Journal of Herbal Drug
journal homepage: www.jhd.iaushk.ac.ir

Interaction of Saponins extracted from *Tribulus terrestris* with Human Serum Albumin

Azadeh Hekmat[*1], Rouya Bromand Gohar[1], Kambiz Larijani[2]

[1] *Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran;*
***Email:** *hekmat@ut.ac.ir*

[2] *Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran;*

ARTICLE INFO

Type: *Original Research*
Topic: *Medicinal Plants*
Received *January 15th 2020*
Accepted *May 21th 2020*

Key words:
- ✓ *Saponins*
- ✓ *Human Serum Albumin (HSA)*
- ✓ *Tribulus terrestris*
- ✓ *Spectroscopy*
- ✓ *Fluorescence*
- ✓ *Förster theory*

ABSTRACT

Background & Aim: Saponins are well-known secondary metabolites with numerous beneficial pharmacological properties. Since the interactions of drugs with blood constituents, in particular with human serum albumin (HSA) may have a major impact on drug pharmacology, the present study designed to provide a fundamental understanding of the interaction of saponins extracted from *Tribulus terrestris* (typically utilized in folk medicine) with HSA in detail.

Experimental: After extraction and purification of saponins; UV-Vis spectroscopy, fluorescence spectroscopy, and far-UV CD spectroscopy were used to examine the effects of saponins from *Tribulus terrestris* on HSA.

Results: Thin-Layer chromatography confirmed the presence of 3 different saponins. The UV-Vis absorption, fluorescence emission and far-UV CD results displayed that saponins from *Tribulus terrestris* could form a complex with HSA. The binding constant for the saponins–HSA complex was found to be $13.4\times10^{4}M^{-1}$. The distance *r* between HSA and *Tribulus terrestris* saponins was also acquired according to the Förster theory.

Recommended applications/industries: Altogether, saponins can bind to HSA and change the secondary and tertiary structure of HSA moderately. The results obtained from this study can help in understanding the pharmacokinetic properties of saponins.

1. Introduction

Tribulus terrestris L. also called puncture vine, caltrop, bull's head and tackweed is an annual plant of the family Zygophyllaceae (Kostova and Dinchev, 2005). It is mostly planted in the Mediterranean and in sub-tropical areas, for example, Iran, South America, Spain, India, Mexico, China, and Bulgaria. Many compounds have been extracted and identified from *Tribulus terrestris* including proteins, amino acids, glycosides, steroidal saponins, flavonoids, tannins, amide derivatives, phytosterols, and terpenoids. These compounds have different chemical structures and bioactivities. Among these compounds, steroidal saponins and flavonoids are the most important metabolites with various biological functions. The most characteristic chemicals in *Tribulus terrestris* are furostanol saponins and spirostanol. *Tribulus terrestris* (typically used in folk medicine) has been widely utilized in the treatment of cardiovascular diseases, rheumatism, treatment of cancer, eye trouble, diabetes, menorrhagia, and sexual dysfunction related disorder Song *et al*., 2016; Zhu, Du, Meng, Dong and Li, 2017).

Saponins are phytochemicals materials that are found largely in plants and consist of polycyclic aglycones attached to one or more sugar side chains. Sapogenin, the aglycone part, is either a steroid or a triterpene. Generally, saponins are thermally and chemically labile, besides, they have high polarity. Saponins have been widely used in the food industry (Guclu-Ustundag and Mazza, 2007), the cosmetic industry (Olmstead, 2002), the pharmaceutical industry and medicine (Waller and Yamasaki, 2013). It has been reported that saponins possess a variety of biological activities such as antiviral, anti-allergic, antimicrobial, anti-mutagenic, antioxidant, adjuvant, anti-inflammatory, neuroprotective, chemopreventive, anti-genotoxic, antifungal, and sedative Guclu-Ustundag and Mazza, 2007). It has also been confirmed that saponins have an effect on cognitive behavior, absorption of minerals and vitamins, as well as animal growth and reproduction (Guclu-Ustundag and Mazza, 2007).

Human serum albumin (HSA) is the most abundant and significant blood plasma protein. HSA is a protein storage component (Purcell *et al*., 2000). HSA crystallographic data revealed that its helical triple-domain structure contains 585 amino acids in a single polypeptide chain with approximately 66 kDa molecular weight. HSA consists of three domains (I, II and III) and each one separated into two subdomains (A, B) (Amézqueta *et al*., 2018; Wang *et al*., 2011). The binding of drugs to HSA can modulate their pharmacokinetics and pharmacodynamics. Modified or new drug binding to HSA is a significant determinant of drug pharmacokinetics, restricting the unbound concentration as well as affecting drug distribution and elimination.

Regarding the various biomedical and biological effects of saponins, the present study is designed to offer a fundamental understanding of the mechanism of interaction between saponins and HSA in detail using multiple spectroscopic instruments at 37˚C. To the best of our knowledge, there is not any considerable research about the saponins' interaction with HSA in physicochemical terms. It is essential for pharmacists and structural biologists to design ideal herbal drugs with fewer side effects, preferably non-covalently binding, and also with accessible clinical value. Due to the fact that the pharmacokinetic properties of drugs count on how they interact with HSA, it becomes crucial to determine the drugs' binding affinity. This study can provide potential insight into the mechanism responsible for the binding of saponins from *Tribulus terrestris* to HSA and improves the understating of its effect during transport and distribution in the blood. We hope the achieved observations deliver valuable information to understand saponins from *Tribulus terrestris* pharmacological behaviors *in vitro*.

2. Materials and Methods

2.1. Chemicals and Reagents

Human Serum Albumin (lyophilized powder, essentially fatty acid-free) was acquired from Sigma Aldrich Co., USA. Tris (hydroxymethyl) aminomethane (Tris-base), were purchased from Sinagen Co, Iran. Thin-layer chromatography plates (silica gel, 60, F254), ethanol, Toluene, n-hexane, and ethyl acetate were acquired from Merck (Darmstadt, Germany). During all experiments, sterile water (Barnstead Nanopure (USA) ER 18.3 MΩ) was utilized and all experiments were carried out at 0.1 M Tris-based buffer (pH 7.4) at 37ºC.

2.2. Plant Material

Tribulus terrestris L. (Stem and Leaves) were harvested in Iran, around Qazvin Province in August 2018. Plants were authenticated by Dr. Majid Ghorbani Nohooji, from the Institute of Medicinal Plants Research Center, Iran. The voucher specimen of plant (Voucher No. 4539) was deposited at the herbarium of the Medicinal Plants Institute (ACECR).

2.3. Extraction of Saponins

The extraction procedure was adapted from Van Dyck *et al*. (2009). Briefly, 100 g of the fresh parts of the plant (Stem and Leaves) were dried in oven and ground to a fine powder with a mechanical grinder. 100 grams of plant powder was then macerated in 500 ml of absolute ethanol for 8 hr and covered with aluminum foil. After 8 hr, the extract was evaporated at low pressure in a double boiler at 30 °C by means of a Laborota 4001 Efficient rotary evaporator (Heidolph, Germany). Afterward, the dry extract was diluted in 90% methanol and partitioned against n-hexane (*v/v*). Then, the water content of the hydroethanolic phase was adjusted to 20% (*v/v*) and 40% (*v/v*) and the solutions partitioned against CCl_4 and $CHCl_3$, respectively. Lastly, the hydroethanolic solution was

evaporated and dissolved in water in order to undergo chromatographic purification.

2.4. Purification of the Extraction

The crude hydroethanolic solution was placed in column chromatography with silica gel (60-120 mesh) as a stationary phase. Firstly, the chromatography column was washed with pure chloroform solvent. Subsequently, chloroform-methanol at a ratio of 10-90, 20-80, 30-70 and 50-50 *v/v* were used for column washing. At the end of the chromatography, 4 solutions were obtained. These solutions were used for the next experiments.

2.5. Thin-layer chromatography (TLC)

TLC was performed for all 4 solutions as follows. First, a TLC plate consisting of a 20 cm ×20 cm aluminum plate pre-coated with a silica gel layer was prepared as a stationary phase. 10 µl of 4 fractions of saponins from *Tribulus terrestris* were loaded onto the TLC plate. D-glucose and L-rhamnose were utilized as a marker. The retention factor (R_f) values of D-glucose and L-rhamnose were 0.25 and 0.42, respectively (Chen *et al*., 2013). Then Toluene/ethyl acetate (93:7) has been utilized as a mobile phase (Bladt, 2009). After 30 minutes, chromatography was completed on the TLC tank. Subsequently, separated fractions were distinguished on UV light (at 254 nm wavelength) in UV Viewing Cabinet. The R_f value can be calculated according to Eq. 1:

$$R_f = \frac{\textit{distance traveled by the compound}}{\textit{distance traveled by the solvent front}} \quad (1)$$

2.6. Frothing Test

One mL of each solution was diluted with distilled water then made up to 6 ml. The test-tubes were shaken for 5 min and were allowed to stand for 15 min (Auwal *et al*., 2014).

2.7. Ultraviolet-Visible (UV-Visible) Measurements

Absorption spectra were recorded using UV-Vis spectrophotometer CARY, 100 Conc, UK. The saponins from *Tribulus terrestris* concentration was calculated spectrophotometrically via a molar extinction coefficient of 16542.5 $nM^{-1}cm^{-1}$ at 330 nm. Diverse amounts of saponins (0.015×10^{-6}-0.21×10^{-6} nM) were added to the HSA solution (4.8 µM) at 37 °C and the absorption spectra were recorded. All experiments were run and verified in a 1 cm quartz cell thermostated.

2.8. Steady-StateFluorescence Measurements

Steady-state fluorescence emission spectra were explored via a Varian Cary Eclipse Fluorescence Spectrophotometer, USA. The thickness of the quartz cuvettes in all experiments was 1 cm and the width of the slits for excitation and emission was 10 nm and 10 nm, respectively. The fluorescence intensity spectrum for each sample was achieved, by a fluorescence cuvette with 1 cm path length. The concentration of HSA was 4.8 µM and a diverse amount of saponins (0.015×10^{-6}-0.21×10^{-6} nM) were added to the HSA solution and fluorescence measurements were taken. The excitation and emission wavelengths were 280 nm and 341 nm, respectively. For inner filter effect correction caused via the excitation and emission signals attenuation produced from the quencher absorption the Eq. 2 was used:

$$F_{corr} = F_{obs}.10^{(Ab_{ex}+Ab_{em})/2} \quad (2)$$

Where F_{corr}, $F_{obs,}$ Ab_{ex} and Ab_{em} are the corrected intensities, the observed fluorescence intensities, the mixture absorption at excitation and the mixture absorption at emission wavelengths, respectively (Maity *et al*., 2016).

2.9. Circular Dichroism (CD) Measurements

By means of Aviv Circular Dichroism Spectrometer, Model 215, USA, the CD spectra were recorded to investigate deviations in the structure of HSA (4.8 µM). Therefore, by adding saponins (0.015×10^{-6} and 0.21×10^{-6} nM) to HSA the far-UV CDspectra (190 to 260 nm) were recorded using a quartz cell, 0.1 cm path length, with a resolution of 0.2 nm and speed of scanning 20 nm min^{-1}. By subtracting the proper baseline, each CD spectra were corrected. The CDNN CD spectra deconvolution software (version 2.1) was applied to deconvolute all far-UV CD spectra.

2.10. Statistical Analysis

All data were demonstrated as the Mean ± SD of three independent experiments.

3. Results and discussion

3.1. Thin-Layer Chromatography (TLC) Results

Thin-layer chromatography is a cost-effective and acceptable methodology for the quantitative and qualitative investigation of herbal products. It should be mentioned that literature data exposes some differences in the saponin composition and the saponin content of *Tribulus terrestris* growing in diverse geographic areas of the world (Kostova and Dinchev, 2005) and there are limited studies on saponins from *Tribulus terrestris* from Iran, Australia and Romania. TLC chromatogram of saponins extract has been shown in Fig. 1. Exposure of the spotted to UV 254 nm can display the presence of any distinct fractions as dark bands in a green background. As shown in Fig. 1, only chloroform-methanol at the ratio of 30-70 *v/v* produced 3 distinct fractions. Also, among the fractions obtained, only the fraction isolated using chloroform-methanol at the ratio of 30-70 v/v produced stable 3 cm layer of foam that persisted for 30 min, which confirmed the presence of saponins. Therefore, *Tribulus terrestris* (Stem and Leaves) displayed the presence of 3 distinct types of saponins with R_f values 0.55, 0.62 and 0.91. Since R_f value (0.91) is in the R_f range of steroidal saponins, so this fraction was identified as steroidal saponins (Bladt, 2009). In addition, the values of 0.55 and 0.62 are in agreement with those of Klyachenko *et al.* (2017). Thus, for the next experiments, the mixture of all extracted saponins was used.

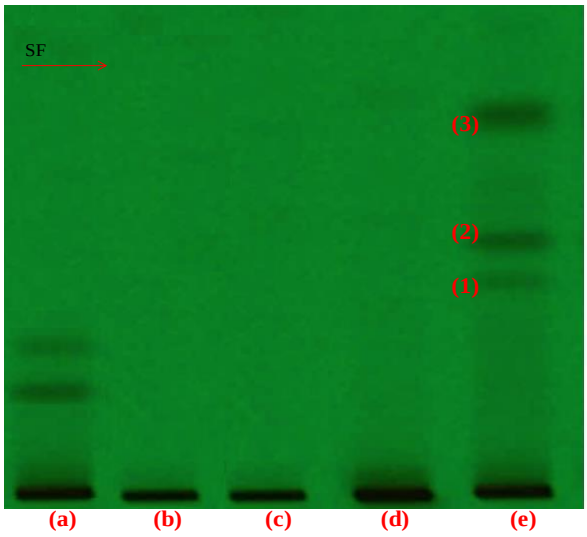

Figure 1. TLC chromatogram of extracts from Tribulus terrestris fractions: (a) D-glucose and L-rhamnose. (b) chloroform-methanol at ratio of 10-90 *v/v*, (c) chloroform-methanol at ratio of 20-80 *v/v*, (d) chloroform-methanol at ratio of 50-50 *v/v* and (e) chloroform-methanol at ratio of 30-70 *v/v*.

3.2. Absorption Studies

Absorption spectroscopy is one of the most significant methods for studying the interaction between proteins and small molecules. With the aim of providing information for the structural effect of saponins on HSA, the UV-Vis absorption spectra of HSA in the absence and presence of saponins were achieved. As illustrated in Fig. 2, HSA has two strong absorption bands: around 213 nm and around 280 nm. The absorption band at around 213 nm is owing to the $n \rightarrow \pi^*$ transition of $C=O$ in the backbone of HSA and it also reflects the α-helix content in the protein (Na *et al.*, 2015; Yuqin *et al.*, 2014). As seen in this figure the addition of saponins to HSA resulted in an increase of absorbance intensity at approximately 213 nm. The weak absorption band at approximately 280 nm is owing to phenyl rings in Trp (Tryptophan), Tyr (Tyrosine) and Phe (phenylalanine) residues absorption (Wang *et al.*, 2011). The HSA absorption spectrum perturbation at 280 nm after the addition of saponins indicates that the aromatic acid residues micro-environment was changed. Since the strong absorption peak at around 213 nm represents the HSA framework structure, so it can be concluded that the interaction between HSA and saponins from *Tribulus terrestris* (at higher concentration) leads to some micro-environmental and conformational variations in HSA (Li *et al.*, 2014). Furthermore, UV-Vis absorption spectra indicated the side chains of aromatic residues exposure to the solvent (inset of Fig. 2) (Hoque *et al.*, 2013). Moreover, this spectral variation can be due to the formation of the complex between HSA and saponins from *Tribulus terrestris.* Since saponins were added to both cuvettes, increment in absorbance is the result of the complex formation between HSA and saponins. According to the literature, no variations in the absorption spectra are expected in dynamic quenching because dynamic quenching only disturbs the excited states of the fluorophores (Han *et al.*, 2009). Therefore, our UV-Vis absorption spectra verify that the quenching mechanism of HSA and saponins from *Tribulus terrestris* can be static quenching.

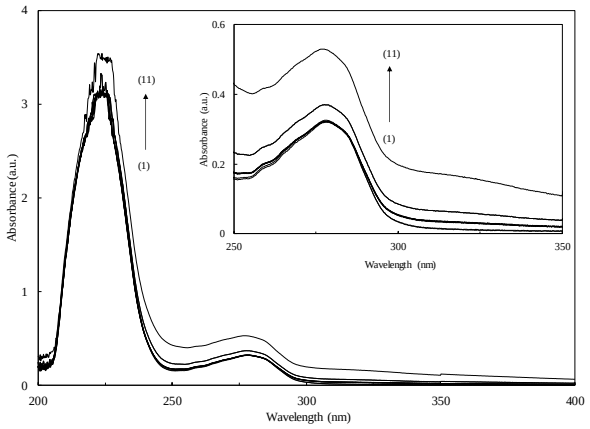

Figure 2. The UV-Vis spectra of 4.8 µM HSA (1) with increasing concentrations of saponins (2-11) in Tris buffer (0.1 M), pH 7.4 at 37 ºC. The inset illustrates the alterations in HSA maximum absorbance (280 nm) in the presence of various concentrations of saponins from Tribulus terrestris (0.015×10-6 and 0.21×10-6 nM) in the wavelength range of 250 to 350 nm.

3.3. Steady-State Fluorescence Studies

One of the most influential methods to discover the protein folding and protein-ligands interaction is fluorescence spectroscopy (Wang *et al*., 2011). There are three intrinsic fluorophores for HSA: Tyr, Trp, and Phe (Amani *et al*., 2011), however, the HSA intrinsic fluorescence is commonly acquired by Trp residue alone. HSA has only one Trp residue (the Trp_{214} residue) (Amani *et al*., 2011) and any variations in polarity around the fluorophore could be evaluated by the shift in the maximum fluorescence emission peak ($\lambda_{max,em}$) (Wang *et al*., 2011). Accordingly, by virtue of the fluorescence spectroscopy, the HSA conformational variations in the presence of saponins from *Tribulus terrestris* were studied. It is clear that HSA has a strong $\lambda_{max,em}$ at 341 nm after being excited with a wavelength of 280 nm. The intensity of HSA decreased regularly with increasing concentrations of saponins from *Tribulus terrestris* with no shifts in $\lambda_{max,em}$ (Fig. 3). It is clear that the reduction in Trp fluorescence intensity is attributable to the decrease in the surface-exposed Trp residues to the polar solvent (Amani *et al.*, 2011; Varlan and Hillebrand, 2010). The comparison of the present data with previous publications leads to the following conclusions: the micro-environment of Trp was affected by saponins from *Tribulus terrestris* barely and a little reduction in the polarity of the micro-environment around the Trp occurred after addition of saponins. Our data correlate with those of Cheng *et al.* (2009) who have established that the $\lambda_{max,em}$ of serum albumin was hardly changed after the addition of berbamine, which indicates that berbamine cannot alter the polarity of the hydrophobic microenvironment around Trp of the protein. This data is also in agreement with Han *et al.* (2009) who have established that Quinclorac could interact with serum albumin, however there was no variation in the local dielectric environment of the protein. Thus, the HSA structure altered partially after the addition of saponins from *Tribulus terrestris.* This data is in agreement with the UV-Vis spectroscopy as mention above.

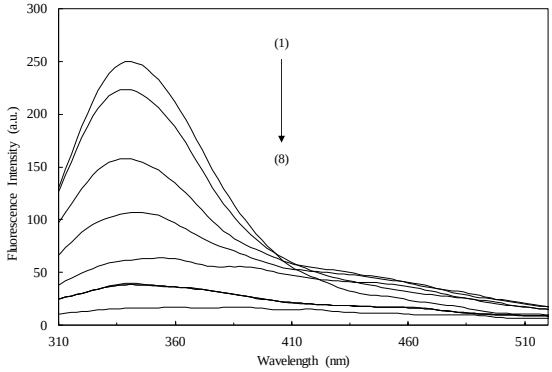

Figure 3. The fluorescence emission spectra of 4.8 µM HSA at 37 °Cafter adding various concentrations of *saponins from Tribulus terrestris* (0.015×10^{-6} and 0.21×10^{-6} nM); the fluorescence emission of HSA alone (1) and the fluorescence quenching with increasing concentrations of saponins from *Tribulus terrestris* (2-8) are shown.

3.4. Determination of Quenching Mechanism and Binding Parameters

Any process that causes a reduction in the fluorescence emission can be considered as quenching. Quenching can arise via a number of molecular mechanisms, such as ground state complex formation (static quenching), dynamic quenching, molecular rearrangement, and energy transfer (Hekmat *et al*., 2013). Thus, in order to find parameters and mechanisms involved in the quenching phenomenon Stern-Volmer equation was used (Eq. 3) (Sprung *et al*., 2017):

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \qquad (3)$$

Where F, F_0, [Q] and K_{SV} are the fluorescence data in the presence of saponins, absence of saponins, the concentration of saponins from *Tribulus terrestris*

(quencher) and the Stern-Volmer quenching value constant, respectively (Amani *et al*., 2011; Hekmat *et al*., 2013; Varlan and Hillebrand, 2010). By means of Eq. 3, the plots for F_0/F versus [saponins] were drawn (Fig. 4A). We achieved a positive deviation for the plots of F_0/F versus [saponins] (Fig. 4A). Thus, the binding of saponins to HSA initiated via non-fluorescence complex formation (static quenching) (Masters, 2008).

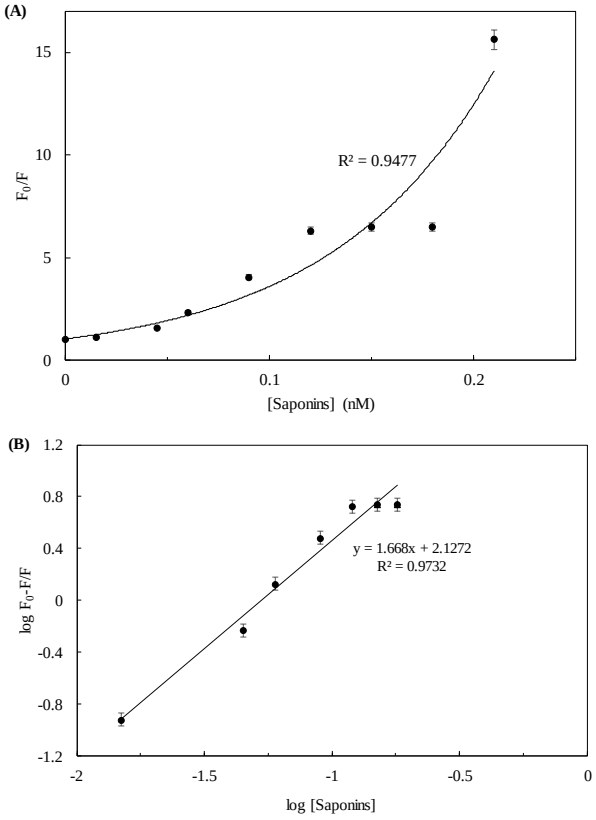

Figure 4. (A) The classic Stern–Volmer plot of HSA in the presence of diverse concentrations of saponins from *Tribulus terrestris* at 37 °C. **(B)** The Modified Stern-Volmer plot of HSA in the presence of diverse concentrations of saponins at 37 ºC. The data are obtained from the Means of three independent measurements ± SD.

Assuming that there were independent and similar binding sites in HSA, with Eq. 4 the binding constant (K_A) and the number of binding sites (n) for static quenching were determined (Yang *et al*., 2013).

$$\log \frac{F_0 - F}{F} = \log K_A + n \log[Q] \quad (4)$$

Apparently, by the intercept and slope by plotting $\log \frac{F_0 - F}{F}$ against log [Q], the n and K_A could be determined. Consistent with Fig. 4B, K_A, and n for HSA-saponins were achieved. It could be realized that the plots displayed a good linear relationship. The value of K_A and n were found to be 13.4×10^4 M^{-1} and 1.6, respectively. The value of n is valuable to distinguish the number of binding sites. It should be mentioned that it is usual in biological systems that the value of n for binding of a ligand to HSA involves more than one binding site. The number of binding sites changes can affect binding affinity (Moradi et al., 2018). The value of n was approximately equal to 2 for saponins, suggesting that saponins bind to HSA, forming 1:2 adduct. It can be suggested the existence of two classes of binding sites for HSA-saponins.

Applying K_A, the standard Gibbs free energy change (ΔG^0) can be calculated from the following equations (Shi *et al*., 2015).

$$\Delta G^\circ = -RT \ln K \quad (5)$$

Where T and $R$are the absolute temperature and the universal gas constant, respectively. The value of ΔG^0 was found to be -27.4 kJ mol^{-1}. The negative values of ΔG^0 point out that the interaction of *Tribulus terrestris* saponins to HSA was spontaneously in nature (Li *et al*., 2017).

3.5. Fluorescence Resonance Energy Transfer (FRET) Studies

By using FRET, the distance between saponins and HSA can be estimated (Han *et al*., 2009). Consistent with the Förster theory, the distance (r) of binding between saponins and HSA and the efficiency (E) of the energy transfer between the donor (HSA) and acceptor (saponins) can be calculated by Eq. 6 (Han *et al*., 2009; Mote *et al*., 2010)

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r_0^6} \quad (6)$$

Where R_0 is the critical distance when the transfer efficiency between the acceptor (saponins) and the donor (HSA) is 50% (Han *et al*., 2009; Mote *et al*., 2010).

$$R_0^6 = 8.79\times10^{-25} k^2 N^{-4} \Phi J \quad (7)$$

In Eq.7, K^2 and N are the dipole special orientation factor and the refracted index of the medium, respectively (Cheng *et al*., 2009; Han *et al*., 2009; Mote *et al*., 2010). Furthermore, Φ and J are the fluorescence quantum yield of HSA and the integral overlap area between the emission spectrum of HSA (the donor) and

the absorption spectrum of saponins (the acceptor), respectively (Cheng *et al*., 2009; Han *et al*., 2009). *J* can be calculated according to Eq. 8 (Han *et al*., 2009; Mote *et al*., 2010).

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 / \sum F(\lambda)\Delta\lambda \quad (8)$$

Where $\varepsilon(\lambda)$ and $F(\lambda)$ are the extinction coefficient of saponins (the acceptor) at wavelength λ and the corrected fluorescence intensity of HSA (the donor) in the wavelength range λ to λ+Δλ, respectively (Cheng *et al*., 2009; Han *et al*., 2009; Mote *et al*., 2010).

The overlap of fluorescence spectra of HSA (4.8 μM) and the UV absorption spectra of saponins from *Tribulus terrestris* (0.21×10^{-6} nM) is shown in Fig. 5. In the present case, using K^2 = 2/3, N = 1.336 and Φ = 0.12 and according to Eqs. (6)-(8), we calculated R_0 = 2.6 nm, E = 0.37 and r_0 = 2.8 nm. Obviously, the donor-to-receptor distances (r_0) is less than 7 nm, consistent with the conditions of the Förster non-radiation energy transfer theory, and also $0.5R_0<r_0<1.5R_0$, indicating that the energy transfer from HSA to saponins happens with a high level of possibility (Han *et al*., 2009; Mote *et al*., 2010).

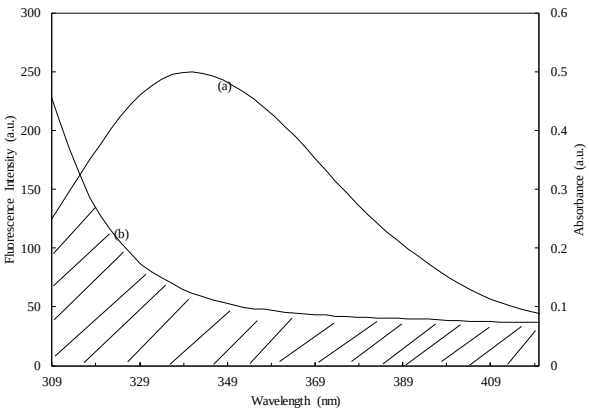

Figure 5. Spectral overlap of absorption spectrum of saponins from *Tribulus terrestris* (a) with fluorescence emission spectrum of HSA (b); [HSA]=4.8 μM and [saponins]= 0.21×10^{-6} nM at 37° C.

3.6. CD Spectroscopic Studies

Circular dichroism (CD) spectroscopy is an ideal spectroscopic technique for analyzing the conformation of macromolecules, mostly proteins. The secondary structure is sensitive to its environment. Therefore, the CD spectra can be utilized extensively to study how secondary structure alters with environmental conditions (Hekmat *et al*., 2017). CD measurements were examined in the absence and presence of saponins to monitor the conformation variations of HSA. As shown in Fig. 6, two negative bands around 208 and 222 nm were detected in the far-UV CD spectrum of free HSA. The far-UV CD spectrum for all α-helical protein has two double minimum around 208 nm (for π→ π* transition) and 222 nm (for n→ π* transition) as well as a stronger maximum around 191-193 nm (for π→π* transition) (Corrêa & Ramos, 2009). The energy and intensity of these transitions related to the peptide bond angles (Ψ and Φ), and therefore the protein secondary structure. The content of the HSA secondary structure was calculated by CDNN software. has consists of 52.87% α-helix, 22.14% β-sheet and 24.97% random coil (Table 1).

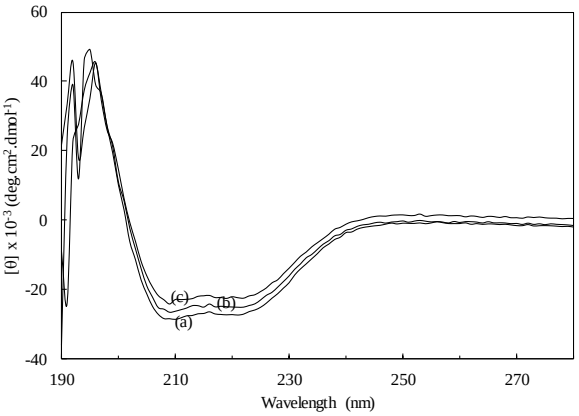

Figure 6. The far-UV CD spectra of 4.8 μM HSA in the absence (a) and presence of 0.015×10^{-6} nM saponins from *Tribulus terrestris* (b) and 0.22×10^{-6} nM of saponins from *Tribulus terrestris* (c) in Tris buffer (0.1 M), pH 7.4 at 37° C.

Table 1. Content of the secondary structure of 4.8 μM HSA in the absence and presence of saponins from *Tribulus terrestris* in Tris buffer (0.1 M), pH 7.4 at 37 °C.

	α-Helix (%)	β-Sheet (%)	Random coil (%)
HSA	52.87	22.14	24.97
HSA+0.015×10^{-6} nM	51.19	22.39	26.39
HSA+0.218×10^{-6} nM	45.01	24.96	30.00

Consequently, our result demonstrated that HSA has an α-helical structure. This finding has been confirmed by other groups (Amani *et al*., 2011; Hekmat *et al*., 2017; Wang *et al*., 2011; Wei, Thyparambil and Latour, 2014). From Fig. 6 and Table 1, the addition of

saponins to HSA solution caused a slight decrement in the ellipticity, which manifested that the α-helical content of HSA decreased (52.87% to 45.01%). Our data also correlate with those of Cheng *et al*. (2009) who have established a decrease in α-helical structure and an increase in β-sheet structure of serum albumin after the addition of berbamine, which indicates that berbamine can alter the conformation of the protein. Wang *et al.* (2011) and Fu *et al.* (2014) reported that when the HSA ellipticity decreases partially after the addition of ligand, demonstrating some loss of α-helical secondary structure. Furthermore, this may specify that the ligand is able to interact with the amino acid residues of the HSA polypeptide chain and abolish the hydrogen-bonding networks moderately. Also, Corrêa and Ramos (2009) published that the band at around 222 nm is associated with the strong hydrogen-bonding environment of α-helical secondary structure and is not dependent on their length. Accordingly, based on these publications we can conclude that because the HSA ellipticity reduces partially after saponins addition, saponins are able to abolish the hydrogen-bonding networks of HSA slightly. Moreover, the loss of α-helical content specifies that saponins binding induced a little unfolding of the polypeptides of HSA. However, HSA preserves its secondary structure and helicity when interacting with saponins (Fu *et al*., 2014). This phenomenon is very essential for biomedical applications.

4. Conclusion

Once drug molecules enter blood vessels, they are picked up via HSA and delivered to distant parts of the body. The absorption, distribution, metabolism, excretion properties and toxicity of drugs can be considerably affected as a result of their binding to HSA. Consequently, HSA has been extensively utilized as a model protein in assessing the interactions of protein and drug. It has been reported that saponins possess a variety of biological activities. In this study, several techniques have been utilized in order to explore the binding properties between saponins from *Tribulus terrestris* and HSA. The UV-Vis data, fluorescence emission data, and CD measurements showed that saponins could form a complex with HSA. Additionally, the values of binding constants and ΔG° showed that saponins interact with HSA spontaneously. The distance *r* between HSA and saponins was found to be 2.8 nm according to Förster's energy transfer theory. Thus, since saponins change the secondary and tertiary structure of HSA moderately we can propose that saponins from *Tribulus terrestris* could be used in pharmacological applications.

5. Acknowledgments

We gratefully acknowledge Ms. Evini at the Institute of Biochemistry and Biophysics of Tehran University for technical support.

Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

6. References

Amani, N., Reza Saberi, M. and Khan Chamani, J. 2011. Investigation by fluorescence spectroscopy, resonance rayleigh scatt ering and zeta potential approaches of the separate and simultaneous binding effect of paclitaxel and estradiol with human serum albumin. *Protein and peptide letters*, 18(9): 935-951.

Amezqueta, S., Bolioli, A.M., Beltran, J.L. and Rafols, C. 2018. Evaluation of the interactions between human serum albumin (HSA) and warfarin or diflunisal by using molecular fluorescence using two approaches. *ADMET and DMPK*, 6(1): 47-54.

Auwal, M.S., Saka, S., Mairiga, I.A., Sanda, K.A., Shuaibu, A. and Ibrahim, A. 2014. *P*reliminary phytochemical and elemental analysis of aqueous and fractionated pod extracts of *Acacia nilotica* (*Thorn mimosa*).*Veterinary research forum*, 5: 95-100.

Bladt, S. 2009. *Plant Drug* Analysis*: A thin layer chromatography atlas*: Springer Science and Business Media.

Chen, G., Su, L., Feng, S.G., Lu, X., Wang, H. and Pei, Y.H. 2013. Furostanol saponins from the fruits of *Tribulus terrestris*. *Natural product research*, 27(13): 1186-1190.

Cheng, X.X., Lui, Y., Zhou, B., Xiao, X.H. and Liu, Y. 2009. Probing the binding sites and the effect of berbamine on the structure of bovine serum albumin. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 72(5): 922-928.

Corrêa, D.H. and Ramos, C.H. 2009. The use of circular dichroism spectroscopy to study protein folding, form and function. *African Journal of Biochemistry Research,* 3(5): 164-173.

Fu, X.B., Liu, D.D., Lin, Y., Hu, W., Mao, Z.W. and Le, X.Y. 2014. Water-soluble DNA minor groove binders as potential chemotherapeutic agents: synthesis, characterization, DNA binding and cleavage, antioxidation, cytotoxicity and HSA interactions. *Dalton Transactions,* 43(23): 8721-8737.

Guclu-Ustundag, O. and Mazza, G. 2007. Saponins: properties, applications and processing. *Critical Reviews in Food science and nutrition,* 47(3): 231-258.

Han, X.L., Mei, P., Liu, Y., Xiao, Q., Jiang, F.L. and Li, R. 2009. Binding interaction of quinclorac with bovine serum albumin: a biophysical study. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,* 74(3): 781-787.

Hekmat, A., Hajebrahimi, Z. and Motamedzade, A. 2017. Structural Changes of Human Serum Albumin (HSA) in Simulated Microgravity. *Protein and peptide letters,* 24(11): 1030-1039.

Hekmat, A., Saboury, A.A., Divsalar, A. and Seyedarabi, A. 2013. Structural effects of TiO_2 nanoparticles and doxorubicin on DNA and their antiproliferative roles in T47D and MCF7 cells. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents),* 13(6): 932-951.

Hoque, M., Dave, S., Gupta, P. and Saleemuddin, M. 2013. Oleic acid may be the key contributor in the BAMLET-induced erythrocyte hemolysis and tumoricidal action. *PLoS One,* 8(9): e68390.

Klyachenko, O. and Likhanov, A. 2017. Content of Triterpene saponins and phenolics compounds in leaves of in vitro *Beta vulgaris* L. genotypes. *Ukrainian Journal of Ecology, 7*(4): 564-568.

Kostova, I. and Dinchev, D. 2005. Saponins in Tribulus terrestris–chemistry and bioactivity. *Phytochemistry reviews,* 4(2-3): 111-137.

Li, S., Pan, J., Zhang, G., Xu, J. and Gong, D. 2017. Characterization of the groove binding between di-(2-ethylhexyl) phthalate and calf thymus DNA. *International journal of biological macromolecules,* 101: 736-746.

Li, X., Wang, G., Chen, D., and Lu, Y. 2014. Interaction of procyanidin B3 with bovine serum albumin. *Rsc Advances,* 4(14): 7301-7312.

Maity, S., Mukherjee, K., Banerjee, A., Mukherjee, S., Dasgupta, D. and Gupta, S. 2016. Inhibition of porcine pancreatic amylase activity by sulfamethoxazole: structural and functional aspect. *The protein journal,* 35(3): 237-246.

Masters, B.R. 2008. Principles of fluorescence spectroscopy. *Journal of Biomedical Optics,* 13(2): 029901.

Moradi, S.Z., Nowroozi, A., Sadrjavadi, K., Moradi, S., Mansouri, K., Hosseinzadeh, L., *et al*. 2018. Direct evidences for the groove binding of the Clomifene to double stranded DNA. *International journal of biological macromolecules,* 114: 40-53.

Mote, U., Bhattar, S., Patil, S. and Kolekar, G. 2010. Interaction between felodipine and bovine serum albumin: fluorescence quenching study. *Luminescence,* 25(1): 1-8.

Na, N., Zhao, D.Q., Li, H., Jiang, N., Wen, J.Y. and Liu, H.Y. 2015. DNA binding, photonuclease activity and human serum albumin interaction of a water-soluble freebase carboxyl corrole. *Molecules,* 21(1): 54.

Olmstead, M.J. 2002. Organic toothpaste containing saponin: Google Patents.

Purcell, M., Neault, J.F. and Tajmir-Riahi, H. A. 2000. Interaction of taxol with human serum albumin. *Biochim Biophys Acta,* 1478(1): 61-68.

Shi, J.H., Chen, J., Wang, J. and Zhu, Y.Y. 2015. Binding interaction between sorafenib and calf thymus DNA: spectroscopic methodology, viscosity measurement and molecular docking. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,* 136: 443-450.

Song, Y.H., Kim, D.W., Curtis-Long, M.J., Park, C., Son, M., Kim, J.Y., et al. 2016. Cinnamic acid amides from Tribulus terrestris displaying uncompetitive α-glucosidase inhibition. *European journal of medicinal chemistry,* 114: 201-208.

Šprung, M., Soldo, B., Orhanovic, S. and Bucevic-Popović, V. 2017. Substrate-Induced Conformational Changes of the Tyrocidine Synthetase 1 Adenylation Domain Probed by Intrinsic Trp Fluorescence. *The protein journal,* 36(3): 202-211.

Van Dyck, S., Gerbaux, P. and Flammang, P. 2009. Elucidation of molecular diversity and body

distribution of saponins in the sea cucumber Holothuria forskali (Echinodermata) by mass spectrometry. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 152(2): 124-134.

Varlan, A. and Hillebrand, M. 2010. Bovine and human serum albumin interactions with 3-carboxyphenoxathiin studied by fluorescence and circular dichroism spectroscopy. *Molecules*, 15(6): 3905-3919.

Waller, G.R. and Yamasaki, K. 2013. *Saponins used in traditional and modern medicine* (Vol. 404): Springer Science and Business Media.

Wang, Y., Wang, X., Wang, J., Zhao, Y., He, W. and Guo, Z. 2011. Noncovalent interactions between a trinuclear monofunctional platinum complex and human serum albumin. *Inorganic chemistry*, 50(24): 12661-12668.

Wei, Y., Thyparambil, A.A. and Latour, R.A. 2014. Protein helical structure determination using CD spectroscopy for solutions with strong background absorbance from 190 to 230 nm. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1844(12): 2331-2337.

Yang, X., Ye, Z., Yuan, Y., Zheng, Z., Shi, J., Ying, Y., et al. 2013. Insights into the binding of paclitaxel to human serum albumin: multispectroscopic studies. *Luminescence*, 28(3): 427-434.

Yuqin, L., Guirong, Y., Zhen, Y., Caihong, L., Baoxiu, J., Jiao, C., et al. 2014. Investigation of the interaction between patulin and human serum albumin by a spectroscopic method, atomic force microscopy, and molecular modeling. *BioMed research international*, 2014: 734850.

Zhu, W., Du, Y., Meng, H., Dong, Y. and Li, L. 2017. A review of traditional pharmacological uses, phytochemistry, and pharmacological activities of Tribulus terrestris. *Chemistry Central Journal*, 11(1): 60.