HSA, indicating the inability of AFB_1 to compete for albumin sharing the same binding site with trans-resveratrol. From Fig. 8b, it is clear that the increasing concentration of AFB_1 (0–20 µM) has no effect on the percentage displacement of trans-resveratrol bound to HSA.
Comparative analysis of the binding potentials of trans-resveratrol and AFB₁ to HSA. We also performed the comparative analysis of the effect of AFB₁ and trans-resveratrol on HSA, as depicted in Fig. 9. From the figure, based on the values of the fluorescence intensities, the emission spectrum of HSA underwent more quenching in the presence of trans-resveratrol than AFB₁. The decrease in the fluorescence intensity is more in HSA + T-res as compared to HSA + AFB₁. To further gain insights into the dislodging potential of trans-resveratrol, competing for HSA, equal concentrations of AFB₁ and trans-resveratrol were used for displacing trans-resveratrol bound HSA and AFB₁ bound HSA, respectively. From Table 4, it is evident that HSA alone (5 µM) showed fluorescence intensity of 47,776.6, the fluorescence intensity in the presence of AFB₁ and trans-resveratrol was decreased to 37,322.6 and 24,784.8, respectively. The fluorescence intensity of HSA for the HSA + T-res + AFB₁ system (Table 4) insignificantly differed from the fluorescence intensity of the HSA + T-res system, thereby confirming the inability of AFB₁ to displace trans-resveratrol from HSA-T-res. Nevertheless, the fluorescence emission intensity of HSA for the HSA + AFB₁ + T-res system (Table 4) significantly changed from
the fluorescence emission intensity of the HSA + AFB1 system, elucidating the potentiality of trans-resveratrol to dislodge the AFB1 bound to albumin and apprehending its binding site.

Molecular docking and amino acid substitution studies. Molecular docking is a powerful computational approach to investigate ligand binding to the protein molecule at the atomic level. These in silico tools

**Table 4.** The effect of AFB1 and trans-resveratrol on the fluorescence intensity of HSA at λex = 280 nm, T = 298.15 K, and pH = 7.4. The concentration of HSA alone was taken as 5 µM and the concentration of T-res = AFB1 = 14 µM.

| System                  | Fluorescence intensity |
|-------------------------|------------------------|
| HSA alone               | 47,776.6 ± 183.17      |
| HSA + AFB1              | 37,322.6 ± 236.79      |
| HSA + AFB1 + T-res      | 17,301.3 ± 250.46      |
| HSA + T-res             | 24,784.8 ± 153.83      |
| HSA + T-res + AFB1      | 22,610.6 ± 356.61      |

Figure 8. Graphical representation of (a) AFB1 displacement by trans-resveratrol in HSA-AFB1 system, (b) trans-resveratrol displacement by AFB1 in HSA-T-res system. At the level of 0.05 (p < 0.05), the data is significant. Error bar represents the standard deviation value (mean ± SD).

Figure 9. Graphical representation of the fluorescence intensity of HSA in the presence of trans-resveratrol and AFB1, excited at 280 nm. The concentration of HSA is 5 µM and the concentration of T-res = AFB1 = 14 µM. At the level of 0.05 (p < 0.05), the data is significant. Error bar represents the standard deviation value (mean ± SD).
Figure 10. Molecular docking of trans-resveratrol with HSA showing the best docked pose, site of binding and the amino acid residues involved in the complex stabilization.

Table 5. Docking parameters like binding energy and amino acids involved in T-res-HSA and T-res- HSA mutant interaction.

| Protein–ligand system | Amino acid residues involved in stabilizing HSA-T-res complex | Binding energy of the complex |
|-----------------------|-------------------------------------------------------------|-------------------------------|
| (a) T-res-HSA         | Ala-215, Ala-210, Phe-211, Ser-202, Val-482, Val-344, Trp-214, Leu-198, Leu-481, Ser-454, Asn-458, Leu-457, Arg-484 and Arg-485 | −7.76 kcal mol$^{-1}$ |
| (b) T-res-HSA (Trp-214 substituted with Gly) | Arg-484, Ser-454, Leu-198, Leu-481, Lys-199, Ser-202, Ala-215, Ala-210, Gly-214, Phe-211, Asn-458, Leu-457, Val-344, Val-482, Leu-347 | −7.06 kcal mol$^{-1}$ |
| (c) T-res-HSA (Trp-214 substituted with Val) | Lys-190, Asn-429, Ala-194, Asp-108, Tyr-148, Ala-194, His-146 | −6.29 kcal mol$^{-1}$ |

were used to explore and recognize the binding site of trans-resveratrol in HSA, corroborate the findings of spectroscopic studies, and get insight into the amino acid residues involved in the binding of trans-resveratrol with HSA. Figure 10 shows the best-docked pose and Sudlow's site 1 as the binding pocket for the binding of trans-resveratrol with HSA. The binding energy for the interaction as calculated from the docking is −7.76 kcal mol$^{-1}$. If we look at Fig. 10, Trp-214 is present in the binding pocket, and it has a role in the stabilization of HSA trans-resveratrol complex. The amino acid residues surrounding the trans-resveratrol molecule are shown in Table 5. Figure 11 depicts the 2D picture of the amino acid residues in the vicinity of the trans-resveratrol and the nature of bonds formed between them. The hydrogen bonding and van der Waals interactions are best illustrated by the 2D pot of the HSA and trans-resveratrol interaction. Previous studies on the interaction of AFB$_1$ with HSA also confirmed Sudlow’s site 1 as the binding pocket for AFB$_1$, using in silico approach$^{17}$, and the amino acids residues involved in the binding are in very close proximity to that involved in HSA and trans-resveratrol binding in the same Sudlow’s site 1. The van der Waals + hydrogen bonding + desolvation energy for HSA and trans-resveratrol complex was −9.07 kcal/mol, much higher than their electrostatic energy of −0.19 kcal/mol, thus proposing hydrogen bonding and van der Waals interaction as the major forces stabilizing the HSA and trans-resveratrol complex. This further corroborates our thermodynamic findings. Since the binding affinity of trans-resveratrol is much higher than the AFB$_1$, trans-resveratrol is able to displace bound AFB$_1$ to HSA for com-
The study investigates the role of Trp-214 during the stabilized complex formation between HSA and trans-resveratrol. We further explored the binding of mutated HSA with trans-resveratrol. In mutated HSA, Trp-214 was substituted with neutral amino acid glycine to elucidate the changes in the binding fashion of trans-resveratrol with mutant HSA as compared to non-mutated HSA. Glycine was substituted in place of Trp-214 because glycine, the simplest amino acid, is also neutral. The molecular docking study revealed that the complex of trans-resveratrol with mutant HSA was less stabilized than the non-mutated complex because the binding energy of the mutated HSA complex was less negative compared to the non-mutated complex. The binding energy of the complex and the amino acid residues involved in the stabilization of the mutated HSA-trans-resveratrol complex is shown in Table 5. Interestingly, the binding site of trans-resveratrol was located at the same subdomain IIA as it was with non-mutated HSA. However, the number of hydrogen bonds was reduced to 2 only, as shown in Fig. 12. Gly-214 was not involved in any kind of bonding with trans-resveratrol. A mutation study was also performed by substituting the hydrophobic amino acid valine in Trp-214. Surprisingly, the binding site of trans-resveratrol on HSA was different as compared to native non-mutated HSA, and HSA with Trp-214 substituted with Gly. The binding energy of the complex was also less negative. The study with mutated HSA was significant since it investigates the role of Trp-214 during the stabilized complex formation between HSA and trans-resveratrol. The substitution of Trp-214 with either Gly or Val did not result in the highly stabilized complex formation as compared to the non-mutated HSA. The binding of the mutated HSA complex with trans-resveratrol was less negative as compared to the native HSA. The amino acid residues that form the binding pocket for mutant HSA were evaluated to explore the protein flexibility. Figure 13b shows the plot of RMSF values versus amino acid residues of the HSA system after binding with trans-resveratrol. During the simulated time of 100 ns, the RMSD value of the HSA system after binding with trans-resveratrol is steadily bound to HSA at subdomain IIA. During the simulated time of 100 ns, the RMSD value of the HSA system reaches equilibrium suggesting HSA and trans-resveratrol interaction are shown in Table 5. Figure 12 shows the best-docked pose of binding of trans-resveratrol with mutated HSA. Table 5 represents the amino acid residues involved and binding energies in the formation of mutated HSA-trans-resveratrol complex.

**Molecular dynamic simulation studies.** Molecular dynamic (MD) simulation studies were carried out to get insights into the dynamic nature of trans-resveratrol binding to HSA over the simulated period up to 100 ns. For the investigation of the system stability, root mean square deviations (RMSD) values of HSA backbone (C–Cα–N) and HSA-T-res complex were calculated, and the graph was plotted from 0 to 100 ns. From Fig. 13a, it is clear that the RMSD value of HSA alone was stable from 0 to 40 ns, and it was increased from 40 to 60 ns and later became stable again from 60 to 100 ns. For HSA-T-res complex, the RMSD value steadily increases from 0 to 20 ns. From 40 to 100 ns, the system becomes stabilized, and no significant increase or decrease in the RMSD value is obtained, and the system reaches the equilibrium suggesting HSA and trans-resveratrol is steadily bound to HSA at subdomain IIA. During the simulated time of 100 ns, the RMSD value of HSA and trans-resveratrol complex was lesser than the RMSD value of HSA alone, this also suggests the stability of the HSA system after binding with trans-resveratrol molecule. The RMSD values of HSA obtained in the present study are in good agreement with the previously reported studies.55,57

Root mean square fluctuation (RMSF) values for HSA alone and HSA-trans-resveratrol complex were also evaluated to explore the protein flexibility. Figure 13b shows the plot of RMSF values versus amino acid residues in HSA. It is inferred that the amino acid residues in subdomain IIA are more rigid than other regions due to the complex formation between HSA and trans-resveratrol. Owing to the presence of random coils at the end of helix, flexibility is observed at the end residues, as shown in Fig. 13b.

Radius of gyration ($R_g$) value for HSA alone and HSA-trans-resveratrol complex was calculated to investigate the compactness of the protein and structural dynamics of the system over the simulated period of 100 ns. From Fig. 14, it is evident that the $R_g$ value of HSA alone initially fluctuates at around 2.80 nm and reaches a maximum fluctuation value of 2.85 nm at around 50 ns. After that, it decreases continuously up to 90 ns, and after that, it remains constant up to 100 ns. The $R_g$ values of HSA obtained are in accordance with the previously reported studies.55,57 On the other hand, the $R_g$ value of HSA-trans-resveratrol complex initially decreases from 0 to 20 ns. Then it increases up to 30 ns, becomes stable, and reaches equilibrium from 30 to 100 ns. Figure 15 shows the
dynamic nature of binding of trans-resveratrol to HSA at 0, 80, and 100 ns time at subdomain IIA. The findings of MD simulation studies corroborate the findings of our site markers displacement assays that also suggest subdomain IIA as the binding site of trans-resveratrol.
Conclusion

The study describes the interacting potential of polyphenol, trans-resveratrol with HSA explored by UV-absorption spectroscopy, fluorescence spectroscopy, and molecular docking. The study also investigated the dislodging potential of trans-resveratrol competing for the binding site on HSA by displacing AFB1 bound to HSA using fluorescence spectroscopic tools. The fluorescence quenching study was used to calculate the Stern–Volmer quenching constant ($K_{SV}$), binding constant ($K_b$), and various thermodynamic parameters ($\Delta G$, $\Delta H$, and $\Delta S$). The $K_{SV}$ and $K_b$ values obtained suggested high fluorescence quenching potential and strong binding constant, respectively, for trans-resveratrol and HSA. Circular dichroism studies confirmed an increase in the alpha-helix content in HSA in the presence of trans-resveratrol. MD simulation studies confirmed the dynamic nature of trans-resveratrol binding at the subdomain IIA of HSA over the simulated time of 0 to 100 ns. In silico amino
acid substitution studies using Trp-214 mutated HSA provided the role of Trp-214 residue in the binding of trans-resveratrol to HSA. The thermal stability or melting temperature (Tm) of the HSA also increased in the presence of trans-resveratrol. The binding constant of AFB1, for serum albumin was lower than trans-resveratrol, signifying the displacing potential of the polyphenol for competing AFB1 acquiring the same binding site i.e. subdomain IIA or Sudlow’s site 1. This is the first investigation on the use of trans-resveratrol to remove AFB1 from serum albumin and thereby to decrease the bound form of AFB1. This is a spectroscopy-based study; however, an in vivo study is required to elucidate the effect of trans-resveratrol and AFB1 competition in animal models. The findings of this study will aid in the understanding of the pharmacokinetics and pharmacodynamics of phytochemical efficacy to compete with bound toxin and quick biotransformation leading to mycotoxin clearance from the body. The study will help explore more phytochemicals and their use in the unloading of the toxic substances from serum albumin, thus reducing the risk of pathological conditions in the human and animal body.

Material and methods

Materials. Aflatoxin B1, trans-resveratrol, human serum albumin, warfarin, and ibuprofen were procured from Sigma Aldrich (USA). All the chemicals were high purity grade and used as such without any further purification.

Methods. Sample preparation. The stock solution of AFB1 of strength (1 mM) was constructed in HPLC grade methanol and later was diluted with 20 mM sodium phosphate buffer of pH 7.4 to maintain the working concentration. The stock solution of HSA (200 µM) was prepared in 20 mM sodium phosphate buffer, pH 7.4, and later it was diluted with the same buffer to make the working concentration. The stock solution of trans-resveratrol (1 mM) was prepared in HPLC grade methanol and distilled water in the ratio 1:1 (v/v) and later diluted with sodium phosphate buffer pH 7.4 to make the desired working concentrations.

UV-absorption studies. The UV-absorption studies were performed using Shimadzu UV-1900 spectrophotometer using quartz cuvettes of 1 cm. Sodium phosphate buffer of pH 7.4 and 20 mM strength was used for baseline correction and as a reference solution. The UV–absorption spectra of HSA, with the increasing concentration of trans-resveratrol, were recorded in the wavelength range of 220–360 nm. The concentration of HSA was fixed to (5 µM), and trans-resveratrol concentration was increased from (0–14 µM).

Fluorescence spectroscopic studies. Shimadzu RF-6000 spectrofluorometer equipped with xenon flash lamp was used for fluorescence spectroscopic studies. Initially, the binding potentials of AFB1 and trans-resveratrol for HSA were evaluated by studying the fluorescence emission spectrum of HSA in the presence of AFB1. HSA was excited at 280 nm, and emissions were recorded against increasing concentration of AFB1 (0–14 µM) in the wavelength range of 300–400 nm. The bandwidth of the excitation and emission wavelength was fixed to 5 nm each. The fluorescence intensity was corrected using Eq. (1) to check the inner filter effect

\[ F_{\text{corr}} = F_{\text{obs}} \times e^{\frac{A_{\text{exc}} - A_{\text{em}}}{2}} \]  

In Eq. (1), Fcorr is the corrected fluorescence intensity; Fobs is the observed fluorescence intensity; Aexc and Aem are the absorbances of ligand molecule at excitation and emission wavelength of HSA.

Binding parameters like Stern–Volmer quenching constant \((K_{SV})\), binding constant \((K_b)\), and bimolecular quenching constant \((K_q)\) were calculated using the following Eq. (2) and (3).

\[ \frac{F_0}{F} = 1 + K_{SV}[Q] = K_q \tau_0(Q) + 1 \]  
\[ K_q = \frac{K_{SV}}{\tau_0} \]  

where \(F_0\) is the fluorescence intensity of HSA alone; \(F\) is the fluorescence intensity of HSA in the presence of ligand; \(Q\) is the concentration of the ligand; \(K_q\) is the bimolecular quenching constant, and \(\tau_0\) is the average integral lifetime of the tryptophan residue in HSA (~10^{-9} s). The binding constant \((K_b)\) for the interaction of trans-resveratrol with HSA was calculated using Eq. (4)

\[ \log \left( \frac{F_0 - F}{F} \right) = \log K_b + n \log(Q) \]  
\[ \Delta G = -RT \ln K_b \]  
\[ \ln K_b = \frac{\Delta S}{R} - \frac{\Delta H}{RT} \]

The calculations of thermodynamic parameters were based on Eqs. (5) and (6). In Eq. (5), \(\Delta G\) is the Gibbs free energy, \(R\) is the universal gas constant (1.987 cal mol^{-1} K^{-1}), \(T\) denotes temperature in kelvin, \(\Delta S\) is the entropy change, and \(\Delta H\) represents enthalpy change of the HSA and trans-resveratrol system.
Investigation of the binding site of trans-resveratrol on HSA using site markers. Most of the small molecule binds to HSA at Sudlow’s site 1 (subdomain IIA) or Sudlow’s site 2 (subdomain IIIA). To confirm the binding location of trans-resveratrol on HSA, a competitive site markers displacement assay was followed using warfarin and ibuprofen. Initially, 5 micro molar concentration of HSA was saturated with an excess of trans-resveratrol (14 µM) followed by titrations with increasing concentration of warfarin and ibuprofen (0–80 µM). The HSA and trans-resveratrol complex was excited at 280 nm, and the emission was recorded in the wavelength range of 300–400 nm. The percentage of trans-resveratrol displaced by the site markers is calculated according to Eq. (7) as follows

\[
\text{Probe displacement (\%) } = \frac{F_2}{F_1} \times 100\%
\]

where \(F_1\) and \(F_2\) are the fluorescence intensities of HSA bound trans-resveratrol in the absence and presence of warfarin and ibuprofen site markers. The fluorescence spectrum was recorded using Shimadzu RF-6000 spectrophotometer with fixed emission and excitation bandwidth of 5 nm each.

Circular dichroism. Jasco J-1500 spectropolarimeter was employed to investigate the secondary structure alterations in HSA in the presence of trans-resveratrol. The spectropolarimeter was equipped with a temperature control Peltier system. Quartz cuvette of path length 0.1 cm was used for taking the reading of the sample and for correcting the baseline. Far-UV CD spectra of HSA (5 µM) in the presence of trans-resveratrol (10 and 20 µM) were taken in the wavelength range of 190–250 nm with a data pitch of 1 nm. The scanning speed was fixed to 200 nm/min, and the bandwidth of 1 nm was set. Each spectrum was an average value of 3 spectra. MRE_208 were taken in the wavelength range of 20–90 °C, to investigate the melting temperature (T_m) of HSA.

\[
MRE_{208} = \frac{\text{Observed CD (mdeg) at 208 nm}}{C_p n l \times 10}
\]

\[
\text{Percentage } \alpha \text{ helix } = \frac{MRE_{208} - 4000}{33000 - 4000} \times 100
\]

In Eq. (8), \(C_p\) represents the concentration of the HSA, \(n\) is the number of amino acid residues in HSA, and \(l\) is the path length of the quartz cuvette in cm. in Eq. (9), MRE_{208} is the mean residual ellipticity value at 208 nm.

Competitive displacement of HSA bound AFB1 by trans-resveratrol. In the first set of experiments, the dislodging potential of trans-resveratrol was investigated to displace AFB1 from HSA bound to AFB1, employing the fluorescence spectroscopic tool. Five micromolar concentration of HSA was added to 14 µM AFB1 and subsequently titrated with the increasing concentration of trans-resveratrol (0–20 µM). The HSA-AFB1 complex was excited at 280 nm, and emission was recorded between 200 and 400 nm.

In the second set of experiments, the dislodging ability of AFB1 was explored by probing AFB1 ability to displace trans-resveratrol from HSA bound trans-resveratrol. The percentage displacement of the AFB or trans-resveratrol was studied by plotting \(F_2/F_1 \times 100\) versus the concentration of trans-resveratrol or AFB. The experiment was performed using Shimadzu RF-6000 spectrophotometer having xenon flash lamp, with excitation and emission bandwidth of 5 nm each. Sodium phosphate buffer of strength 20 mM and pH 7.4 was used to construct the desired concentration of the sample.

Molecular docking and in silico amino acid substitution studies. Autodock 4.2 tools were employed for performing molecular docking to explore the binding sites of trans-resveratrol on HSA. The chemical structure of trans-resveratrol was obtained from Pubchem (CID: 445154). The crystal structure of HSA was obtained from the RCSB protein data bank (PDB ID: 1AO6). The energy optimization of ligand was performed using Avogadro software, whereas Swiss PDB viewer was used for energy minimization of the protein molecule. PDBQT files were created for both ligand and protein after adding polar hydrogen and removing water molecules. Grid files were created with the grid dimension of 68 × 68 × 44 in xyz axis with grid point spacing of 0.375 Å, and all other parameters were used as default set values. Total 100 GA runs were processed for docking analysis, and Lamarckian genetic algorithm 4.2 with a maximum of 2,500,000 energy evaluations were used for docking calculations. In silico amino acid substitution studies were performed by substituting the desired amino acid in place of Trp-214 followed by saving the mutated HSA molecule in .pdb format. Molecular docking of the mutated HSA with trans-resveratrol was performed using the above-mentioned protocol for native HSA. The final docked complex was visualized using Discovery studio visualizer and PyMOL.

Molecular dynamic simulation studies. The MD simulation studies were carried out to explore the binding mode of trans-resveratrol to HSA. MD simulations were performed by Desmond v4.1 applied in Schrodinger-Maestro v11. The side-chain bumps and steric clashes were fixed. The PDB structure of HSA (1AO6) and trans-resveratrol (CID: 445154) were optimized for GROMOS96 54a7 force field. These prepared structures were then optimized by GROMOS96 54a7 force field. Simple point charge water model was employed for adding the solvent molecules in the dodecahedron box with 1 Å distance from the protein surface, followed by the addition of four Na+ ions for the system neutralization. MD runs were set for 100 ns in three replicas with a time steps of
2 femtoseconds. The root mean square deviation (RMSD), root mean square fluctuation (RMSF) and radius of gyration (Rg) values were calculated post completion of the MD simulation runs.

**Statistical analysis.** Standard deviations (mean ± SD) were calculated wherever required in the experiment. One Way ANOVA analysis was conducted and the data with the p value < 0.05 were considered statistically significant. The statistical analysis was performed using OriginPro 2021 software.

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**Authors contributions**

M.A.Q. carried out the experimental work and collected the data. S.J. conceptualized the study and finalized the manuscript.

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**Competing interests**

The authors declare no competing interests.

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