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Binding and drug displacement study of colchicine and bovine serum albumin in presence of azithromycin using multispectroscopic techniques and molecular dynamic simulation



Tanveer A. Wani^{a,*}, Ahmed H. Bakheit^a, Abdulrahman A. Al-Majed^a, Nojood Altwaijry^b, Anwar Baquaysh^b, Ashwaq Aljuraissy^b, Seema Zargar^b

^a Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia

^b Department of Biochemistry, College of Science, King Saud University, PO Box 22452, Riyadh 11451, Saudi Arabia

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ABSTRACT

The binding and displacement interaction of colchicine and azithromycin to the model transport protein bovine serum albumin (BSA) was evaluated in this study. Azithromycin, a macrolide antibiotic, has antiviral properties and hence, has been used concomitantly with hydroxychloroquine against SARS-CoV-2. Colchicine, a natural plant product is used to treat and prevent acute gout flares. Some macrolide antibiotics are reported to have fatal drug-drug interactions with colchicine. The displacement interaction between colchicine and azithromycin on binding to BSA was evaluated using spectroscopic techniques, molecular docking and molecular dynamic simulation studies. The binding constant recorded for the binary system BSA-colchicine was 7.44×10^4 whereas, the binding constant for the ternary system BSA-colchicine in presence of azithromycin was 7.38×10^4 and were similar. Azithromycin didn't bind to BSA neither did it interfere in binding of colchicine. The results from molecular docking studies also led to a similar conclusion that azithromycin didn't interfere in the binding of colchicine to BSA. These findings are important since there is possibility of serious adverse event with co-administration of colchicine and azithromycin in patients with underlying gouty arthritis and these patients need to be continuously monitored for colchicine toxicity.

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1. Introduction

As of March 10, 2021 more than 2.62 million deaths have been reported due to COVID-19 infection worldwide. Currently there are 21 million active cases of which around 90,000 are critical [1]. Lack of any specific therapeutic regimen and vaccine led to experimental evaluation of other alternatives [2,3]. One of the drugs suggested in the treatment of COVID -19 is azithromycin (Fig. 1A) which is a macrolide antibiotic and has antiviral properties. Antiviral mechanism of action of azithromycin involves reducing viral replication, blocking viral entry to host cell and possible immunomodulation [4]. Several studies have demonstrated that combination of hydroxychloroquine and azithromycin are effective against SARS-CoV-2 [2,5]. A French study reported six-day treatment with hydroxychloroquine alone in combination with azithromycin resulted clearance of SARS-CoV-2 [3]. In addition, some conflicting results were also reported for hydroxychloroquine alone or in combination with azithromycin [2,3,6].

Colchicine (Fig. 1B) is a plant based natural product obtained from *Colchicum autumnale* and *Gloriosasuperba* L. Several molecular pathways are involved in the inflammation triggered by gouty arthritis. Colchicine is suggested to modulate the pro-inflammatory pathways related to gouty arthritis. Various modulations carried out by colchicine include prevention of microtubule assembly thus inhibiting inflammasome activation, chemotaxis of inflammatory cell, production of leukotrienes and cytokines, and phagocytosis. Patients with renal and hepatic impaired function need to be monitored for colchicine toxicity [7,8].

Clarithromycin a macrolide antibiotic is known to inhibit P-glycoprotein [9,10], which can lead to increased gastric absorption and reduced excretion of colchicine. This might lead to high serum and intracellular colchicine concentrations and possibility of tubulin inhibition and toxicity [11]. High colchicine concentration can affect the myocardial cells and is also reported to lead to multi-organ failure in overdose cases [12–14]. Azithromycin and clarithromycin belong to

* Corresponding author.

E-mail address: twani@ksu.edu.sa (T.A. Wani).

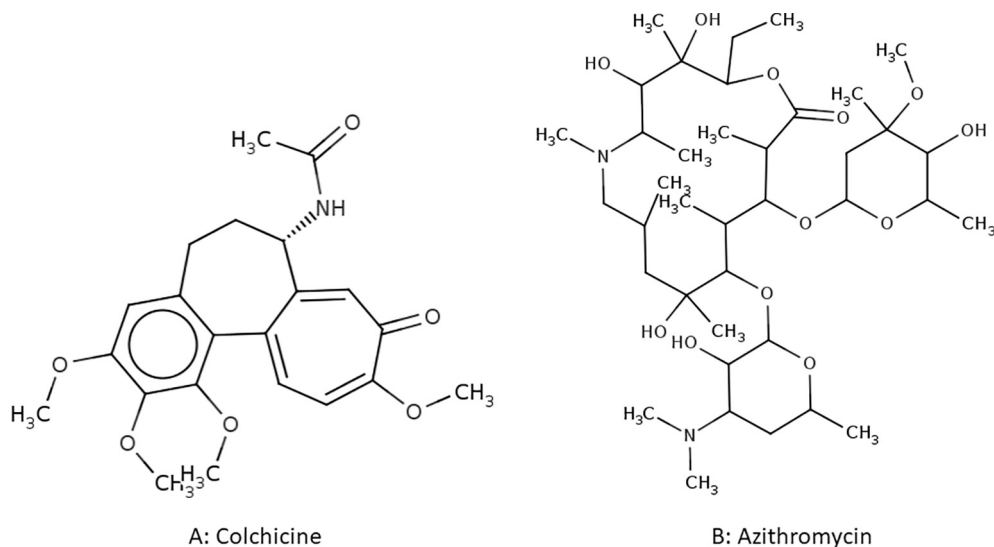


Fig. 1. Chemical structures of A: colchicine; and B: azithromycin.

the same class of antibiotics. An enhanced toxicity of colchicine with azithromycin or clarithromycin has been reported [9,15].

The therapeutic and/or toxic effects of most drugs are related to the free active drug plasma concentrations and is proportional to the tissue distribution of the drug. Plasma protein binding of drugs is a reversible dynamic process [2]. The determination of bound and unbound drug fractions is most important step during clinical pharmacokinetic and pharmacodynamics of the drug [4,5]. The plasma protein binding to drugs is necessary to maintain effective therapeutic concentration [16,17]. Displacement of drugs from their binding sites on plasma protein can lead to increased concentration of free drugs in systemic circulation leading to undesired or toxic effects [18–20]. In this study, *in-vitro* displacement interaction between azithromycin and colchicine was investigated based on the previous literature where concomitant use of macrolide antibiotics and colchicine resulted in some serious adverse events [9,10,15].

Bovine serum albumin (BSA) is the most commonly used model serum albumin protein to study such protein ligand interactions. This protein has a homologous structure to that of human serum albumin [21]. The interaction between the ligand and the BSA leads to some micro-environmental changes in the vicinity of fluorophore residues present in the protein [22–26]. The interaction mechanism, quenching and binding constants were determined using fluorescence spectroscopy and UV absorbance studies. In addition, circular dichroism (CD), synchronous fluorescence spectroscopy and 3D fluorescence studies provided information about the conformational changes in the protein on interaction with azithromycin and colchicine. Molecular docking studies were conducted to verify the experimental results. Molecular dynamic simulation provides information about the stability of the complex formed during the protein ligand interaction.

2. Experimental

2.1. Materials and methods

Colchicine and BSA (fatty acid free) were obtained from Sigma Aldrich (USA), and azithromycin standard was obtained from Weihua Pharma Co., Limited (China). The BSA was prepared in phosphate buffered saline (PBS) at physiological pH (pH 7.4). Stock solutions for azithromycin and colchicine were prepared in ethanol and water, respectively. The working standards were prepared in PBS. Type IV

water was used in preparation of the reagents all of which were of analytical grade.

2.2. Fluorescence measurements

Jasco FP-8200 spectrofluorometer (JASCO, Japan) was used to record the fluorescence spectra at 298, 303 and 307 K [27]. The spectra were recorded at $\lambda_{ex} = 280$ nm and $\lambda_{em} = 300$ –500 nm. The BSA concentration of 1.5 μ M was constant for all the measurements both in the binary and the ternary system. In the binary system, the concentrations for azithromycin was in range of 0–200 μ M and that for colchicine varied from 0 to 20 μ M. For the ternary systems: i) BSA-colchicine in presence of azithromycin fixed concentration (BSA 1.5 μ M –azithromycin 100 μ M) + colchicine (0 to 20 μ M) and for ii) BSA-azithromycin in presence of fixed concentration colchicine (BSA 1.5 μ M –colchicine 15 μ M) + azithromycin (0 to 200 μ M) system. The inner filter effects were decreased by correction of fluorescence intensities for absorption and reabsorption of excited and emitted light, respectively. The inner filter effects were decreased using the following equation [28,29].

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

In the equation, F_{cor} and F_{obs} represent corrected and observed fluorescence intensities, respectively. The absorbance value at excitation and emission wavelength are given as A_{ex} and A_{em} , respectively.

2.3. UV absorption measurements

The UV-absorbance measurements were carried out with Shimadzu-1800 UV-spectrophotometer (Japan). The BSA concentration of 1.5 μ M was fixed for the measurements whereas the concentrations of colchicine varied from (0–20 μ M) in presence of a constant azithromycin concentration of 200 μ M or in its absence. All the spectra were recorded at room temperature. The spectra were also recorded for azithromycin in the presence of different concentrations of BSA (0.5–5.0 μ M). In addition, UV spectra was also recorded for colchicine alone.

2.4. Synchronous fluorescence

In the synchronous fluorescence spectra, the excitation and emission monochromators scan at a fixed wavelength interval. The spectra were

recorded using Jasco FP-8200 spectrofluorometer (JASCO, Japan). Wavelength intervals ($\Delta\lambda$) of 15 nm and 60 nm that are characteristic for tyrosine and tryptophan residues, respectively, were used to record spectra at 298 K. The synchronous fluorescence spectra recorded for both the binary and the ternary system. For the binary system and ternary system BSA concentration was fixed (1.5 μM). The variable concentration of colchicine (0–20 μM) was used for binary system and a fixed concentration of colchicine (15 μM) was used for ternary system. A variable azithromycin concentration was used (0–200 μM) in binary system and a fixed (100 μM) in the ternary system.

2.5. Docking study

The structure for the protein was obtained from the protein data bank (PDB ID: 6QS9) (<http://www.pdb.org>). The docking analysis was performed on molecular operating environment (MOE) software. The structures for ligands azithromycin and colchicine were drawn within the software. The docking parameters were as follows: energy minimization set to default: field force MMFF94X, $\epsilon_{\text{ps}} = 1$ and the cutoff (8–10). The scoring function was set to function 1 (London dG) and function 2 (GBVI/WSA dG).

2.6. Molecular dynamic simulation (MDS)

The MDS study was performed with NAMD 2.13 Suite (<http://www.ks.uiuc.edu/Research/namd>) [30] and MOE [31]. A TIP3P water box was used to immerse the ligand structures by solvation algorithm in order that all proteins atoms are not less than 10 Å. The charge was neutralized by addition of Na^+ or Cl^- counter ions for which the concentration for these ions didn't exceeded 0.15 M. The system temperature was raised to 310 K for 0.1 ns. The temperature increment was of 50 K at each step. A run of 10 ns was followed after an equilibration of 0.1 ns at 310 K. The system was later cooled and decreased the temperature from 310 K to 0 K in 0.1 ns. Particle mesh Ewald method was used to evaluate the long range electrostatics interactions and the time step was set to 2 fs. The root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (R_g) were calculated for both the BSA and BSA-Ligand complexes with MDS. The stability of the complex formed was established by MDS. The VMD1.9.3 analysis tool was used for trajectory calculation in the analysis.

3. Results and discussion

3.1. Fluorescence quenching

Protein ligand interactions studied with fluorescence spectroscopy is effective, convenient and a highly sensitive technique providing means to work with very small concentrations. A reduced fluorescence intensity or quenching of fluorescence is because of the molecular interaction that might lead to excited state reactions, complex formation, dynamic quenching, energy transfer and molecular rearrangement [29,32]. Colchicine was observed to reduce the fluorescence intensity of BSA, whereas, azithromycin did not influence BSA quenching. The fluorescence spectra are given in Fig. 2. As the concentration of colchicine increased a reduction in the fluorescence intensity was observed in both binary and ternary systems. Both ligands azithromycin and colchicine have no intrinsic fluorescence at these wavelengths.

A blue shift of 4 nm in the emission wavelength of colchicine was observed after excitation at 280 nm. The blue shift suggests reduced polarity around the aromatic amino acids of protein and an increase in hydrophobicity [29].

3.2. Stern–Volmer analysis

The fluorescence quenching is determined with Stern–Volmer equation:

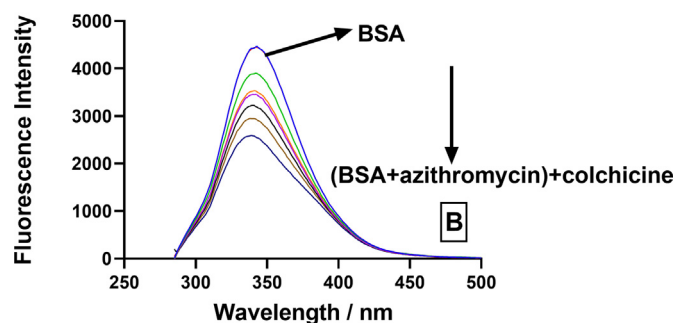
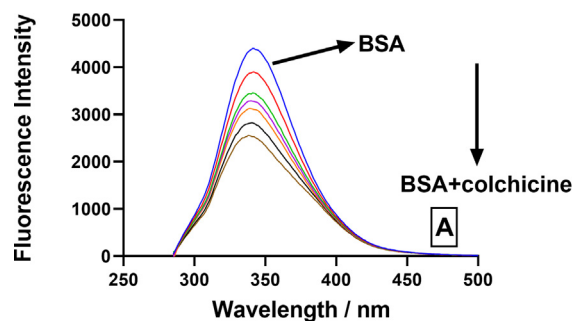


Fig. 2. Fluorescence spectra of BSA (1.5 μM) with colchicine (0–30 μM); BSA (1.5 μM) + azithromycin (100 μM) with colchicine (0–20 μM).

$$\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + k_q\tau_0[Q]$$

$$k_q = K_{sv}/\tau_0$$

where, F_0 and F represent the fluorescence intensity of BSA prior to and after quencher addition (Fig. 3A). K_{sv} is the Stern–Volmer quenching constant, k_q is the biomolecular quenching constant and $[Q]$ represent the quencher concentration. An average life time of biomolecule without quencher τ_0 is 10^{-8} s. The approachability of the quencher to the fluorophore is determined from the bimolecular quenching constant k_q . In case of dynamic quenching the k_q does not attain values beyond $2 \times 10^{10} \text{ M}^{-1}\text{S}^{-1}$. The k_q values higher than the diffusion-controlled limit of $2 \times 10^{10} \text{ M}^{-1}\text{S}^{-1}$ suggest a binding interaction between the ligand and the protein [29]. The k_q values obtained for the interaction of BSA with colchicine (Table 1) were higher than those of diffusion controlled limit suggesting complex formation between BSA and colchicine. The Stern Volmer constant values obtained with the binary system BSA–colchicine were similar to those obtained with the ternary system (BSA–azithromycin)–colchicine (Table 2). These results lead to the conclusion that the presence of azithromycin didn't interfere in the BSA and colchicine interaction.

The K_{sv} values are helpful in understanding the quenching mechanism involved in the protein ligand interaction. For BSA–colchicine the quenching mechanism was found to be static quenching and also inferred that a complex formation occurred between BSA and colchicine. Static quenching was also predicted on the basis that at higher temperatures the K_{sv} values decreased suggesting unstable complex at high temperatures.

3.3. Binding constants

Binding parameters of protein ligand interaction is important in providing valuable information regarding the pharmacokinetics and

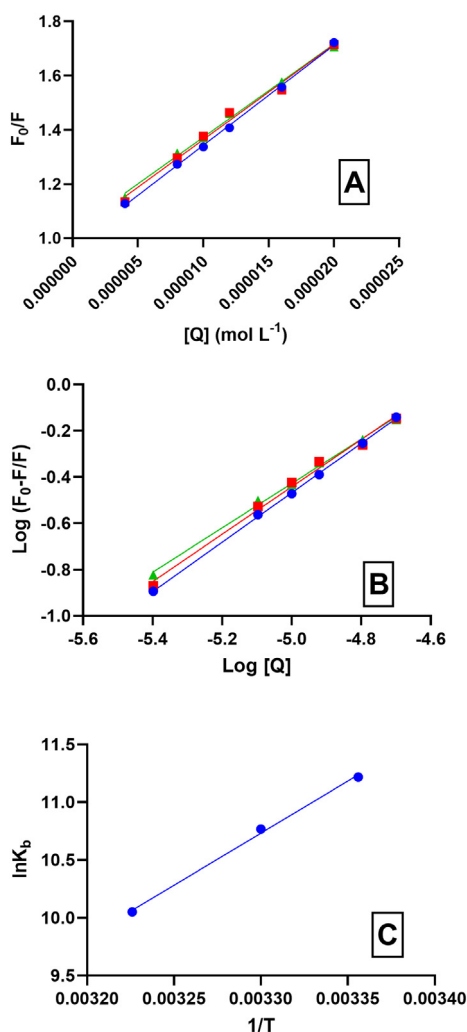


Fig. 3. Calculated fluorescence parameters for BSA-colchicine at three studied temperatures of 298, 303 and 310 K; A: Quenching constants from Stern-Volmer plot; B: Binding constants from double reciprocal plot $[(F_0 - F)/F]$ versus $\log [Q]$; C: Thermodynamic parameters from Van't Hoff plot.

Table 1

Stern-Volmer constant K_{sv} and the values for the BSA-colchicine system r quenching constant k_q .

T(K)	R	$K_{sv} \pm SD^a \times 10^4 (M^{-1})$	$k_q \times 10^{12} (M^{-1}s^{-1})$
298	0.998	3.69 ± 0.04	3.69
303	0.986	3.51 ± 0.03	3.51
307	0.995	3.43 ± 0.05	3.43

^a SD is the standard deviation.

Table 2

Quenching constants of the binary and ternary systems of BSA-colchicine system.

System	$K_{sv} \pm SD^a (M^{-1})$	$K_b \pm SD^a$
BSA-Colchicine	$3.69 \times 10^4 \pm 0.04$	$7.44 \times 10^4 \pm 0.14$
(BSA + azithromycin)-colchicine	$3.60 \times 10^4 \pm 0.05$	$7.38 \times 10^4 \pm 0.11$

^a SD is the standard deviation.

pharmacodynamics of ligands [33]. The binding constants (K_b) and the binding stoichiometry (n) are determined with the double logarithm equation:

$$\log \frac{(F_0 - F)}{F} = \log K_b + n \log [Q]$$

Binding constant K_b is determined from plot of $\log [(F_0 - F)/F]$ versus $\log [Q]$ (Fig. 3B). The binding constant values for the binary system are provided in the (Table 3). Earlier studies for binding interaction of BSA with colchicine predicted similar binding constant values [34,35]. No change in the binding constant was observed between the binary and the ternary system Table 2. Thus, azithromycin does not interfere with the binding of colchicine to BSA. Binding between BSA- colchicine is suggested on the basis of the binding constant values that were in the range of $10^4 M^{-1}$ [26,36]. Colchicine has low systemic bioavailability as it undergoes extensive hepatic first-pass metabolism.

Since colchicine has high tissue distribution and binds to intracellular moieties. The plasma protein binding reported for colchicine is 10–50% [37]. The plasma protein binding is an important parameter in drug pharmacokinetics as the strong or weak binding of drug to plasma protein will ultimately affect the excretion and metabolism of drugs [38–40]. These effects can thus lead to either toxicity of the drug or may give a sub therapeutic response [41,42]. At higher temperatures the binding stoichiometry decreased as high temperatures increase the disorder of molecules and they vibrate at higher speed. Such a disorder results in higher diffusion coefficient leading to the instability of the BSA-colchicine complex. Similar dynamic motions are displayed by proteins of similar topologies even though they have different distribution of molecular population due to different sequences [43].

3.4. Thermodynamic interaction

The enthalpy change ΔH° , the entropy change ΔS° and the free energy ΔG° thermodynamics parameters were calculated for the BSA – colchicine system. Van't Hoff plot (Fig. 3C) and equation were used for the calculation of these parameters:

$$\ln K_b = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}$$

In the above eq. R is the universal gas constant and T is the temperature in kelvins.

The following equation was used to calculate the ΔG° value:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

The ΔG° values (Table 3) suggest spontaneous interaction between the BSA and colchicine based on its negative values. The negative values for the enthalpy and the entropy suggest the involvement of Van der Waals force and hydrogen bonds in the BSA-colchicine interaction [44]. The binding was enthalpy driven for the BSA-colchicine interaction.

3.5. Binding site

Markers used for the binding site identification were phenylbutazone for site I and ibuprofen for site II [45]. Drug displacement studies were conducted for colchicine with BSA-site mark ligand. Equimolar concentration of BSA and phenylbutazone/or ibuprofen were interacted with variable concentrations of colchicine (0–20 μM) at 298 K. The binding constants obtained for the system in presence of phenylbutazone were lower (1.1×10^3) compared to either in presence of ibuprofen (6.6×10^4) of no site marker (7.4×10^4) (Table 3). Thus, the displacement of colchicine from its binding site by phenylbutazone suggest that colchicine interacts with BSA at site I.

Table 3
Binding parameters and thermodynamic parameters for BSA–colchicine system.

T(K)	$K_b \pm SD^a$	n	$\Delta G^\circ \pm SD$ (kJ mol ⁻¹)	$\Delta H^\circ \pm SD$ (kJ mol ⁻¹)	$\Delta S^\circ \pm SD$ (J mol ⁻¹ ·K ⁻¹)
298	$(7.44 \pm 0.14) \times 10^4$	1.068	-27.83	-75.02	-158.34
303	$(4.74 \pm 0.11) \times 10^4$	1.023	-27.04		
307	$(2.31 \pm 0.07) \times 10^4$	0.959	-26.41		

^a SD is the standard deviation.

3.6. Binary and ternary system interaction

At excitation wavelength of 280 nm both the tryptophan and the tyrosine residues are responsible for the BSA fluorescence. Previous studies suggested Site I (subdomain IIA) of BSA to bind colchicine [35]. The displacement interaction between azithromycin and colchicine at the BSA binding site was studied with the changes in the quenching behavior and binding constants in the binary and the ternary system. The binary and the ternary system quenching curves also provided the change in affinity of drugs towards BSA in presence of each other. Colchicine reduced the fluorescence intensity of BSA in the binary system. There was no difference in the quenching behavior in the binary (BSA + colchicine) and the ternary system where interaction took place in presence of azithromycin for (BSA + colchicine). In the BSA–azithromycin system, azithromycin didn't shift the BSA fluorescence. The non-binding of azithromycin to BSA can be attributed to several factors. One of the factors is that azithromycin is a basic drug [46]. Basic drugs primarily bind to the plasma protein alpha-1-acid glycoprotein (AGP or orosomucoid). The AGP binding properties are different from the

albumin as it has only one available binding site and is present in the lower amount in comparison to serum albumin. Potential drug–drug interactions are suspected for AGP binding drugs since, binding to AGP is saturable and bound drugs can be displaced from the binding site and can lead to toxicity. The AGP concentration in plasma increases in cancer, burns, surgery and inflammatory diseases. Azithromycin binds to AGP, thus, such conditions may change azithromycin free drug concentration, affecting the pharmacokinetics and pharmacodynamics of azithromycin [47,48]. The AGP consists of 183 amino acid residues and has a molecular weight of approximately 44 kDa. AGP contains \approx 11% sialic acid and is considered responsible for the negative charge on its surface [49].

Azithromycin has low serum and high tissue concentration. A low plasma concentration of 0.4–0.45 mg L⁻¹ was detected after an oral dose of 500 mg azithromycin within 2–3 h post-dose [48,50]. The intestinal absorption of azithromycin was increased in presence of nelfinavir which is a substrate of P-glycoprotein and inhibits P glycoprotein. Along with this study several other studies have proved that azithromycin is a substrate for P glycoprotein [51].

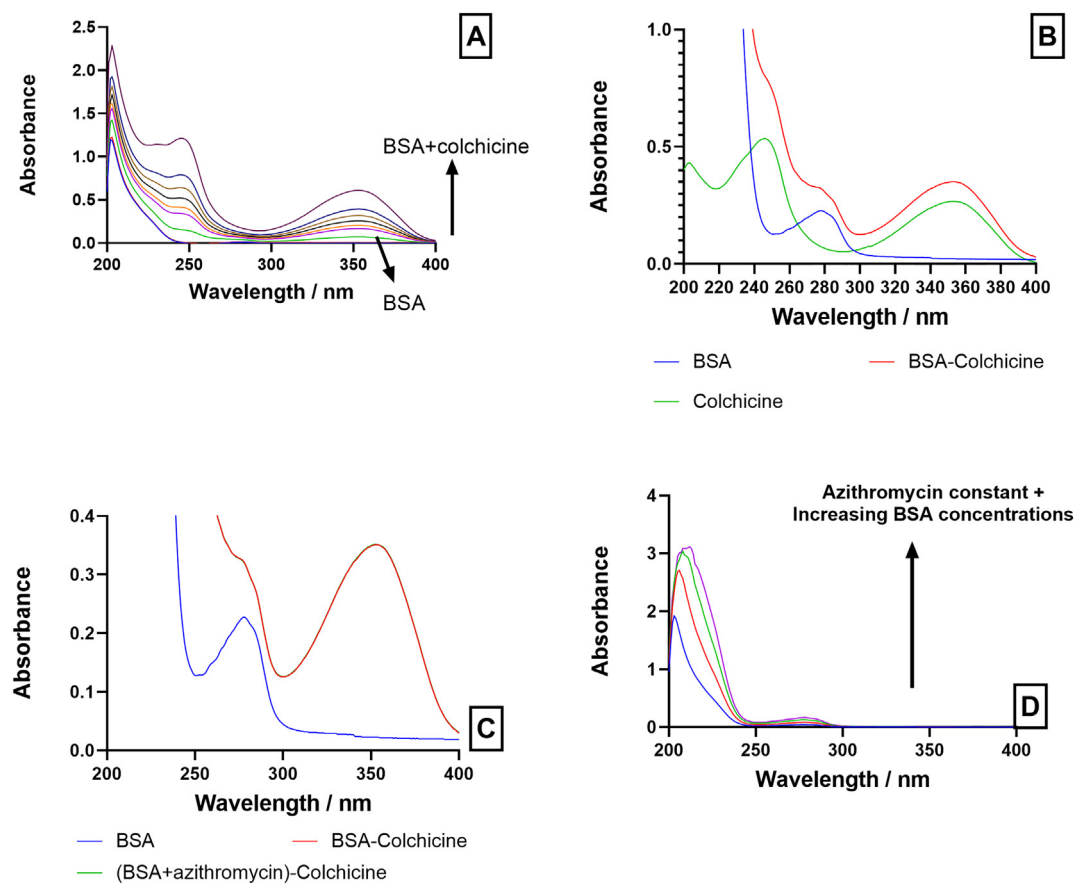


Fig. 4. A: Ultraviolet visible spectra for BSA (1.5 μM) in presence and absence of colchicine (0–20 μM); B: Comparison UV Spectra of BSA(1.5 μM), colchicine (12 μM) and BSA (1.5 μM)–colchicine (12 μM); C: UV Spectra for the binary and ternary system where BSA is (1.5 μM), BSA(1.5 μM)–Colchicine (16 μM) and (BSA 1.5 μM–azithromycin 100 μM) + colchicine (16 μM) D: UV spectra for azithromycin (200 μM) in presence of increasing BSA (1.5 μM–5 μM) concentrations.

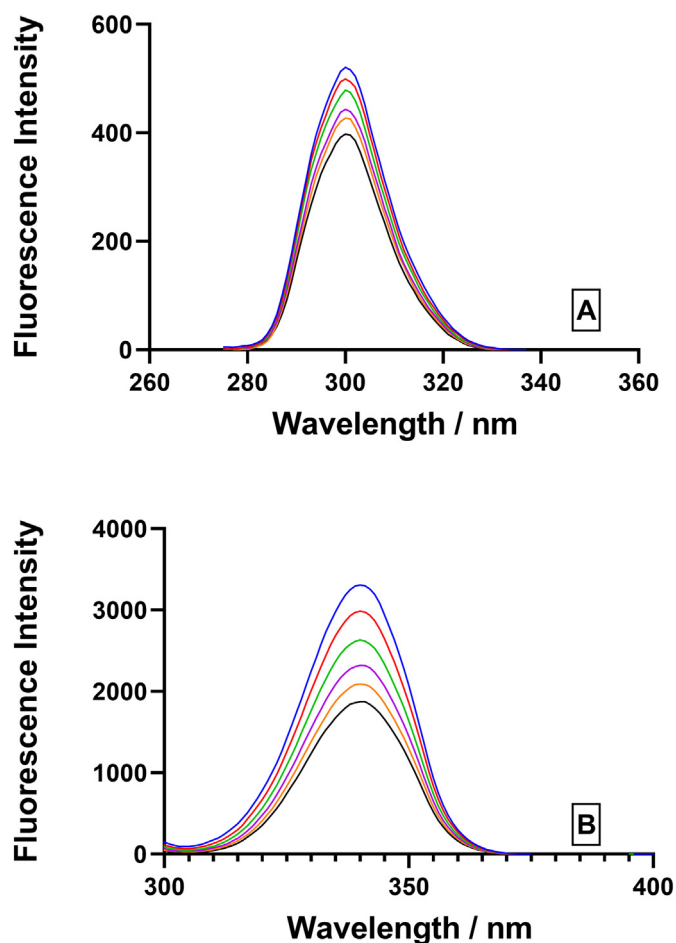


Fig. 5. Synchronous fluorescence spectra of BSA-colchicine system at A: $\Delta\lambda = 15$ nm; B: $\Delta\lambda = 60$ nm.

3.7. UV absorption measurements

The UV absorption spectra for BSA, azithromycin and colchicine (binary and ternary system) are provided in Fig. 4. These spectra provided additional information to verify the protein-ligand interaction. The absorption bands of near 280 nm are because of amino acids Trp, Tyr and Phe. Whereas, the absorption bands at 205–210 nm are suggested due to BSA frame work conformation. Higher absorption of BSA at

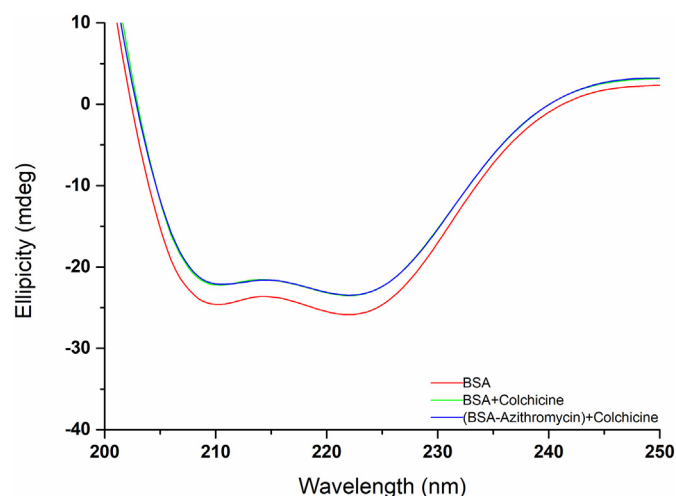


Fig. 7. Circular dichroism spectra for BSA in presence and absence of colchicine and azithromycin.

280 nm suggest an interaction occurs between BSA and colchicine. The absorption increased further on increasing the concentration of colchicine with constant BSA concentration (Fig. 4A). The absorption spectrum for colchicine was also recorded without BSA and two absorbance maxima were observed for colchicine at 245 nm and 353 nm. The spectra comparing colchicine, BSA-colchicine and BSA alone are given in Fig. 4B. These results corroborate to the suggested static quenching mechanism based of Stern-Volmer analysis results between BSA and colchicine [52].

Azithromycin was found spectrophotometrically inactive in this study, as reported by Haleem et al. [53], because of its structure, it doesn't show any absorption in the UV/visible region. The UV-spectra for BSA in presence and absence of azithromycin doesn't show any changes in the BSA spectrum. These findings are in contrast to that reported by Wu et al. [54], where they suggested the interaction between BSA and azithromycin. The study reported that on interaction of azithromycin with BSA the maximum absorbance for azithromycin at 210 nm disappears and a new maxima of complex is formed at 230 nm. The study also reported that on increasing the BSA concentration a red shift occurred in the absorption spectrum [54].

The absorbance spectra for (BSA-azithromycin) + colchicine and BSA-colchicine were found to be similar (Fig. 4C) and are superimposed on each other. We repeatedly tried to verify the interaction between BSA and azithromycin by keeping the concentration of azithromycin

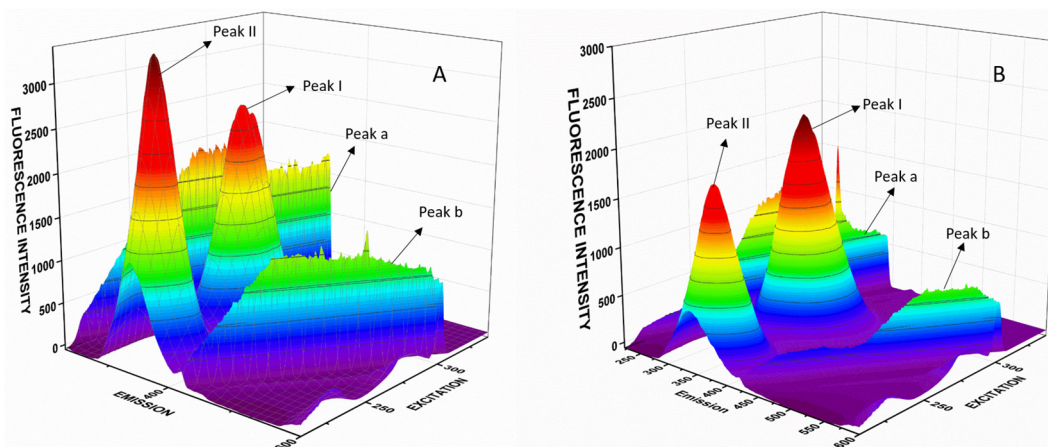


Fig. 6. Three dimensional fluorescence spectra for A: BSA; B: BSA-colchicine system.

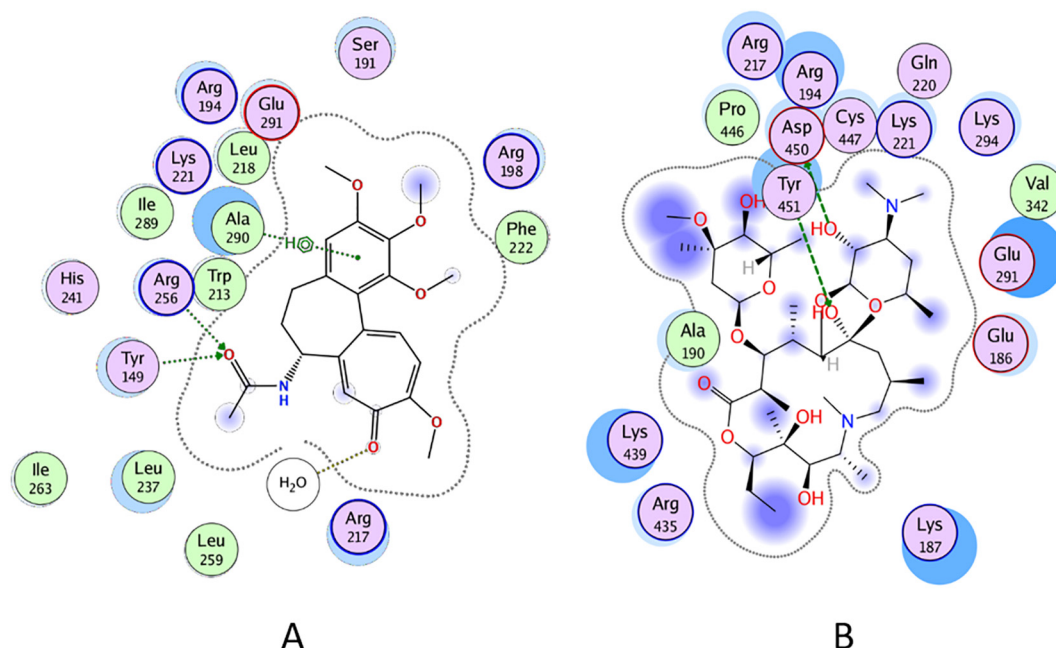


Fig. 8. Two dimensional molecular docking conformation for BSA; A: colchicine; B: azithromycin.

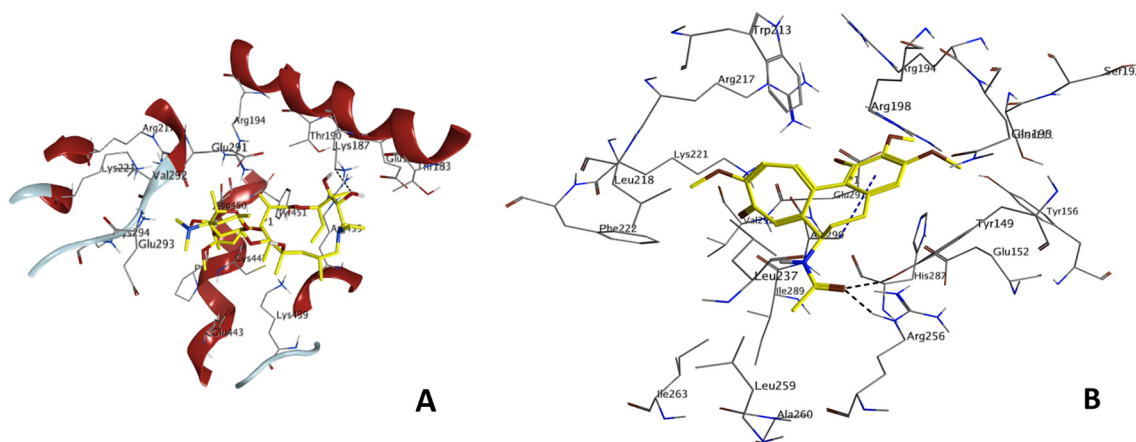


Fig. 9. Three dimensional blind docking conformation for A: BSA-azithromycin B: BSA-colchicine.

constant as 200 μM and increasing the concentration of BSA from 1.0–4.0 μM (Fig. 4D). The absorbance maxima occurred at 280 nm and increased as the concentration of BSA increased, however no other peak between 230 and 240 nm appeared as reported by Wu et al. [54].

3.8. Analysis of BSA conformation upon ligand binding

The synchronous fluorescence technique provides information about the micro-environment of the chromophores present in the protein molecule. The synchronous fluorescence spectroscopy has an advantage of being sensitive, reduced spectral band width and free of several perturbing effects [18,55]. Micro-environmental changes in the Tyr and Trp amino acid residues are suggested if a shift occurs in the emission wavelength. The shift in wavelength suggests a polarity change round the chromophore molecule [28]. The Fig. 5A and B represent synchronous fluorescence spectra at $\Delta\lambda = 15$ nm and 60 nm, respectively. The fluorescence intensity was reduced in presence of

colchicine (0 to 20 μM). Whereas azithromycin had no effect on the fluorescence of BSA.

The binary and ternary system showed a similar decrease in the fluorescence intensity at both $\Delta\lambda = 15$ and 60 nm. No shift in the emission wavelength was observed either at $\Delta\lambda = 15$ or 60 nm. Thus, no changes in the polarity around the fluorescent amino acid residues Trp and Tyr both in binary and the ternary system is suggested.

Three dimensional (3D) fluorescence spectroscopy was conducted to access microenvironmental changes around Trp and Tyr amino acid residues in the BSA [28]. In the 3D spectra, four peaks were identified for BSA-colchicine (Fig. 6 A-B). Peak I represent Trp and Tyr residues; peak II represents the aromatic moieties higher excited state [56]. In addition, Peak a is the Rayleigh scattering peak; peak b is the IInd order scattering peak. Peak I had a $\lambda_{\text{ex}} = 278$ nm and $\lambda_{\text{em}} = 340$ nm and Peak II had a $\lambda_{\text{ex}} = 226$ nm and $\lambda_{\text{em}} = 340$ nm. The micro-environmental changes in the fluorophore residues of Trp and Tyr on interaction with colchicine are suggested based on the changes in the fluorescence intensities of BSA.

3.9. Circular dichroism (CD) measurements

Conformational changes were also studied with CD since proteins have a certain characteristic far-UV CD spectra (178–250 nm) [18]. The CD spectra were recorded for the BSA in both the binary and the ternary system. Two negative minima were recorded for the BSA at 208 nm and 222 nm. These wavelengths provided information about the protein α -helix structure. There was a change in the CD spectra of BSA on addition of colchicine and the result is in agreement with an earlier study [35]. The changes in the CD spectra on addition of colchicine suggests an alteration in the secondary structure of BSA (Fig. 7). The BSA-colchicine spectra were similar to the ternary system CD spectra BSA-colchicine in presence of azithromycin. Thus, presence of azithromycin didn't influence the interaction between colchicine and BSA. Thus, microenvironmental changes in BSA are suggested on interaction with colchicine.

3.10. Docking study

The ligands colchicine and azithromycin blindly docked to BSA (Fig. 8). The blind docking confirmed that azithromycin did not bind to any of the available binding sites present on BSA and instead was found to be present on the outer surface of BSA (Fig. 9A). However, in case of colchicine the majority of the clusters with high affinity were found to be at site I (Fig. 9B). [40,57]

The amino acid residues surrounding colchicine Fig. 8A in the binding pocket at site I [35] were Arg217, Arg256, Arg194, Arg198, Leu259, Leu237, Leu218, Ile 263, Ile289, Tyr149, His241, Trp213, Ala290, Lys221, Glu291, Ser191 and Phe222. The fluorescent residues Tyr 213 were found in close proximity of colchicine. Two hydrogen bonds were found between an oxygen of the carbonyl group and the amino acid residues. One of the bonds was with Tyr149 (2.12 Å) and the second one was with Arg256 (1.89 Å). In addition, a pi-hydrogen bond was found between 6-ring-Ala290 (4.72 Å). The free energy for the protein ligand system was calculated on the basis of thermodynamic principles and the free energy at room temperature was found to be equal to $-27.83 \text{ kJ mol}^{-1}$. The free energy calculated by molecular docking for the binding interaction between colchicine and BSA was found to be $-27.46 \text{ kJ mol}^{-1}$. The difference between the thermodynamic experiment based binding energy and docking study binding energy is due to the fact that the experimental conditions for both are dissimilar. The experimental binding is conducted in a solution whereas the docking is performed in cyberspace. The binding energy obtained from the experimental and molecular docking analysis are similar and thus validate each other.

Hydrogen bonds between colchicine and the residues Tyr 149 and Arg 256, indicate that complex formed is stable. Further, the molecular docking results also suggest that colchicine and BSA interaction is dominated by hydrogen-bonding forces and is in accordance with thermodynamic experiments [28]. Circular dichroism studies suggest a decrease in the amplitude of the peaks at 208 and 222 nm for BSA on interaction with colchicine (Fig. 7). Also, no alteration in the peak amplitude was observed for BSA on interaction with colchicine in presence of azithromycin. The peaks with both BSA-colchicine in presence and absence of azithromycin were similar suggesting no effect of azithromycin on the BSA-colchicine interaction. Since, the biological activity of protein is related to its secondary structure, studies have shown that the α -helix content decreases whereas an increase in the β -sheet occurs when a ligand interacts with protein [58,59]. Thus, the changes in the secondary structure of BSA indicate that colchicine binds to amino acid residues present on the main polypeptide chain of protein destroying the hydrogen bonding networks as suggested in the molecular docking to obtain a more relaxed conformation. [60,61].

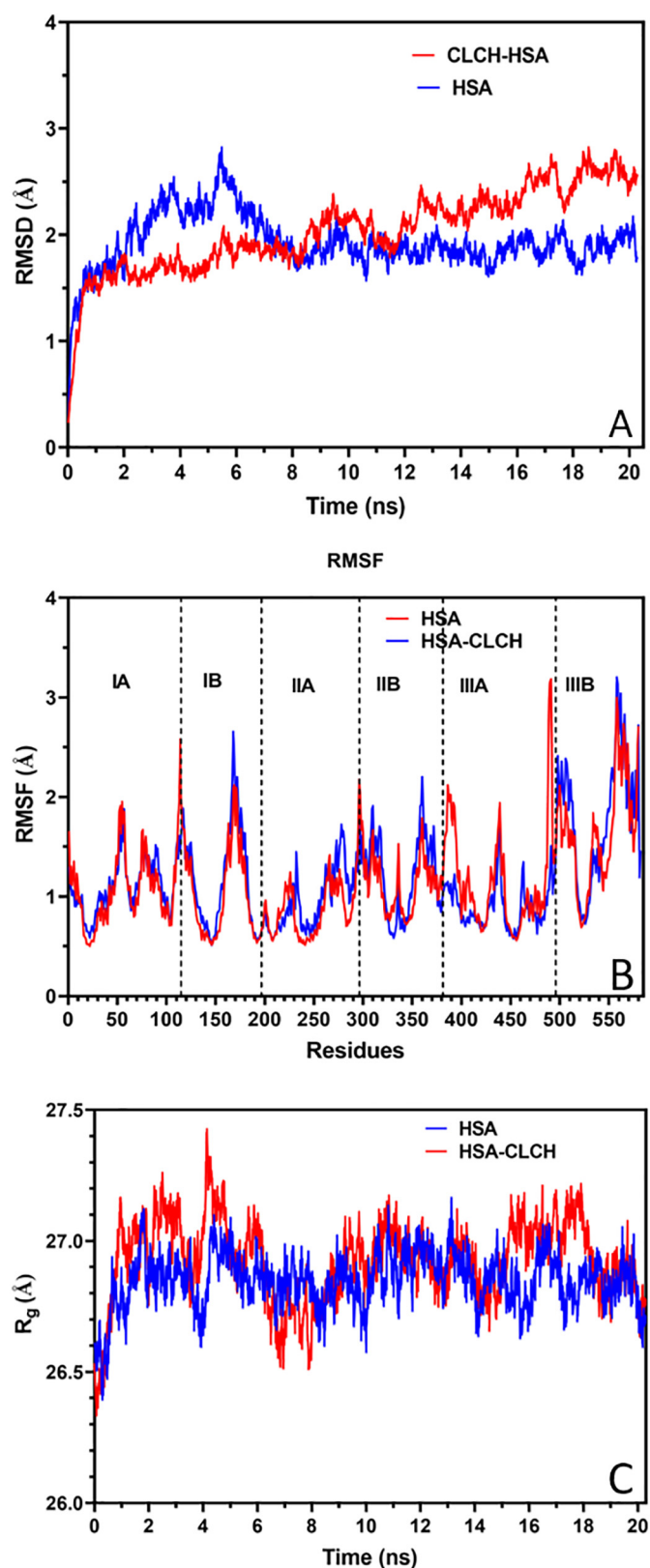


Fig. 10. Molecular dynamic simulation plots for BSA-colchicine system, A: Plot for root mean square deviation; B: root mean square fluctuation; C: Radius of gyration plot.

3.11. Molecular dynamic simulation (MDS)

Molecular dynamic simulation was used to further understand the docking results [62]. The suggested binding site for both drugs (site I)

was investigated for its interaction with colchicine using MDS. The MDS run was conducted for 10 ns. The RMSD plot for MDS is given in Fig. 10A. The structural changes in the BSA on its interaction with the ligands can be interpreted for the changes in the RMSD values of the protein alone and the protein-ligand complex. The RMSD plot for BSA and BSA-colchicine did not suggest any significant changes in the RMSD. Thus, it is inferred from the RMSD values that the complex formed is stable.

The RMSF values indicate the residual flexibility. A 10 ns RMSF plot for BSA and BSA-colchicine revealed a stable complex formation. The fluctuations in the BSA and the BSA-colchicine complex were similar in nature. Therefore, the RMSF plot Fig. 10B suggests a stable complex formation between BSA and colchicine.

The protein compactness folding and stability were indicated from the radius of gyration (R_g) plot. The R_g plot provides information about the atomic distribution around the protein axis, which in turn provides information about the stability and integrity of protein backbone. The R_g plot given in Fig. 10C suggests the BSA and BSA-colchicine were similar indicating stability of the formed complex between BSA and colchicine.

The overall results from the MDS indicate that the BSA-colchicine did not show too much fluctuation and the complex remained compact and stable inferring a stable complex formation between BSA and colchicine.

4. Conclusions

Interactions between colchicine, azithromycin and BSA were evaluated in this study. Azithromycin did not bind to BSA and had no influence on BSA and colchicine interaction. These results were confirmed by various spectroscopic techniques. The presence of azithromycin didn't influence the BSA conformation either also inferred from the CD results. Molecular docking confirmed the experimental results. Even though BSA is the most abundant protein in the plasma, azithromycin did not bind to BSA. Therefore, no competition occurred between colchicine and azithromycin for binding to BSA. However, interaction studies between azithromycin and colchicine with alpha-1-acid glycoprotein are recommended since azithromycin binds primarily to alpha-1-acid glycoprotein which has only one site for binding and competition for the binding site on alpha-1-acid glycoprotein may lead to altered the pharmacokinetics of colchicine.

CRedit authorship contribution statement

Tanveer A. Wani: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Resources, Writing - review & editing, Project administration. **Ahmed H. Bakheit:** Software, Validation, Formal analysis, Investigation. **Abdulrahman A. Al-Majed:** Methodology, Writing - review & editing. **Nojood Altwaijry:** Software, Writing - review & editing. **Anwar Baquaysh:** Investigation. **Ashwaq Aljuraissy:** Investigation. **Seema Zargar:** Conceptualization, Formal analysis, Resources, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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