Evaluation of the antiglycating potential of thymoquinone and its interaction with BSA

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ABSTRACT
Thymoquinone (TQ) is a bioactive component of medicinal plant, Nigella sativa. It has been identified as promising anti-inflammatory and anti-analgesic properties. In the present study, the TQ has been investigated for physiological interaction as well as binding properties with serum albumin and their thermodynamic parameters at different temperatures. Glycation process was checked with the measurement of fructosamine content, carbonyl content and total advanced glycated end products. The aggregation of amyloid β-structure was measured with Thioflavin-T and the secondary structure of BSA was observed by circular dichroism (CD) in glycated and thermal treated samples. The results indicate that the TQ showed binding interaction (both static and dynamic) with BSA (K_D = 18.31 × 10^7 M^-1 at 293 K) and suppression of glycated products. The glycation-induced and thermal aggregation were prevented and the secondary structure of BSA was maintained. Therefore, these findings suggest that TQ may be used for a therapeutic drug for antiglycation as well as anti-aggregation.

1. Introduction
In today's modern and fast-paced world, new age diseases are also taking shape. Scientists are persistent to develop curative drugs that are more impactful and at the same time less noxious (Jha et al., 2018). However, understanding the underlying mechanism of drug and protein interaction remains elementary and very essential for the development of any therapeutic molecule (Jiao et al., 2018). The drug stability is greatly influenced by the binding of a drug to protein and its affinity for the same (Jiao et al., 2018). This significantly affects the passage of the drug into the target tissue and the compound’s efficacy and toxicity.

The circulatory system of mammals consists of approximately 60% of albumin (Yasseen et al., 2014), which is the major soluble protein constituent as well as has many important physiological roles. They are actively involved in the transportation of diverse metabolites, drugs as well as dyes. It is essential to study interactions between drug and serum albumin, as the administered drugs are first associated with proteins in the serum and then are transported across the body to reach their target sites. This binding also helps in defining adsorption, distribution, metabolism, along with excretion (ADME) properties of drugs for rational drug designing (Yasseen et al., 2014). Bovine serum albumin (BSA) is immensely used as a model plasma protein for these studies mainly because of its structural similarities to Human Serum Albumin (HSA). They are about 76% homologous with each other and the main difference between the two proteins lies in tryptophan amino acid residues (in HSA- Trp-214 and in BSA- Trp-134 and Trp-212) (Togashi & Ryder, 2008). Moreover, BSA has several advantages such as remarkable ligand-binding properties, low cost and ease of availability.

BSA protein entails about 583 amino acid residues and with a molecular mass of 66,400 Da (Jahanban-Esfahlan et al., 2015; Roufegarinejad et al., 2018). These amino acid residues are cross-linked with 17 cysteine residues and are bound in a single chain (Majorek et al., 2012). This amino acid chain shows three domains (I, II and III) that are homologous but structurally discrete (Jalali et al., 2014). BSA also shows binding sites with diverse specificities, the vital ones are described as Sudlow site-I and Sudlow site-II (Jahanban-Esfahlan et al., 2016). Various molecular forces present in the binding sites, number of binding sites, and their affinity towards the ligand (drug) determine the overall association which can be achieved by obtaining the equilibrium constants between ligands and protein (Du et al., 2016).

Thymoquinone (TQ) is a bioactive compound of Nigella sativa, constituting about 30–48% of the total oil components (Figure 1). Nigella sativa shows promising antioxidant, anti-inflammatory, anti-analgesic, anti-bacterial, anti-fungal, anti-allergic and anti-ulcer activities (Lupidi et al., 2010). Studies with human colorectal cancer cells have shown the
anti-tumor properties of TQ (Ahmad et al., 2013). This study aimed to explore the interaction mode of TQ with model protein BSA by fluorescence spectroscopy. The results have been interpreted based on mathematical models and equations leading to evaluate the binding as well as thermo-dynamic parameters. In order to get into the deeper insight of these interactions, CD spectroscopy and Molecular docking was performed. Glycation of proteins especially is another very significant interaction model in the cellular system wherein sugars interact with proteins and lead to generation of harmful products. The glycated products production has been highly increased and involvement of these products noticed in the diabetes and neurodegenerative disorders like Alzheimer’s, Parkinson’s etc. (Miranda et al., 2016).

Researchers have been trying to design a drug to prevent the formation of glycation products and safeguard the cells from deleterious effects of glycation mediated processes like aggregation and free radical generation. Therefore, studies were also designed to study the role of TQ in this process of glycation and some established parameters like fructosamine content, carbonyl content, total advanced glycated end (AGEs), aggregation and structural alterations were evaluated. The thermal aggregation was carried out at 60 °C and analyzed with Thioflavin-T (ThT). These results indicate towards a static as well as dynamic interaction between TQ and BSA. The glycation products were also significantly reduced in the presence of TQ.

2. Materials and methods

2.1. Materials

BSA and TQ were procured from Sigma-Aldrich. All other reagents used were of analytical grade.

2.2. Fluorescence quenching studies

The fluorescence emission spectra of TQ were recorded at a range of 300–500 nm with a fixed excitation wavelength at 280 nm. Fluorescence titrations of BSA with constant concentration (0.3 mg/mL) were carried out by varying the TQ concentrations (0–50 μM) at marked temperatures. All fluorescence emission spectra were recorded on Agilent Cary Eclipse spectrophotometer (Model No.-G-9800AA, Agilent Technologies, Victoria, Australia) equipped with Xenon lamp and path length of 1 cm. Fluorescence emission spectra were recorded at four different temperatures, 293, 298, 303 and 310 K and the temperature was maintained with Cary single cell Peltier accessory (Type-SPVF-1 × 0, Agilent Technologies, Victoria, Australia). Slit width for excitation as well as emission was fixed at 5 nm (Kumar et al., 2020).

The influence of the inner filter effect was eliminated by correcting the steady-state fluorescence spectra with the following formula for Stern–Volmer plots (Shi et al., 2017)

$$F_{cor} = \frac{F_{obs}}{10(A_{ex}A_{em})/2}$$

where, \(F_{obs}\) and \(F_{cor}\) are the measured and corrected fluorescence intensity, respectively. \(A_{ex}\) and \(A_{em}\) are the optical density at \(\lambda_{ex}\) and \(\lambda_{em}\), respectively.

2.3. Glycation sample preparation

BSA (10 mg/mL) and glucose (100 mg/mL) and/or TQ (10 μM and 20 μM) were incubated along with phosphate buffer (100 mM, pH 7.4) and sodium azide (NaN₃) (3 mM) for 28 days at 37 °C. All samples containing proteins were dialyzed overnight in 10 mM phosphate buffer and stored at –20 °C for further analysis (Kumar et al., 2020).

2.4. Thermal aggregation sample preparation

BSA and glucose (10 mg each) were incubated along with the TQ (10 μM and 20 μM) for 3 and 6 h at 60 °C. These samples were collected in aliquots at 3 and 6 h for analyzing aggregation and structural modification further (Rondeau et al., 2010).

2.5. Measurement of fructosamine and carbonyl content

The incubated samples have been used to check fructosamine content by Nitroblue terazolium (NBT) method at 530 nm, and carbonyl content by the 2,4-Dinitrophenylhydrazine (DNPH) method at 370 nm according to our previous study (Kumar et al., 2020).

2.6. Determination of total AGEs

The generation of AGEs was checked with a quantitative method (Gutierrez, 2012). Glucose mediated glycated samples were excited at 370 nm and recorded emission between 400 and 600 nm. The % inhibition was calculated as below.

![Figure 1. Molecular structure of Thymoquinone (TQ). (PubChem CID 10281).](image-url)
Total AGES inhibition (%) = \[ \left[ 1 - \left( \frac{F.I._{Sample}}{F.I._{Control}} \right) \right] \times 100. \]

F.I. Sample and F.I. Control represented the fluorescence intensity of sample and control, respectively.

2.7. Aggregation of amyloid β-structures by ThT assay

The protein aggregation has been observed with ThT assay fluorometrically (Khan et al., 2020; Shamsi et al., 2020). The amyloid cross β-structure aggregation was checked in glucose-mediated glycation samples as well as thermally treated samples in the presence/absence of TQ. The samples were mixed with 20 µM ThT at room temperature for 30 min. These reaction mixtures were scanned from 450 to 600 nm with excitation at 440 nm. The % inhibition of aggregation was calculated as below:

Aggregation inhibition (%) = \[ \left[ 1 - \left( \frac{F.I._{Sample}}{F.I._{Control}} \right) \right] \times 100. \]

F.I. Sample and F.I. Control represented the fluorescence intensity of sample and control, respectively.

2.8. Structural analysis by circular dichroism

The CD spectra in the far UV region (180–260 nm) were generated with the Jasco-815 CD spectrometer (Easton, Northampton, USA) at 25 °C. To check the structural stability of TQ-BSA complex, glycated samples and thermally treated samples with or without TQ, the CD study was carried out with a 1 cm path length cell. The spectrometer was purged with nitrogen gas prior to and throughout the experiment. The plots were scanned at 100 nm/min. The BSA concentration was kept fix at 0.3 mM. The molar ellipticity \( \theta \) was calculated from the observed ellipticity \( \theta \) and plotted against the corresponding wavelength (Kelly & Price, 2000).

2.9. Molecular docking

The crystal structure of BSA with PDB id 4OR0 (Bujacz et al., 2014) was downloaded from Protein Data Bank (Berman et al., 2000). Furthermore, ligands like TQ and glucose were obtained from the crystal structures of 4HCO (Yin et al., 2013) and 2B3B (Cuneo et al., 2006), respectively. The active site for the binding of TQ on BSA was identified based on the PDBSum Ligplot report of 6QS9 (BSA in complex with ketoprofen) (Castagna et al., 2019). Hex 8.00 (Ritchie & Kemp, 1999) was considered for docking of BSA with TQ. For correlation type, and Post-processing we opted for ‘Shape + Electro + DARS’ and for ‘DARS Minimization’, respectively. Standard docking with a grid dimension of 0.6 Å and solution of 2000 was preferred. After docking, top ten listed docked poses were considered for amino acid interaction analysis using Discovery Studio standalone software (BIOVIA, 2019). Each docked pose was evaluated for their binding site based on the reference crystal structure of 6QS9. The BSA-TQ pose which harmonized with the reference structure was considered for hydrogen bond analysis.

2.10. Statistical analysis

Four distinct sets of experiments were performed and evaluated with one way ANOVA test for statistical significance. The standard error was represented and p-value < 0.05 was employed for the significance of probability of occurrence.

3. Results

3.1. Fluorescence quenching studies

BSA shows intrinsic fluorescence properties and emits intensely upon excitation. By monitoring intrinsic fluorescence quenching, we can evaluate the interaction between TQ and BSA. The effect of TQ on the fluorescence intensity of BSA has been shown in Figure 2. It was observed that the fluorescence intensity of BSA quenched with the increasing concentration of TQ and a slight blue shift was observed, from these data it can be said that TQ can interact with BSA.

To determine the type of quenching mechanism, we analyzed the obtained fluorescence data at different temperatures (293, 298, 303 and 313 K) with a well-known mathematical Stern–Volmer equation (Bhogale et al., 2013; Jalali et al., 2014).

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

where, \( F_0 \) is the Relative fluorescence intensity without a quencher; \( F \) is the Relative fluorescence intensity with a quencher; \([Q]\) is the Concentration of quencher; \( K_{SV} \) is the Stern–Volmer quenching constant; \( k_q \) is the Biomolecular quenching rate constant; \( \tau_0 \) is the Average lifetime of the fluorophore in the excited state usually for a biomacromolecule.

The Stern–Volmer plot of \( F_0/F \) vs. [TQ] is presented in Figure 3. \( K_{SV} \) value determined by the Stern–Volmer equation with linear regression of plot \( F_0/F \) versus concentration of TQ. The corresponding values of parameters \( K_{SV} \) and \( k_q \) were shown in Table 1. The values of \( K_{SV} \) and \( k_q \) decreased with the increasing temperature.

The quenching process is further confirmed by the value of \( K_{SV} \) which was estimated using the following equation...
where $\tau_0$ is the average lifetime of the molecule without a quencher. The maximum scatter collision quenching constant is $2.0 \times 10^{16} \text{ M}^{-1} \text{s}^{-1}$, apparently, in this case, the value of $K_q$ has been found to be in the range of $10^{16} \text{ M}^{-1} \text{s}^{-1}$ (Table 1) which is much greater than the maximum scatter collision value (Bhogale et al., 2013).

On applying the modified form of Stern–Volmer equation,

$$\frac{F_0}{F} = \frac{1}{f_a} + \frac{1}{f_a K_a [Q]}$$

where $K_a$ and $f_a$ represent the effective quenching constant and fraction of accessible fluorophores, respectively (Jahanban-Esfahlan et al., 2015). The value of $K_a$ has shown the decreasing trend against increasing temperature was supported the $K_{sv}$ dependency on temperature (Table 1, Supplementary material, Figure S1).

There was always an equilibrium condition for binding interaction of protein (P) and drug (D) as follow:

$$P + nD \rightleftharpoons P - nD.$$  

The binding stoichiometry (n) and binding constant can be evaluated by following another modified Stern–Volmer equation (Kou et al., 2021):

$$\log \frac{F_0 - F}{F} = \log [K_b] + n \log \left\{ \frac{[D]_0 - n[P]_0 (F_0 - F)}{F_0} \right\}$$

where, $[D]_0$ and $[P]_0$ are the total concentration of drug (ligand) and total concentration of protein, respectively. A plot of $\log(F_0 - F/F)\text{ vs. } \log([D]_0 - n[P]_0 (F_0 - F)/F_0)$ (Figure 4) at different temperatures gave a straight line, in that slope was equivalent to binding stoichiometry (n) and the y-intercept corresponds to binding constant ($K_b$), respectively.

The $K_b$ was calculated and it was found to be $1.83 \times 10^7 \text{ M}^{-1}$ at 293 K (Table 2) and there was only one binding stoichiometry. The obtained values were then further evaluated using thermodynamic and van’t Hoff equations to understand thermodynamics and the binding forces of TQ-BSA interaction;

$$\log K_b = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}$$

where, $R$ is the Gas Constant; $T$ is the Temperature in Kelvin; $\Delta H$ is the Change in Enthalpy; $\Delta S$ is the Change in Entropy.

Other thermodynamic parameters were obtained from the following equation,

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

Figure 5 showed the plot of $\log K_b$ versus $1/T$ from the van’t Hoff equation provided a straight line, the value of $\Delta S$ and $\Delta H$ were obtained from the slope and the intercept respectively. The calculated values of thermodynamic parameters ($\Delta H$, $\Delta S$ and $\Delta G$) were represented in Table 3, and which is found to be negative.

### 3.2. Measurement of fructosamine content

The presence of TQ (10 μM and 20 μM) reduced the fructosamine content to 10.60 μmol/mg protein and 8.34 μmol/mg protein on increasing TQ concentration manner as compared to glycated BSA (19.34 μmol/mg protein) at day 28 (Figure 6). The reduction in fructosamine content suggested the initial hindrance of the glycation process in presence of TQ significantly.
3.3. Measurement of carbonyl content

The glucose-mediated glycation of BSA generated 7.69 μmol/mg protein of carbonyl content at day 28. But it was decreased to 4.73 and 3.84 μmol/mg protein on the addition of TQ concentration on 10 μM and 20 μM to glycation system at day 28 (Figure 7). The reduced carbonyl content on the addition of TQ indicated suppression of Amadori products as well as AGEs formation.

3.4. Determination of total AGEs

The generated glycation products were measured cumulatively with the fluorometric spectrum. The spectrum of total AGEs was clearly indicated the presence of TQ suppressed their formation with emission peaked 446 nm at day 28 (Figure 7). A reduction of total AGEs formation to 33.91% and 47.20% in the presence of 10 μM and 20 μM TQ at 28 days incubation respectively was observed in comparison of glycated BSA as control (Figure 8).

3.5. Determination of aggregation of amyloid β-structures by ThT

The ThT study of glycated samples resulted in the reduced β-amyloid aggregates in the presence of 10 μM and 20 μM TQ to 33.62% and 43.50%, respectively, as compared to glycated BSA at 28 days. The BSA with TQ (10 μM and 20 μM) has shown an equivalent spectrum position as native BSA (Figure 9(a)). Similarly, the thermally treated samples have observed the reduction in β-amyloid aggregates on the addition of TQ at 6 h incubation. The β-amyloid aggregates have reduced to 21.41% by 10 μM TQ and 35.37% by 20 μM TQ at 60 °C (Figure 9(b)). The BSA only treated with both concentration

| Temperature (K) | ΔH (kJ mol⁻¹) | ΔS (JK⁻¹ mol⁻¹) | ΔG (kJ mol⁻¹) |
|-----------------|---------------|-----------------|---------------|
| 293             | −14.58        | −29.05          | −6.07         |
| 298             | −14.58        | −29.05          | −5.92         |
| 303             | −14.58        | −29.05          | −5.78         |
| 310             | −14.58        | −29.05          | −5.57         |
of TQ showed very reduced aggregates of β-amyloid. However, glucose treated BSA showed more aggregation than native BSA. It has indicated that TQ reduced the β-amyloid aggregates in the glycation process as well as thermal aggregation.

3.6. Structural analysis by circular dichroism

The complex of TQ-BSA was analyzed for the secondary structure of the protein. The presence of TQ (10 μM and 20 μM) caused no alteration in the secondary structure of native BSA (Figure 10(a)). TQ maintained the native structure of BSA significantly.

The CD spectrum of the Far-UV range has indicated that glycated BSA showed alteration in the secondary structure of BSA (Figure 10(b)). However, TQ maintained the structure of BSA as compared to native BSA. Similarly, thermally treated samples have also maintained the α-helical structure of BSA in the presence of TQ (10 μM and 20 μM) (Figure 10(c)). However, the glucose treated BSA in thermal condition has been found with modification in secondary structure.

3.7. Molecular docking

TQ interacts with BSA with a binding affinity of −194.34 kcal/mol. There are five hydrophobic interactions like Trp213, Leu237, Lue259, Ile289 and Ala290 observed between the ligand and the protein. Furthermore, three hydrogen bonds viz. Tyr149, His241 and Arg256 were observed between TQ and BSA (Figure 11). These docking results were found in
accordance with the interaction results showing the hydrogen bonding between BSA-ketoprofen complex (Castagna et al., 2019).

4. Discussion

The structure of BSA contains amino acid residues like tryptophan, tyrosine and phenylalanine which are accountable for these fluorescent properties (Hao et al., 2017). However, the fluorescence of BSA is majorly dominated by tryptophan (Trp) residue which is extremely sensitive to the surrounding environment. Any alterations in the fluorescence spectra of BSA can be due to various reasons like ground state complex formation, molecular interactions, molecular rearrangements, or energy transfer (Bhogale et al., 2013). TQ prefers to interact with the drug site 1 similar to that of ketoprofen, naproxen27 or 3,5-diiodosalicylic acid. Thus TQ is well fitted within the hydrophobic pocket with residues like Trp213, Leu259, Leu237, Ile289 and Ala290 (Castagna et al., 2019).

A fluorescence quenching experiment was executed at four marked temperatures (293, 298, 303 and 310 K) to understand the mechanism and thermodynamics of binding between TQ and BSA. There are two types of the mechanism by which quenching can occur by dynamic and/or static quenching. In dynamic quenching, the quencher and fluorophore interact for a brief amount of time. In contrast, in static quenching, a stable non-fluorescent complex is formed between quencher and fluorophore. This quenching behaviour can be easily differentiated by studying the fluorescence quenching at marked temperatures. An increase in the temperature causes a faster rate of diffusion that is molecules tend to collide more resulting in an interaction between for a brief amount of time, with the increasing temperature the bimolecular quenching constants are anticipated to increase. Thereby, providing higher probabilities for dynamic quenching. However, in the case of static quenching, increased temperatures can dwindle the stability of complexes thereby leading to low values of bimolecular quenching constants (Jiao et al., 2018). Obtained $K_{SV}$, $K_q$ and $K_a$ values (Table 1) designate that the probable quenching mechanism of BSA by TQ is static quenching and potentially there is the formation of a non-fluorescent complex of TQ with BSA (Cheng et al., 2013; Kou et al., 2021).

Considering the $K_q$ values (Table 2), it is confirmed that the interaction between TQ and BSA appears through a static quenching process by orderly interaction and not due to the collision effect. The decrease in values of $K_q$ and $n$ with a rise in temperature suggests unstable complex formation that may degrade at higher temperatures.

There are three models of interaction between drug and biomolecules (Alam et al., 2018):

1. Hydrophobic forces, $\Delta H > 0$ and $\Delta S > 0$;
2. Van der Waals interactions and hydrogen bonds, $\Delta H < 0$ and $\Delta S < 0$;
3. Electrostatic interactions, $\Delta H < 0$ and $\Delta S > 0$

According to the data obtained in our case, the negative $\Delta H$ and $\Delta S$ values suggested that there were van der Waals interactions and hydrogen bonds mainly involved in the
stabilization of the TQ-BSA complex. Besides, the negative values of ΔG suggested that the interaction of BSA and TQ was a spontaneous process.

In the early stage of the glycation process, glycated products such as fructosamines, also known as Amadori products, are generated. The Amadori products get converted to late-stage glycated products or AGEs via various metabolic and physiological processes. The carbonyl content provides insight into the extent of late-stage product generation in glycation (Khan et al., 2013). Anwar et al. (2014) have been also reported that TQ has suppressed the formation of AGEs by glucose and MG induced glycation in superoxide dismutase (SOD). Losso et al. (2011) have been found that TQ reduces the Amadori products and AGEs generation in Hb-glucose glycation. The represented result has supported the anti-AGEs formation properties of TQ. In another report Kumar and Ali (2019) have suggested the role of thymoquinone in the inhibition of total AGEs.

The aggregation of protein may get accumulated in the tissues due to interaction with drugs, metabolic reaction, and thermal denaturation. These aggregates of β-amyloid have been noticed in metabolic and neurodegenerative disorders. TQ has been observed to have reduced aggregates formation in glucose/MG-mediated glycation in SOD (Anwar et al., 2014; Khan et al., 2014).

The glucose-mediated protein glycation leads to the alteration in the native structure of the protein which may affect the active sites leading to functional loss of protein. The BSA exhibits the two strong negative bands at 208 nm and 222 nm which provide the α-helical characteristics of proteins (Satish et al., 2017). The maintenance of these bands resulted in the treatment of TQ in binding interaction, glycation process as well as the thermal process as native protein, but not as glycated protein.

5. Conclusion

TQ has been studied well for its biological benefits as anti-inflammatory, anti-analgesic and other activities. This study showed fine results of binding interaction of TQ-BSA, thermodynamic parameters, glycation process, and thermal aggregation. The mixed quenching (dynamic and static) behaviour of TQ with BSA for binding interaction was noticed with established plots and equations. Thermodynamic parameters (ΔH, ΔS, and ΔG) suggested van der Waals interactions, hydrogen bond and spontaneity of TQ-BSA complex formation. The AGes formation was suppressed in the presence of TQ in glucose-mediated glycation. The glycation and thermally induced aggregation of the β-amyloid structure were reversed and the native structure of protein was maintained with TQ treatment. Hydrogen bonding was also supported in binding interaction by molecular docking. These observed findings indicate that thymoquinone may play a promising role as drug for antiglycation and antiaggregating purposes.

Disclosure of statement

We declare that we have no conflict of interest.

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