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Binding Interaction Characterization between Antimalarial Artemisinin (Art) and Bovine Serum Albumin (BSA) Using Fluorescence Emission Spectroscopy

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ABSTRACT
Artemisinin (ART) has been widely used as antimalarial drug for the treatment of multiple drug-resistant strains of plasmodium falciparum. In this resarch, the binding interaction of ART with bovine serum albumin (BSA) under the limited physiological conditions (pH 7.4) was conductedusing fluorescence emission spectroscopy. The results revealed that there was a static quenching of BSA induced by ART due to the formation of ART–BSA complex. The binding constants and number of binding sites of ART with BSA under simulated physiological condition (pH=7.4) were -0.61 and 2.5 at 288 K, -2.81 and 2.0 at 298 K, -3.21 and 1.8 at 300 K respectively. Based on the signs and magnitude of the enthalpy and entropy changes, it could be suggested that the binding process of ART and BSA was spontaneous and the main interaction forces of ART with BSA were van der Waal's forces and hydrogen bonding interactions.
Keywords: Fluorescence Emission Spectroscopy, Bovine Serum Albumin, Artemisinin,Protein, Antimalarial.

I. INTRODUCTION

Malaria is a mosquito borne disease caused by plasmodium species, whose multiplication is highly dependent on the availability of folate derivatives for nucleotide synthesis (Nzilaet al., 2005; Francis et al., 2020). Chloroquine, the first antimalarial drug was found to be successful due to it interference with folate (Gregson and Plowe, 2005). Drug resistance developed by the malaria parasite against chloroquine lead to the emergence of several antimalarial drugs (Dondorp, 2011).

Artemisinin (ART), was isolated from Artemisia annua in the early 1970s. Because of its outstanding antimalarial activity, it has broad use as the most important weapon against malaria (Chen et al., 2013). Moreover, it possesses a wide range of biological activities, such as anti-cytomegalovirus (Mott et al., 2013), anti-cancer (Goswami et al., 2013), anti-schistosomal (Li et al., 2011), anti-angiogenesis (Anfossoet al., 2006), and anti-helminth infections (Keiser and Utzinger, 2007).

Serum albumin, the most abundant protein constituent in circulatorysystem of diverse species, performs vital physiological functions suchas maintaining plasma colloid osmotic pressure and blood pH, sequesteringoxygen free radicals, and inactivating various toxic metabolites (Zhou et al., 2018). Among the albumins, bovine serum albumin (BSA) and human serum albumin (HSA) have been studied extensively because of their similar folding and well-known primary structure (Cheng et al., 2013).BSA shows 76% similarity with HSA, which is composed of a single polypeptide chain of 585 amino acid residues, with three α-helical domains (I–III), each containing two subdomains (A and B). It has two tryptophan residues (Trp134 and Trp212) that possess intrinsic fluorescence, that is to say, Trp134 and Trp212 are located on the surface of subdomain IB and the hydrophobic binding pocket of subdomain IIA, respectively (Li et al., 2011).

Shi et al.,(2015) reported the binding interaction of sorafenib with bovine serum albumin using spectroscopic methodologies and molecular docking. The results revealed that there was a static quenching of BSA induced by sorafenib due to the formation of sorafenib–BSA complex. The binding constant and number of binding sites of sorafenib with BSA under simulated physiological condition (pH=7.4) were 6.8×10^{4} M^{-1} 12 and 1 at 310 K, respectively. Based on the sign and magnitude of the enthalpy and entropy changes (ΔH^{0}=-72.2 kJ mol^{-1} and ΔS^{0} =-140.4 J $mol^{-1}K^{1}$) and the results of moleculardocking, it could be suggested that the binding process of sorafenib and BSA was spontaneous and the main interactionforces of sorafenib with BSA were van der Waal's forces

and hydrogen bonding interactions. From the results of site markercompetitive experiments and molecular docking, it could be deduced that sorafenib was inserted into the subdomain IIA (site I) of BSA and leads to a slight change of the conformation of BSA. The significant change of conformation of sorafeniboccurred in the binding process with BSA to increase the stability of the sorafenib-BSA system, implying that the flexibilityof sorafenib played an important role in the binding process.

The interaction of BSA with other molecules may change its intrinsic fluorescence. Fluorescence owing to aromatic residues reveals a wealth of information about the structure, folding and binding interactions of proteins. The intensity of intrinsic fluorescence of protein can be decreased by adding a quencher (Dezhampanahet al., 2018).

The objectives of this work are to determine the interaction mechanism of ART with BSA, to obtain the binding constant (Kb) of ART-BSAcomplex, to locate the specific binding site of ART on BSA, to study effect of ART binding on BSA conformation, among others. To achievethe objectives, the fluorescence spectroscopy was used for the study. It can be expected that this study will provide basic data for clarifying the binding mechanisms of ART with BSA and has great significance in helping to elucidate the store and transport process of ART in the body and its pharmacodynamics and pharmacokinetics.

II. MATERIALS AND METHODS

Preparation of Reagents and Solutions

Bovine serum albumin (BSA fraction V) and Artemisinin (analytical grade), were obtained from Sigma Chemical Company, St. Louis, USA. 75% Ethanol, Tris-HCl buffer (0.1M, pH 7.40), Tris–Base (purity of no less than 99.5%), NaCl all of analytical grade, were obtained from the department of Biochemistry of theUsmanuDanfodiyo University, Sokoto, Nigeria.

A concentration of 1.0×10^{-3}molL^{-1} of Artemisinin was prepared in ethanol by dissolving 0.03g in 100ml of ethanol, while a 1.5×10^{-5}molL^{-1} of BSA was prepared by dissolving 12.0g in 100 ml of 0.1M Tris- HCl.

Fluorescence Spectroscopy Measurements

Fluorescence emissions of ART and BSA were measured using spectrofluorimeter equipped with 1.0 cm quartz cell. Emission was taken from 300-450nm wavelength and excitation at 280nm. The Spectrofluorimeter was firstly left on for 30 minutes to warm up and then zeroed using Tris-HCl buffer and 5.0 ml of BSA was titrated manually into the cuvette and the emission spectrum was obtained. The emission of ART was obtained by taking out 2.0 ml into a cuvette and ran between the wavelengths of 300-450, first at 289K, pH 7.4. This was repeated thrice. It varied from 2μM to 8μM while the concentration of BSA remained constant. The experiment was repeated two more times at temperatures of 298K and 310K. The temperatures of the samples were changed at different levels using ice block to recycle the water in the thermostat. The data obtained was analysed by the Stern- Volmer equation and used to calculate the binding constants and the thermodynamic parameters were obtained. The reaction is as below:

$$\ln K = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (1)$$

In equation (1),InK is the natural logarithm of the binding constant at the corresponding temperature (T) and R is the gas constant. The enthalpy change (ΔH) is calculated from the slope in the Vant's Hoff relationship. The free energy change is estimated from the following relationship:$\Delta G = \Delta H - T\Delta S$ (2)

In equation (2), ΔG is the free energy change, ΔH is the change in enthalpy, T is the temperature and ΔS is change in entropy.

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III. RESULTS AND DISCUSSION

Results

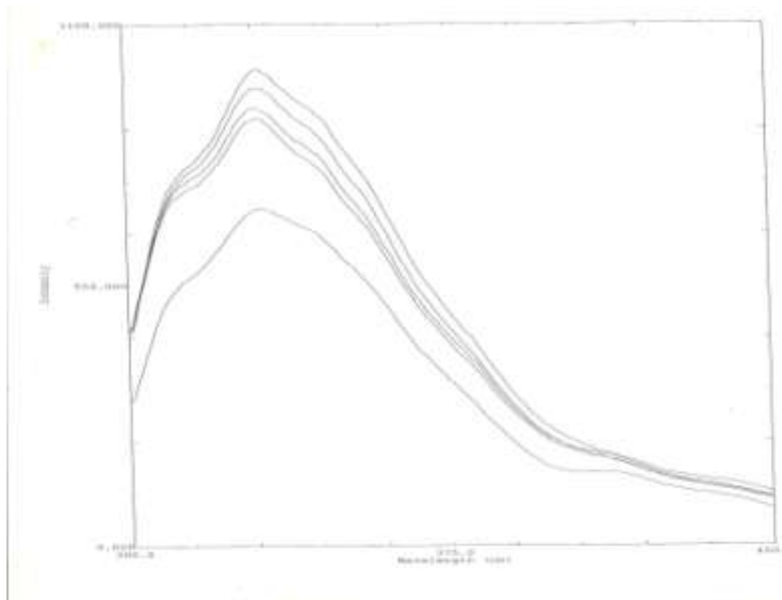

Figure1: Emission spectra of BSA and Artemisinin at 37°C $C_{BSA} = 1.5 \times 10^{-6}$ mol L^{-1}, C_{ART} = 0 - 8μM, pH 7.4

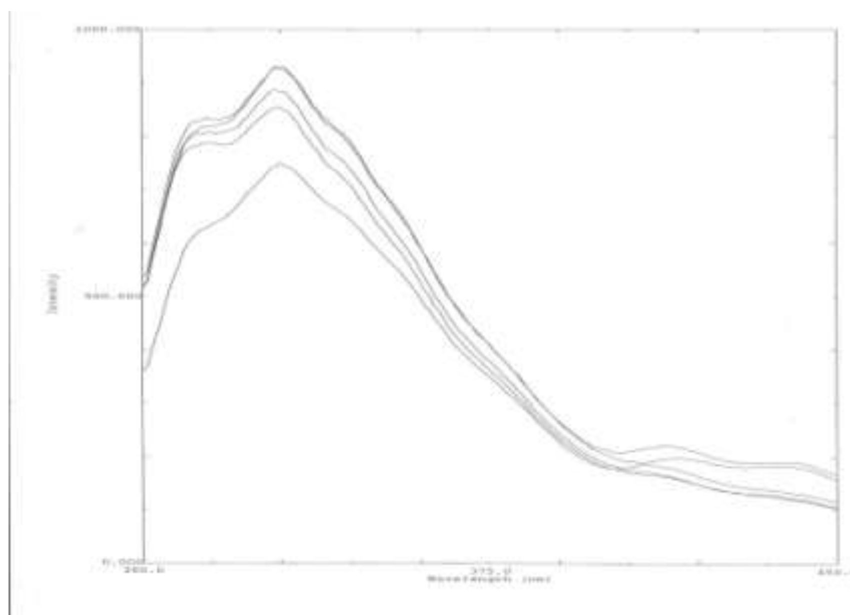

Figure2:Emission spectra of Bovine serum albumin and Artemisinin at 25°C $C_{BSA} = 1.5 \times 10^{-6}$ mol L^{-1}, C_{ART} =0 -8μM, pH 7.04

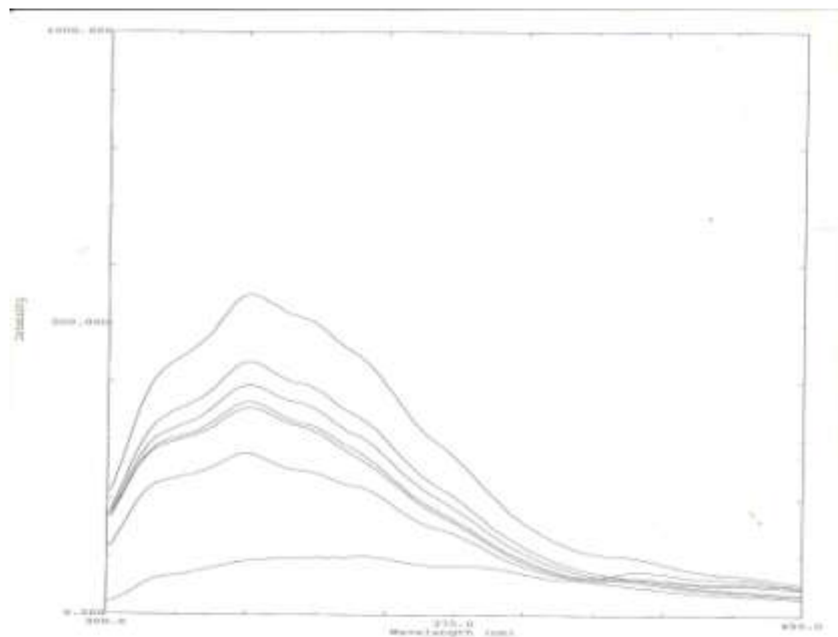

Figure3: Emission spectra of BSA and Artemisinin at 15°C,$C_{BSA} = 1.5 \times 10^{-6}$ mol L^{-1}, C_{ART} = 0 - 8μM, pH 7.4

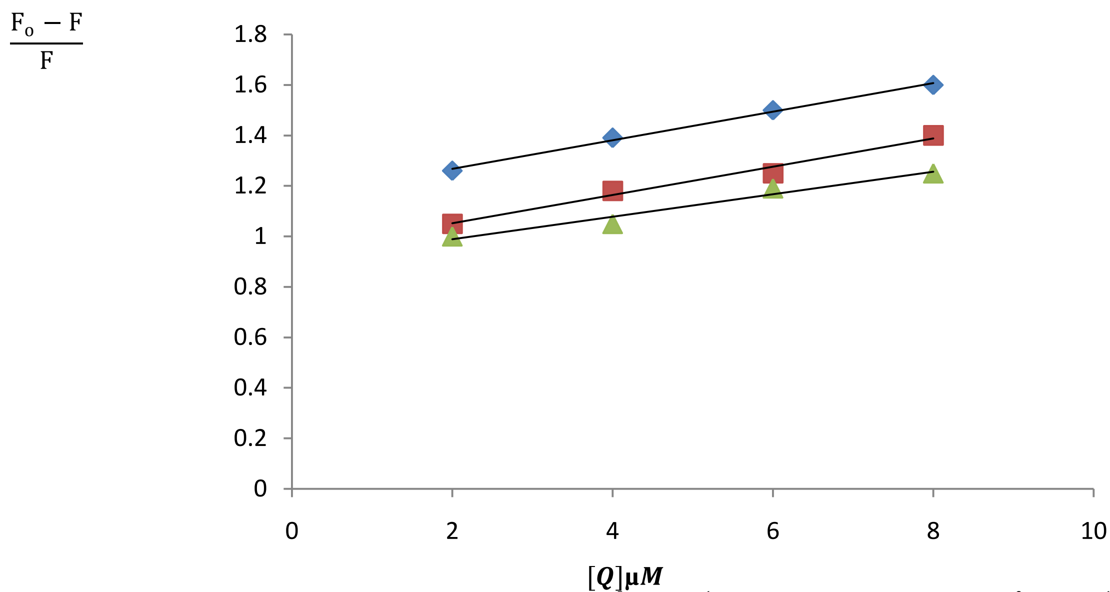

Figure 4: Stern-Volmer's plot of BSA (1.5×10^{-5} mol L^{-1}) and Artemisinin (1.0×10^{-3} mol L^{-1}).

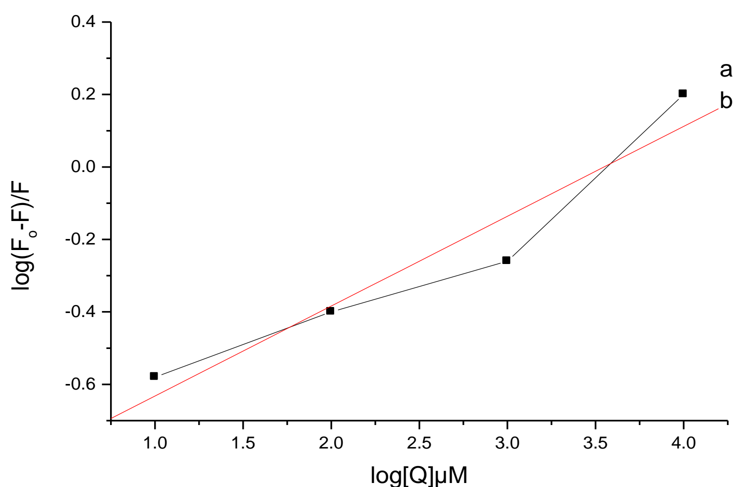

Figure 5: The plot of log(F_o-F)/F vs. log[Q] for quenching of BSA's fluorescence by ART.
$C_{BSA} = 1.5 \times 10^{-6}$ mol L^{-1} (5μM) C_{ART} = 0 - 8μM,pH 7.4; λ_{ex} = 280 nm, λ_{em} = 331 nm at 289K.

a (the black line) is the plot of (log F_o-F)/F vs. log[Q] for quenching of BSA's flouresenceby ART
b (the red line) is the linear plot made to obtain the intercept of the plot.

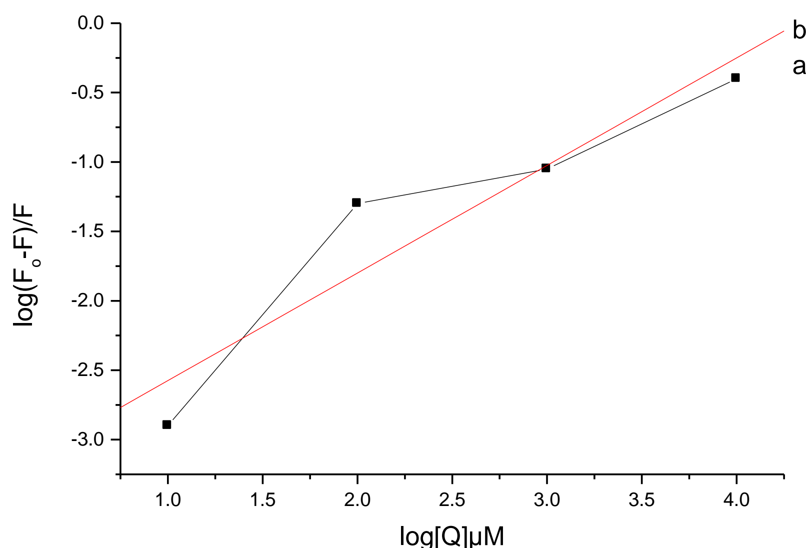

Figure 6: The plot of log(F_o-F)/F vs. log[Q] for quenching of BSA's fluorescence by ART.
$C_{BSA} = 1.5 \times 10^{-6}$ mol L^{-1} (5μM) C_{ART} = 0 - 8μM,pH 7.4; λ_{ex} = 280 nm, λ_{em} = 331 nm at 298K.

a is the plot of log F_o-F)/F vs. log[Q] for quenching of BSA's fluorescence by ART
b is the linear plot made to obtain the intercept

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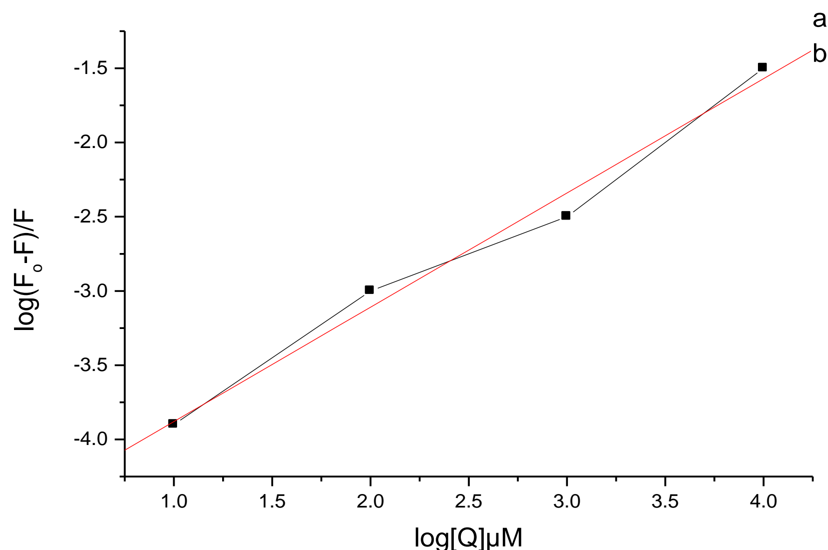

Figure7:The plot of log(F_o-F)/F vs. log[Q] Artemisinin (ART) for quenching of Bovine Serum Albumin (BSA) with artemisinin. (ART). $C_{BSA} = 1.5 \times 10^{-6}$ mol L^{-1} (5µM) C_{ART} = 0 - 8µM,pH 7.4; λ_{ex} = 280 nm, λ_{em} = 331 nm at 310K.

Where line **a** is the plot of log F_o-F)/F vs. log[Q] for quenching of BSA's fluorescence by ART
Line **b** is the linear plot made to determine the intercept of the plot

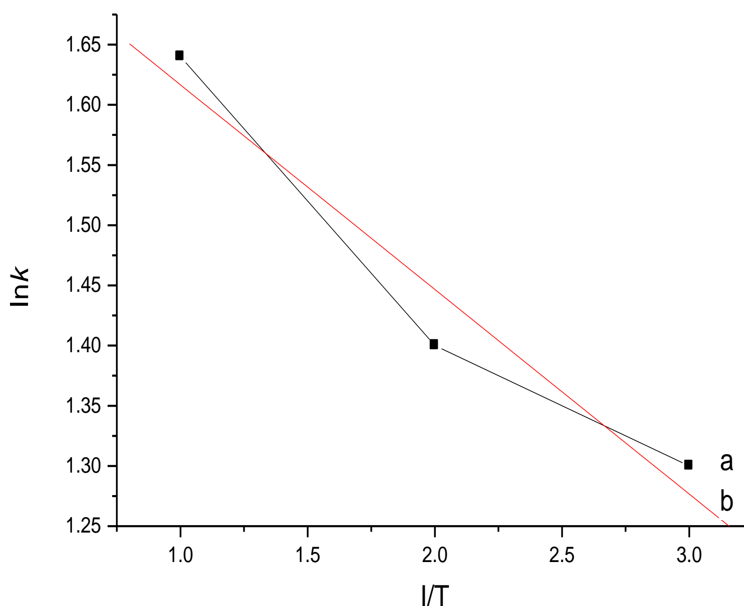

Figure8: The Van't Hoff linear plot of natural logarithm of the binding constant (K)versus temperature.
Where a is the plot of Ink versus I/T
b is the linear plot

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Table 1: Thermodynamic parameters of interaction of Bovine Serum Albumin and artemisinin

pH	T(^{o}c)	ΔG	ΔS	ΔH
	15	-0.208		
7.4	25	-0.307	1.99	-0.071
	37	-0.415		

Table 2: Binding constant (Kb) and binding sites (n)at different temperatures

pH	T(^{o}C)	k_b	n
	15	-0.61	2.5
7.4	25	-2.81	2.0
	37	-3.21	1.8

Table 3: Stern-Volmer quenching constant (K_{sv}) of the interaction of artemisinin and Bovine serum albumin at different temperatures.

pH	T(^{o}C)	K_{sv}
	15	0.060
7.4	25	0.045
	37	0.022

IV. DISCUSSION

Fluorescence Quenching Spectra

The conformational changes of BSA were evaluated by the measurement of intrinsic fluorescence intensity of BSA before and after addition of artemisinin in the range of 300–450 nm upon excitation at 280nm. Fluorescence measurements give information about the molecular environment in a vicinity of the chromophore molecules (Lui et al., 2014). The effect of ART on BSA's fluorescence intensity is shown in Figures 1-3. When different amounts of artemisinin solution were titrated with a fixed concentration of BSA at different temperatures, a remarkable decrease in the fluorescence intensity of BSA was observed, which indicated that artemisinin binds to BSA and quench the Tryptophan intrinsic fluorescence intensity. Furthermore, the maximum emission wavelength of BSA decreased upon addition of different concentrations of artemisinin indicating that the chromospheres of BSA was placed in a more hydrophobic (water repelling) environment after the addition of artemisinin (Bogale et al., 2013).

Mechanisms of Fluorescence Quenching

Fluorescence quenching can occur by different mechanisms, which are usually classified into dynamic and static quenching. They can be distinguished by the values of K_{SV} (Stern–Volmer quenching constant), which is decreased with increasing temperature for static quenching, whereas the reverse result is observed for dynamic quenching (Liu et al., 2011). In order to confirm the quenching mechanism induced by ART, the fluorescence quenching data were analyzed using the Stern–Volmer equation (Li et al., 2011).

It could be observed from Figure4 above that the Stern-Volmer plots were linear and the slope decreased when temperature rose, indicating that it was static quenching interaction between artemisinin and BSA and formation of a complex between quencher and fluorophore. This is observed from the value of K_{sv}(Table 3). For static quenching, the quenching constant decreases with rise in temperature which may be owing to the decrease in BSA-ART complex stability. Similar result has been observed in the study of the interaction between Human Serum Albumin (HSA) and antimalarial drug dispiro-tetraoxanes (Yadav et al., 2020). The fluorescence of BSA originates from tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues (Dezhampanahet al., 2018). The fluorescence intensity ratio of Trp, Tyr and Pheis 100:9:0.5 because of the difference in their structure (Chenget al., 2013).

Binding Constant and Binding Sites

For the static quenching interaction, if we assume that there are independent binding sites in the biomolecule, the binding constants (Kb) and the number of binding sites (n) can be obtained from the following equation (Lui et al., 2015):

$$log\frac{F_o-F}{F} = logk_b + nlog[Q] \quad (3),$$

where F_0, and F where F0 and F denote the fluorescence intensities in the absence and presence of quencher, respectively, [Q] is the concentration of quencher(ART), K_b is thebinding constant of BSA with ART and n is the number ofbinding sites. The binding constants and the number of bindingsites can be calculated using the values of intercept and

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slopeobtained from the plot of log [(F_0– F)/F] versus log [Q]. The binding constants (Kb) and the number ofbinding sites (n) for the interaction of BSA with ART are summarized in Table 2. As shown in Table 2, the values of $K_b$were -0.61, -2.81 and -3.21 M^{-1} and those of n 2.05, 2.00 and 1.80 at 289, 298 and 310K, respectively. The binding constant decreased with increase in temperature, resulting in the reduction of the stability of BSA-ART complex. This is similar to the report made by Shi et al., (2015) for binding interaction of sorafenib with bovine serum albumin. The values of number of binding sites of ART on BSA were almost equal. Therefore, it may be inferred that there is a single class of binding site on BSA for artemisinin. Hence, artemisinin most likely binds to the hydrophobic pocket located in subdomain IIA; that is to say, Trp – 214 is near or within the binding site ofthe binding constants calculated ART–BSA (Lui et al., 2015).

Thermodynamic Parameters and Binding Forces

In general, the active force between a drug and a biological macromolecule may be a hydrogen bond, van der Waal's force, electrostatic force or hydrophobic interaction force. The signs and magnitudes of the thermodynamic parameters of enthalpy change (ΔH) and entropy change (ΔS) may account for the main forces involved in the binding process. For this reason, binding studies were carried out at four temperatures (288, 298, and310 K) and evaluated using the Van't Hoff's equation (Zhou et al., 2011).

The values of ΔH^o, ΔS^o and $\Delta G^o$are summarized in Table 1. The negative value of ΔGo reveals that the binding processes are spontaneous. According Naveenraj and Anandan (2013), when $\Delta H<0$ or $\Delta H \sim 0$ and $\Delta S>0$, the main force is due to electrostatic interactions; when $\Delta H<0$ and $\Delta S<0$, the main force is due to van der Waal's or hydrogen bonding, and when $\Delta H>0$ and $\Delta S>0$, the main force is due to hydrophobic interactions (Shi et al., 2015).

V. CONCLUSION

Bovine serum albumin was used as protein model to explore the protein binding properties of artemisinin in this research. It provided an approach in studying the interactions of BSA with artemisinin using fluorescence techniques.The quenching constant (k_{sv}), the binding constant (k_b)and thermodynamic parameters; changes in the enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) were calculated.The result revealed that the interaction between ART and BSA is a static process. The experimental and theoretical methods used indicate that artemisinin binds to BSA through hydrophobic force and hydrogen bond interaction mostly via the binding sites. Investigating the interaction of drugs to protein can elucidate the property of drug – protein complexes.

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