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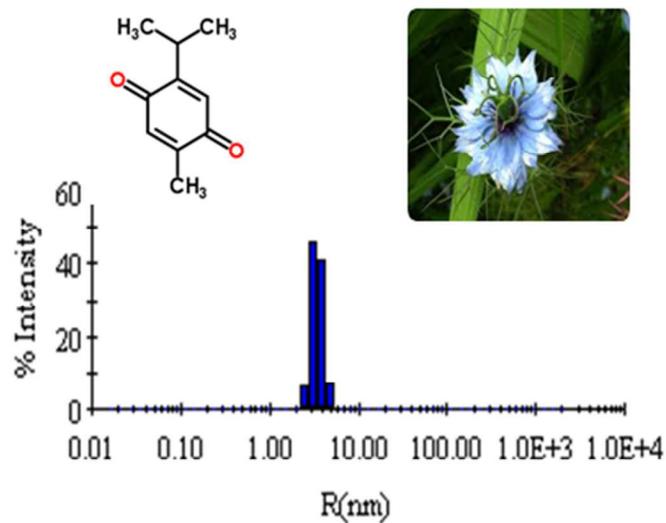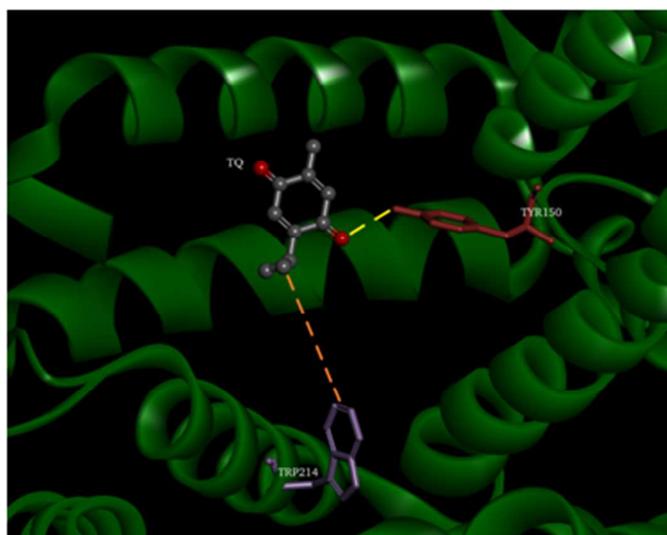
Research Article

Biophysical investigation of thymoquinone binding to 'N' and 'B' isoform of human serum albumin and explore the interaction mechanism and radical scavenging activity

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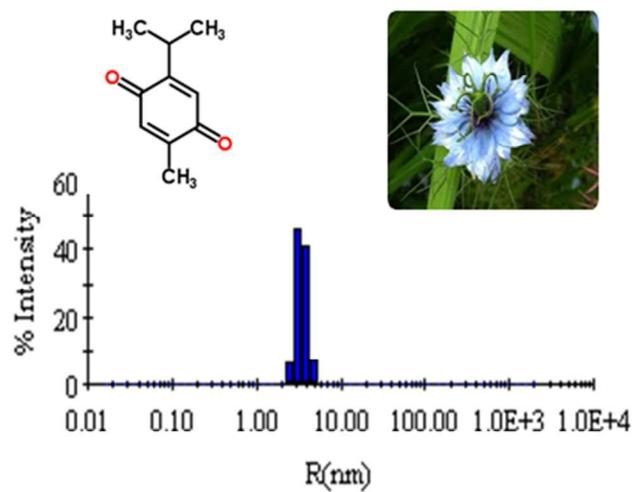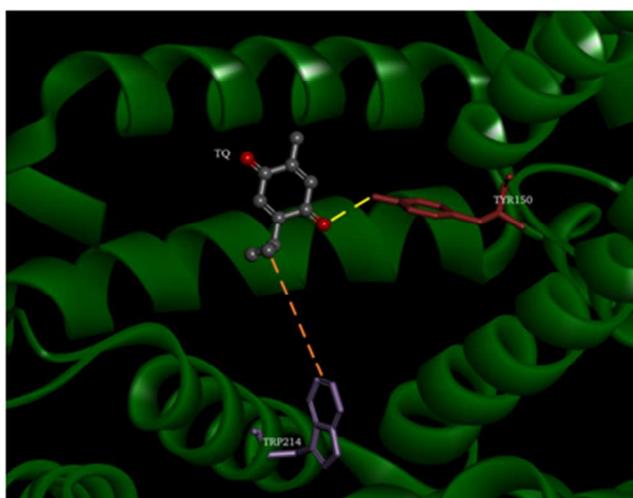
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16   Thymoquinone more strongly interact with the 'N' isoform in comparison to 'B' isoform of HSA

17   and also increase its thermal stability but the antioxidant activity is significantly higher at the 'B'

18   isoform of HSA.

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<center>2</center>

1 **Abstract**

2 Thymoquinone (TQ) is the main constituent of *Nigella sativa* and is traditionally used

3 as folk medicine. Our aim was to investigate the binding mechanism of TQ to human

4 serum albumin (HSA) isoforms ('N' form at pH 7.4 and 'B' form at pH 9.0) using

5 biophysical methods such as intrinsic tryptophan fluorescence quenching, isothermal

6 titration calorimetry (ITC), circular dichroism (CD), dynamic light scattering (DLS),

7 Förster resonance energy transfer (FRET) and antioxidant activity in the absence and

8 presence of TQ. We have calculated the binding and thermodynamic parameters from

9 spectroscopic and calorimetric methods. CD and DLS were respectively used to

10 monitor the changes in the secondary structure and hydrodynamic radii of HSA as a

11 result of its interaction with TQ. The esterase and antioxidant or radical scavenging

12 activities of both the isoforms of HSA were investigated in the absence/presence of TQ.

13 The antioxidant activity of TQ was remarkably enhanced upon its interaction with

14 HSA.. Therefore, the efficiency of HSA to scavenge the free radical ions was increase

15 in the presence of TQ which are generated in the body by various metabolic processes.

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20 **Abbreviations:** HSA: Human serum albumin; TQ: Thymoquinone; ABTS: 2,2'-Azino-

21 bis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt; DLS: Dynamic light

22 scattering; FRET: Förster resonance energy transfer; BHA: Butylated hydroxyanisole;

23 MRE: Mean residual ellipticity.

Keywords: Antioxidant activity; Esterase activity; Fluorescence spectroscopy; Human serum albumin; Radical scavenging activity; Thymoquinone; Isothermal titration calorimetry.

Introduction

Thymoquinone (TQ), a main constituent of *Nigella sativa* (*Ranunculaceae*) essential oil, has been traditionally employed in folk medicine and is now recognized as a herbal remedy by a number of pharmacopoeias [1]. It is an annual plant that grows in the mediterranean area of India and Pakistan. The biochemical activities of *N. sativa* has been ascribed to quinones specifically TQ or 2-isopropyl-5-methyl-1, 4-benzoquinone [2]. TQ shows antimicrobial [3], anti-inflammatory [4], neuroprotective [5], antidiabetic, anticancerous [6], antihypertensive, and mast cell stabilizing effects [7], in addition to a protective role against *in vitro* induced ischemia [8, 9]. Moreover, the therapeutic potential of TQ has been confirmed in cancer research also [10-15]. TQ has a promising role as antineoplastic growth inhibitor against various tumor cell lines [16, 17]. It has protective effects against rheumatoid arthritis [18], induces telomere shortening, DNA damage, apoptosis in human glioblastoma cells [19] and triple-negative breast cancer (TNBC) cells [20]. TQ also shows anti-oxidative properties against oxidative damage induced by a variety of free radical generating agents (including carbon tetrachloride, *cis*-platin, doxorubicin and recently HIV-1 protease inhibitor) [2, 21], analgesic and anti-inflammatory role against renal injury [22]. Because of its immense biological importance, there is an escalating interest to test it in pre-clinical and clinical researches for assessing its health benefits.

1   Human serum albumin (HSA) is a highly abundant serum protein that comprises 50-

2   60% of the total plasma protein in humans [23]. Albumin is responsible for the transport,

3   storage and metabolism of many therapeutic drugs in the blood stream thereby

4   restricting their free, active concentrations and therefore can significantly affect their

5   pharmacokinetics [24]. There are four pH dependent isoforms of HSA that have been

6   characterized in the past. At physiological pH 7.4, HSA assumes the normal form (N)

7   which changes to fast migrating form (F) at pH <4.3 and at pH <2.7 it changes to the

8   fully extended form (E). Whereas on the basic side at pH >8 the N form changes to

9   basic form (B) [25]. Polyphenols interact with HSA through its binding sites at different

10   domains. HSA has two primary binding sites for various ligands commonly referred as

11   binding site I and II which are located in subdomain IIA and IIIA, respectively [10]. HSA

12   binds a variety of molecules, a property that can have profound effects on their

13   pharmacokinetics and pharmacodynamics [26]. Binding of polyphenols to albumin alters

14   the pattern and volume of distribution, lowers the rate of clearance, and increases the

15   plasma half-life of the drug.

16   The work presented here was focused on dissecting the spectroscopic and

17   thermodynamic basis of HSA-polyphenols interactions investigating the mode and

18   forces responsible for binding. Specifically, the aim of this study was to explore the

19   binding of polyphenols to HSA under normal as well as alkaline conditions. Trp

20   fluorescence quenching was monitored at different temperatures to elucidate the

21   mechanism of TQ binding to HSA. CD and DLS were used to study the effect of TQ

22   binding on the overall conformation of HSA, while ITC was used to determine the

23   thermodynamic of TQ-HSA interaction. We have also monitored the esterase activity

5

1   and radical scavenging activity of HSA in the presence of TQ. Antioxidant property of

2   TQ plays an important role in various types of diseases and it also dependent on

3   condition such as alkalosis where it's antioxidant activity increases and esterase activity

4   reduces. Therefore, the binding and thermodynamic studies of TQ-HSA interaction

5   shall provide useful information on the structural features that determine the therapeutic

6   efficacy of TQ.

7   **Materials and methods**

8   **Materials**

9   Fatty acid free human serum albumin (A1887), Thymoquinone (274666), Glycine

10   (G8898), *p*-nitrophenyl   acetate,   4-*p*-NPA   (N8130),   2,2'-Azino-bis   (3-ethyl

11   benzothiazoline-6-sulfonic   acid)   diammonium   salt,   ABTS   (A1888),   potassium

12   peroxodisulfate, $K_2S_2O_8$ (P5592), Trolox (238813) were purchased from Sigma Aldrich

13   and MOPS (134894) buffer was purchased from SRL.

14   **Sample preparation**

15   A stock solution of HSA was made in 20 mM MOPS pH 7.4 and glycine-NaOH pH 9.0

16   buffers and the protein concentrations was determined spectrophotometrically using

17   $E_{280\ nm}^{1\%}$ of 5.30 at 280 nm [7, 8] on a Perkin-Elmer Lambda 25 spectrophotometer.

18   Buffers used throughout the experiments were filtered by 0.45 μm Millipore Millex-HV

19   PVDF filter and pH was measured using Mettler Toledo (model S20) pH meter. The

20   stock solution of TQ (5mM) was prepared in 10% ethanol and finally adjusted to 1.0 ml

21   by diluting with respective buffers.

22   **UV-visible absorption measurements**

1   UV-visible spectra were recorded between 250 and 350 nm on Perkin-Elmer Lambda

2   25 double beam spectrophotometer attached with Peltier temperature programmer

3   (PTP-1) to maintain temperature at 37 °C throughout the experiments. HSA (6 µM) was

4   titrated by 0-30 µM TQ in a 1 cm path length cuvette of 3 ml. All HSA-TQ absorbance

5   spectra were corrected with respective blank, which consist same concentration of TQ

6   in buffer in the absence of HSA.

7   **Fluorescence quenching measurements**

8   All the fluorescence measurements were carried out on Schimadzu (RF-5301PC)

9   fluorescence spectrophotometer equipped with a constant temperature holder attached

10   with Neslab RTE-110 water bath with an accuracy of ±0.1 °C. Intrinsic fluorescence

11   was measured by exciting HSA (2 µM) at 295 nm and the emission spectrum was

12   measured in the range of 300-450 nm, because tryptophan fluorescence is used as a

13   probe of local environment in a protein for determination of protein structure, dynamics

14   as well as ligand binding. The decrease in fluorescence intensity at particular

15   wavelength was analyzed according to the Stern-Volmer equation 1 [8]:

16
$$\frac{F_o}{F} = K_{sv}\,[Q]+1$$

17   (1)

18   where F_o and F were the fluorescence intensities in the absence and presence of

19   quencher (TQ), K_{sv} is the Stern-Volmer quenching constant. Binding constants and

20   binding stoichiometry were obtained from equation 2 [27]:

21
$$K_{sv} = k_q.\tau_o$$

                                                                                                           (2)

7

1   where k_q is the bimolecular rate constant of the quenching reaction and τ_o is the average

2   integral fluorescence life time of tryptophan which is $\sim 5.78 \times 10^{-9}$ s [28].

3 $$\log\left(\frac{F_o}{F} - 1\right) = \log K_b + n \log[Q] \qquad (3)$$

4   where K_b is the binding constant and n is binding stoichiometry.

5   The thermodynamic parameters i.e. change in enthalpy (ΔH^o) and change in entropy

6   (ΔS^o) were determined after measuring K_b at different temperatures and the results were

7   analyzed according to van't Hoff equation 4:

8 $$\ln K_b = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \qquad (4)$$

9   where R is universal gas constant (1.987 cal mol^{-1} K^{-1}).

10   The change in Gibbs free energy (ΔG^o) can be further determined from separate terms

11   of enthalpy change (ΔH^o) and entropy change (ΔS^o) according to the equation 5:

12 $$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \qquad (5)$$

13   The three-dimensional fluorescence measurement were performed on Hitachi F-4500

14   spectrofluorometer under the following condition: the emission wavelength was

15   recorded between 200 and 600 nm, the initial excitation wavelength was set to 200 nm

16   with increment of 5 nm, the excitation and emission slit widths were fixed at 10 nm [29].

17   **Isothermal titration calorimetric measurements (ITC)**

18   The energetics of the binding of TQ to HSA at 37 °C was measured by using a VP-ITC

19   titration microcalorimeter (MicroCal Inc., Northampton, MA). Prior to begin the

20   titration experiment, all samples were degassed on a thermovac. The sample cell loaded

21   with 20 μM HSA dissolved in pH 7.4 and pH 9.0 and reference cell filled with the

22   respective buffer. Multiple injections of 10 μL of TQ solution (3.0 mM) were made into

1   the sample cell containing serum albumin. Each injection was made over 20 s with an

2   interval of 180 s between successive injections. The reference power and stirring speed

3   were set at 16 μcal s^{-1} and 307 rpm, respectively. Heats of dilution for the ligands were

4   determined by the control experiments, and these were subtracted from the integrated

5   data before curve fitting. The first derivative of temperature dependence of enthalpy

6   change is used for the calculation of experimental heat capacity change calculated from

7   equation 6 [30]:

8
$$\Delta C_P^{exp} = \frac{d\Delta H}{dT} \tag{6}$$

9   Temperature dependent van't Hoff enthalpy (ΔH_{vH}) at particular temperature is

10   calculated by the equation:

11
$$\Delta H_{vH} = \left[\frac{\left\{ \ln\frac{K(T_2)}{K(T_1)} - \frac{\Delta C_P}{R}\ln\frac{T_2}{T_1} + \frac{\Delta C_P T_1}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \right\} \times R}{\left(\frac{1}{T_1} - \frac{1}{T_2}\right)} \right] \tag{7}$$

12   where, T_1 and T_2 are the maximum and minimum experimental temperature, $K(T_1)$ and

13   $K(T_2)$ are the values of binding constant at respective temperatures.

14   **Circular dichroism (CD) measurements**

15   CD measurements were carried out with a Jasco spectropolarimeter (J-815) attached

16   with a Peltier-type temperature controller. The instrument was calibrated with D-10-

17   camphorsulfonic acid. All The CD measurements were carried at pH 7.4 and pH 9.0 at

18   physiological temperature 37 °C. Spectra were collected with a scan speed of 50

19   nm/min, data pitch 0.1 nm and a response time of 2 s. Each spectrum was the average

20   of 2 scans. Far-UV CD spectra (190-250 nm) were taken at TQ concentrations from 0-

1   50 µM with protein concentrations of 2 µM and 0.1cm path length cells. The results

2   were expressed as mean residue ellipticity (MRE) in degree cm^2 $dmol^{-1}$, which is

3   defined as:

$$\text{MRE} = \frac{\theta_{obs}\ (m\ \text{deg})}{10 \times n \times C \times l} \tag{8}$$

5   where θ_{obs} is the observed ellipticity in degrees, C is the molar concentration of HSA, n

6   is the number of amino acid residues (585-1= 584) and l is the pathlength of cuvette in

7   centimeter. Helical content of HSA was calculated from the MRE values at 222 nm

8   using the following equation as described by Chen $et\ al.$ [31]:

$$\%\alpha\text{-helix} = \left(\frac{\text{MRE}_{222nm} - 2{,}340}{30{,}300}\right) \times 100 \tag{9}$$

10   The thermal denaturation experiments were carried between 25-90 °C with 1 °C min^{-1}

11   temperature slope probed by far-UV CD at 222 nm. The curves were normalized,

12   assuming a linear temperature dependence of the base lines of native and denatured

13   states.

14   **Data analysis of thermal denaturation**

15   Thermal denaturation data from CD spectroscopy were analyzed on the basis of two-

16   state unfolding model. For a single step unfolding process, N⇌U, where N is the native

17   state and U is the unfolded state, the equilibrium constant K_u is:

$$K_u = \frac{f_u}{f_n} \tag{10}$$

19   with f_u and f_n being the molar fraction of U and N, respectively.

$$f_d = \frac{(Y_{obs} - Y_n)}{(Y_u - Y_n)} \tag{11}$$

10

1 where Y_{obs}, Y_n and Y_u represent the observed property, the property of the native state,

2 and the property of unfolded state, respectively.

Dynamic light scattering measurements

4 DLS measurements were carried out at 830 nm by using DynaPro-TC-04 dynamic light

5 scattering equipment (Protein Solutions, Wyatt Technology, Santa Barbara, CA)

6 equipped with a temperature-controlled micro sampler. HSA (30 µM) was incubated

7 with the different concentration of TQ for 8 h, before scanning the samples were spun

8 at 10,000 rpm for 10 min and were filtered serially through 0.22 and 0.02 µm Whatman

9 syringe filters directly into a 12 µl quartz cuvette. For each experiment, 20

10 measurements were taken. Mean hydrodynamic radius (R_h) and polydispersity were

11 analyzed using Dynamics 6.10.0.10 software at optimized resolution. The R_h was

12 estimated on the basis of an autocorrelation analysis of scattered light intensity data

13 based on translation diffusion coefficient by Stoke's-Einstein relationship-

$$R_h = \frac{kT}{6\pi\eta D} \qquad (12)$$

15 where R_h is the hydrodynamic radius, k is Boltzmann constant, T is the absolute

16 temperature, η is the viscosity of water and D is the diffusion coefficient [32].

Tryptophan fluorescence resonance energy transfer (FRET) to TQ

18 The fluorescence spectra of HSA (2 µM) and absorption spectra of TQ (2 µM) between

19 300 to 400 nm were scanned in similar way as given in method sections 'Fluorescence

20 quenching' and 'UV-visible' experiments at 37 ºC. If the emission spectrum of donor

21 (Trp214) significantly overlaps with the absorption spectrum of acceptor (TQ), these

22 donor-acceptor pairs will be considered in Förster distance and then we could ascertain

23 the possibility of energy transfer [33]. Therefore, the degree of energy transfer depends

11

1   upon the area of overlap and the distance between these donor-acceptor molecules. The

2   efficiency of energy transfer (E) is calculated using the following equation [34]:

3
$$E_{FRET} = \left(1 - \frac{F}{F_o}\right) = \frac{R_o^6}{R_o^6 + r^6}$$
(13)

4   where F_o and F were the fluorescence intensities of HSA in absence and presence of TQ

5   respectively; r is the distance between donor and acceptor and R_o is the critical distance

6   at which transfer efficiency equals to 50% which can be calculated from the following

7   equation:

8
$$R_o^6 = 8.79 \times 10^{-25} K^2 n^{-4} \varphi J$$
(14)

9   where K^2 is the orientation factor related to the geometry of the donor and acceptor of

10   dipoles, n is the refractive index of the medium, φ is the fluorescence quantum yield of

11   the donor in absence of acceptor; and J expresses the degree of spectral overlap

12   between the donor emission and the acceptor absorption which can be evaluated by

13   integrating the overlap spectral area in between 300 to 400 nm from following

14   equation:

15
$$J = \frac{\int_o^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_o^\infty F(\lambda)d\lambda}$$
(15)

16   where $F(\lambda)$ is the fluorescence intensity of the donor at wavelength range λ which is

17   dimensionless, and $\varepsilon(\lambda)$ is the molar absorptivity (extinction coefficient) of the acceptor

18   at wavelength λ in M^{-1} cm^{-1}. In the present study, K^2, n and φ were taken as 2/3, 1.336

19   and 0.118, respectively [35].

20   **Molecular docking parameters**

1   The three dimensional X-ray crystal structure of HSA (PDB ID: 1AO6, resolution 2.5

2   Å) was downloaded from the RCSB Protein Data Bank.   The three dimensional

3   structure of TQ was retrieved from pubchem [CID: 10281]. We performed docking

4   studies using docking program AutoDock version 4.0 [36, 37]. AutoDock works on

5   Lamarkian genetic algorithm and calculate all possible conformations of the ligand that

6   binds to the protein.   Polar hydrogen atoms, Kollman charges were merged to the

7   protein and Gasteiger charges were added to the ligands using graphical user interface

8   program AutoDock Tools (ADT) and then prepared file was saved in PDBQT format.

9   For the preparation of the grid map using a grid box Auto-Grid was used. Size of grid

10   was set to 70 Å × 70 Å × 70 Å xyz points with spacing of 0.375 Å, which covers all the

11   available active site residues. To encompass two binding sites (subdomain IIA and

12   IIIA, respectively) during the docking process, the two different grid centers along the

13   x-, y-, z-axes were set for subdomain IIA and for subdomain IIIA, respectively. To

14   achieve our goal the complex showing lowest binding energy with best fitness score

15   was used.   For visualization purpose we used Pymol version 1.3 and chimera version

16   1.8 [38].

17   **Enzyme activation kinetics by esterase activity (*Determination of esterase-like**

18   *activity*):**

19   The reaction of *p*-nitrophenyl acetate with HSA was followed spectrophotometrically

20   by monitoring the appearance of *p*-nitrophenol [39] at 405 nm for time duration of 2 min

21   on Perkin-Elmer Lambda 25 double beam spectrophotometer attached with Peltier

22   temperature programmer (PTP-1) to maintain temperature at 25 °C throughout the

23   experiments. The reaction mixtures contained 5 μM *p*-nitrophenyl acetate and 5 μM

protein in 20 mM MOPS (pH 7.4) and 20 mM glycine-NaOH (pH 9.0) buffers. The

Michaelis-Menten equation was used to get the rectangular hyperbolic pattern of a

typical enzyme-substrate reaction-

$$V_\circ = \frac{V_{max} \cdot [S]}{K_m + [S]} \qquad (16)$$

where V_o and V_{max} is the initial and maximum velocity respectively, [S] is the substrate

concentration, K_m is the Michaelis-Menton constant. The reciprocal of catalytic

velocity was plotted against the reciprocal of substrate concentration at a constant

activator concentration according to the equation 18 (Lineweaver-Burk plot):

$$\frac{1}{V_\circ} = \frac{K_m}{V_{max} + [S]} + \frac{1}{V_{max}} \qquad (18)$$

Antioxidant or Free radical scavenging activity (*a decolorization assay***):**

The antioxidant activity experiments were performed on the Perkin-Elmer Lambda 25

double beam spectrophotometer attached with Peltier temperature programmer (PTP-1)

to maintain temperature at 37 °C throughout the experiments. The TEAC assay was

performed as described by Re *et al*.[40] with minor modifications. ABTS was dissolved

in respective buffers (pH 7.4 and pH 9.0) to a 7 mM concentration. ABTS radical

cation (ABTS$^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM

potassium persulfate (final concentration) and allowing the mixture to stand in the dark

at room temperature for 12-16 h before use. Because ABTS and potassium persulfate

react stoichiometrically at a ratio of 1:0.5, this will result in incomplete oxidation of the

ABTS. Oxidation of the ABTS commenced immediately, but the absorbance was not

maximal and stable until more than 6 h. The radical was stable in this form for more

than two days when stored in dark condition at room temperature. For the study of

1 phenolic compounds, the ABTS$^{•+}$ solution was diluted with respective buffers (pH 7.4

2 and pH 9.0) to an absorbance of 0.71 (±0.02) at 734 nm and equilibrated at 37 °C. After

3 addition of antioxidant the absorbance was measured at 734 nm at 37 °C exactly 30 min

4 after initial mixing. Appropriate solvent blanks were run in each assay and subtracted,

5 respectively. All determinations were carried out at least three times, and in triplicate,

6 on each occasion and at each separate concentration of the standard and samples.

7 **Results and discussion**

8 **UV-visible absorption studies**

9 Ultraviolet/visible absorption spectroscopy is a powerful tool for steady-state studies of

10 protein-ligand interaction. In proteins, we distinguish different internal chromophores

11 that give rise to electronic absorption bands. From Fig. 1 we can see that the absorption

12 peak of HSA centers at ~280 nm mainly due to absorption of tryptophan residue.

13 However, after addition of the TQ, the maximal absorption peak as well as absorption

14 intensity of HSA is slightly affected. We observed that upon increasing the

15 concentration of TQ, the conformation of HSA was slightly affected. It was evident

16 from the disrupted absorption spectra of HSA around 280 nm (corresponding to Trp

17 residue) and 256 nm (corresponding to transition region of disulphide bond and Phe

18 residues absorption) in the presence of TQ.

19 **Tryptophan fluorescence quenching by TQ**

20 To avoid the effect of phenylalanine, we explored Trp fluorescence quenching

21 experiments to determine the interaction of TQ with 'N' and 'B' isoforms (at pH 7.4

22 and pH 9.0, respectively) of HSA at 37 °C. We observed a strong fluorescence peak of

23 HSA around 340 nm when excited at 295 nm in the absence of TQ. The fluorescence of

1   HSA was quenched in the presence of increasing concentrations of TQ, clearly

2   indicating an interaction between TQ and HSA (Supplementary Fig. S1). The intensity

3   of tryptophan fluorescence emission decreases continuously and gets saturated at higher

4   TQ concentrations, proving that TQ binding sites on HSA was fully occupied. The

5   decrease in fluorescence intensity upon addition of polyphenols was analyzed

6   according to the Stern-Volmer equation 1. There is a linear dependence between F_o/F

7   and molar concentration of the TQ (1:1). The qualitative emission spectral features

8   were slightly affected upon interaction of TQ to HSA which suggests about the minor

9   ligand-induced conformational changes in the protein occurs due to increase in

10   molecular closeness of TQ. The same experimental procedures were also followed at

11   15, 25 and 45 °C where we found that upon increasing the temperature, the quenching

12   also decreases, or in other words, the extent of lowering in fluorescence emission was

13   higher at lower temperatures (Fig. 2 A-I and B-II). The K_{sv} values for TQ at different

14   temperature as well as at different pH are given in the Table 1.

15   **Determination of binding constant and binding stoichiometry**

16   The binding constant (K_b) and the number of binding sites (*n*) can be calculated using

17   the equation 2. A plot of log $[(F_o /F) -1]$ vs log [TQ] gives a straight line, whose slope

18   equals to binding stoichiometry (*n)* and the intercept on y-axis equals to binding

19   constant (K_b), respectively (Fig. 2A-II and B-II). The values of K_b and *n* at 15, 25, 37

20   and 45 °C are listed in Table 1. For TQ, the values of K_b and *n* at pH 7.4 and 9.0 were

21   calculated at different temperatures as well as in presence of salt (NaCl). The data

22   shows that K_b decreases on increasing the temperature in both 'N' and 'B' isoforms but

23   these values are greater for 'N' (1.63×10^4 M^{-1}) than the 'B' (0.28×10^4 M^{-1}) form at

1 physiological temperature. It implies that under basic conditions the binding capacity of

2 TQ reduces up to ~6 times than that under neutral (physiological) conditions.

3 Conclusively, it shows that pH induced conformational change in the protein affects the

4 mode and mechanism of quenching and hence TQ binding to the HSA molecules.

5 Moreover, in the presence of NaCl, the extent of binding was not significantly changed

6 thus giving the clues that the electrostatic force doesn't play any role in TQ-HSA

7 interactions.

8 **Mechanism of HSA-TQ interaction**

9 Fluorescence quenching can be either dynamic or static in nature. To know about the

10 quenching mechanism of HSA by TQ, the values of k_q obtained from equation 3 was

11 closely observed and found that it was of the order of 10^{12}, which was 100 times higher

12 than the maximum scatter collision quenching constant of various quenchers with

13 biopolymers (2×10^{10} M^{-1} s^{-1}) [41]. This shows that quenching is not initiated by

14 dynamic diffusion but occurs by formation of a strong ground state complex between

15 HSA and TQ. Further, the temperature dependency of K_{sv} was studied and we observed

16 that the slopes (K_{SV} values) decreased with increase in temperature, further confirming

17 that the binding of TQ to HSA was due to complex formation (static quenching). In

18 static quenching, K_{sv} decreases due to the formation of complex between ligand and

19 protein, which undergoes dissociation on increasing temperature [29].

20 **Thermodynamics of HSA-TQ interaction**

21 According to the binding constants of TQ to HSA at all the four temperatures, the

22 thermodynamic parameters were determined from linear van't Hoff plot (equation 4)

23 and the observed data are shown in Table 1 and supplementary Fig S2. For the

17

1   determination of enthalpy-entropy relation in protein-ligand interaction we considered

2   only three temperatures *viz*. 15, 25 and 37 °C (NOT 42 °C) to ensure that the integrity

3   of protein conformation was not affect, otherwise it would to lead false interpretation of

4   thermodynamic parameters for the interaction studies. It is very well documented that at

5   42 °C, the domain III of HSA starts to melt, hence, major structural changes occurs

6   beyond this temperature. But we had included the data of 45 °C to assumed the

7   property of protein unchanged [27]. In other words, obtained enthalpy-entropy changes

8   are mainly caused by the binding of the TQ molecule to HSA. The negative values of

9   ΔG^o manifested in each condition suggest that the interaction was spontaneous. ΔH^o

10   and ΔS^o for the complex formation between TQ and HSA were found to be -5.10 kcal

11   mol^{-1} and 0.80, 0.83, 0.87 and 0.89 kcal mol^{-1} K^{-1}, respectively at 15, 25, 37 and 45 °C

12   at pH 7.4 and a similar pattern was also obtained at pH 9.0. Thus, the formation of TQ-

13   HSA complexation is an exothermic reaction accompanied by positive ΔS^o value. The

14   role of bound water to the protein molecule in or near the binding pockets may be

15   disturbed as a positive $T\Delta S^o$ value is a strong indication that water molecules have been

16   excluded from the binding site interface. From the point of view of water structure,

17   these thermodynamic signatures of protein-ligand interactions impersonate the type of

18   forces responsible for ligand association. A positive ΔS^o value is frequently taken as a

19   typical evidence for hydrophobic interaction whereas a negative ΔH^o is taken for

20   hydrogen binding [43]. Therefore, binding of TQ to HSA might involve H-bonding as

21   well as hydrophobic interaction as evidenced by the above thermodynamic signatures

22   (Supplementary Fig S2). Furthermore, it was found that the major contribution to ΔG^o

23   arises from the ΔH^o rather than from ΔS^o, so binding process was enthalpy driven.

18

1 **Isothermal titration calorimetric measurements**

2 The associated thermodynamic and binding parameters were further investigated

3 through ITC measurements. Representative calorimetric measurements to determine the

4 mode of binding of TQ with HSA isoforms at 37 °C are shown in Fig. 3. In the ITC

5 profiles, the lower panel shows the plot of heat librated per injection as a function of

6 molar ratio of the drug to the protein and upper panel, each peak represents the binding

7 isotherm of a single injection of the drug into the protein solution. The titration of TQ

8 to HSA shows negative heat deflection indicating that the reactions were mainly

9 exothermic. The association constant (K_a) and enthalpy change ($\Delta H°$) were directly

10 obtained after the best fitting for the integrated heats was obtained using single set of

11 binding model with lowest χ^2 value. The Gibbs free energy and entropy changes were

12 calculated from equations 6 and 7, respectively and obtained thermodynamic or binding

13 parameters are summarized in Table 1. The binding of TQ shows exothermic process

14 that are the characteristics of hydrogen bond and conformational changes [44] and the

15 values of binding constant were varying in the range of 10^3 to 10^4. Moreover, the

16 negative value of $\Delta G°$ suggests that the TQ-HSA complex formation was spontaneous

17 at both pH values (pH7.4 and pH 9.0). The negative values of $\Delta H°$ and positive value

18 of $\Delta S°$ values advocate that the involvement of hydrogen bond and hydrophobic

19 interaction in the formation of the protein-TQ complex [45], which indicates the

20 occurrence of enthalpy-entropy compensation effect in which enthalpy loss due to the

21 deformation of H-bond is counter balanced by entropic penalty due to the burial of

22 involved groups. This effect is common in protein-ligand interactions [46].

23 **Circular dichroism measurement**

19

1   It is possible to estimate the contents of secondary structure of protein using far-UV CD

2   spectra (190-250 nm). A positive peak near 195 nm and two negative peaks near 208

3   and 222 nm is characteristic feature of α-helical protein. The far-UV CD studies were

4   performed on the protein and protein-TQ complexes in order to investigate the

5   possibility of any structural change of the protein upon complexation with the TQ. Fig

6   4-I shows that the CD spectra of HSA with various TQ concentrations at pH 7.4 and

7   9.0. From Fig. 4-IA (pH 7.4) and 4-IB (pH 9.0) the spectra of HSA in the absence and

8   presence of TQ, as the TQ concentration increases a notable spectral rearrangement

9   occurs in HSA with increase in major minima (208 nm) as well as slight changes in the

10   shape of spectra due to intramolecular H-bonding rearrangement as justified by

11   fluorescence quenching experiments. The TQ induced alterations in secondary

12   structures of HSA were quantified by Chen *et al.* method [31], and the calculated values

13   are summarized in Table 2. In the presence of TQ, a significant increase in the α-helical

14   content of both HSA isoforms was observed. Using equation 9, the α-helical content in

15   'N' isoform of HSA was calculated. It increased from 4% and 9% while in case of 'B'

16   isoform of HSA it increased from 9% and 18% as compared to native structure,

17   correspondingly. Overall, the 'B' isoform of HSA is more stable in the presence of TQ.

18   **Thermal stability of albumin was enhanced by TQ**

19   The existence of intermediates in the thermal unfolding pathway of a protein can also

20   be evidenced by observing the changes in its secondary structure. Fig. 4-II shows the

21   change in the ellipticity at 222 nm of the HSA:TQ at molar ratios 1:0, 1:5 and 1:25,

22   respectively as obtained from CD experiments. The mid-point temperature (T_m) was

23   determined by fitting the ellipticity values in to a two-state folding-unfolding model

1   equation 10 and 11. This shows that the protein loses a considerable fraction of its

2   secondary structure during thermal denaturation. The decrease in ellipticity at 222 nm

3   of HSA is shown as a function of temperature and the denaturation profile is found to

4   be consistent with the earlier reports [47]. It is noted that the presence of intermediate

5   unfolded states in the thermal denaturation of bovine serum albumin (BSA), which is

6   structurally similar to HSA, has already been indicated by calorimetric studies [48]. The

7   CD thermal profiles, including the 1:25 molar ratio of HSA:TQ showed more thermal

8   stability, indicating that the protein is more stable in the presence of TQ as compared to

9   the native condition. Thus, the higher stability of HSA in the presence of 1:25 molar

10   ratio corresponds to increase in the secondary structure. The obtained T_m values of TQ-

11   bound HSA are significantly higher than the native one (Table 2). Denaturation of HSA

12   at high temperature occurs by weakening of hydrophobic as well as polar interactions,

13   which may also facilitate the TQ binding property of HSA. Here, enhancement in

14   thermal stability is also implied by better interaction of TQ at high temperature which

15   induces to more helical structure formation in unordered protein segments of 'B'

16   isoform as compare to 'N' isoform of HSA.

17 **Dynamic light scattering studies**

18   Unfolding of a protein is usually marked by a change in the secondary and globular

19   structure of the protein. The change in the globular structure of a protein can be studied

20   by DLS measurements. It is clear from the above findings that the interaction of TQ

21   with HSA causes conformational changes. So, we decided to measure the molecular

22   sizes of HSA in the absence and presence of TQ by determining the hydrodynamic radii

23   using DLS. The Fig. 5-I & 5-II shows that the change in the globular structure of the

1   protein at 25 °C. The hydrodynamic radii (R_h) of native HSA and HSA in the presence

2   of TQ were calculated and values are shown in Table 3. The R_h values of native HSA

3   were 4.0 nm and 3.6 nm at pH 7.4 and 9.0, respectively. These results are in excellent

4   agreement with previous observations at pH 7.4 and pH 9.0, respectively [49]. The R_h

5   values of HSA complexed with TQ were higher than the native one. The increase in

6   hydrodynamic radii upon ligand binding may be due to the "expansion of domains"

7   which may lead to an increase in the molecular volume as a result of conformational

8   changes. Similar results were also observed previously in the presence of atropine

9   (4.1%), propranolol (11.1%), clonidine (14.4%), phenylephrine (16.6%) and carbachol

10 (15.5%) [50] The lower values of polydispersity (< 20) were indicative of homogenous

11 species in the solution. Fig. 5-II shows the characteristic examples of the dependencies

12 of globule size and compressibility upon the HSA:drug molar ratios. The principal

13 structural rearrangements were displayed at each HSA:drug molar ratios and it

14 increased with an increase in HSA:drug ratio. We observed that at extremely higher

15 molar ratio of HSA:drug (1:25), the hydrodynamic radii were either slightly changed or

16 remain unaffected (Fig. 5-II).It suggested that the structural changes in HSA occurred

17 only by the molecules which were bound to the protein and affected its secondary

18 structures near the binding site [51].

19 **Energy transfer between TQ and HSA**

20 In order to estimate the binding of TQ to the model transporter protein HSA, we have

21 also explored the possibility of energy transfer between donor to acceptor using

22 Förster's resonance energy transfer (FRET) method. In Fig. 6, the emission spectrum

23 from the single tryptophan (Trp214) of HSA and the absorption spectrum of TQ are

22

1   shown. According to FRET theory as describe in the method section, efficiency of

2   energy transfer (E_{FRET}), spectral overlap (J), Förster's distance (R_o) of the donor

3   (Trp214), and r value were derived from the overlaid spectra and the value of energy

4   transfer between TQ and HSA was calculated from the equations 13-15. At pH 7.4, the

5   energy was efficiently transferred from Trp214 of HSA to the bound TQ as indicated

6   by a large spectral overlap between the emission spectra of Trp214 and the absorption

7   spectrum of TQ. Hence, the probability of TQ binding was stronger with the 'N' isomer

8   of HSA at pH 7.4. Further, it is evident from the Fig. 6B that the spectral overlap of the

9   HSA-TQ system was significantly lower at pH 9.0due to minimum contribution from

10   the Trp214. We calculated the energy transfer parameters and found that E_{FRET} =

11   0.3048, J= $3.24{\times}10^{-14}$ cm^3 M^{-1}, R_o = 2.981 nm, r = 3.42 nm for HSA at pH 7.4 and

12   E_{FRET} = 0.1400, J= $2.72{\times}10^{-15}$ cm^3 M^{-1}, R_o = 1.97 nm, r = 2.66 nm for HSA at pH 9.0,

13   respectively (Table 4). The average distance between the donor and acceptor

14   fluorophores was on the 2-8 nm scale and $0.5R_o < r < 1.5R_o$. In our study, the donor to

15   acceptor distance was less than 8 nm, indicating that the energy transfer from HSA to

16   TQ occurred with high probability. These results were also in accordance with a static

17   quenching mechanism.

18   **Three-dimensional conformational investigation of TQ binding with HSA**

19   Three-dimensional fluorescence spectroscopy is a new analytical technique that is

20   applied to investigate the conformational changes of proteins. The excitation and

21   emission wavelength of the fluorescence intensity can be used as the axes rendering the

22   investigation of the characteristic conformational changes of proteins more scientific

23   and credible [29]. The maximum fluorescence emission wavelength of amino acid

1   residues in a protein is related to the polarity of the environment. Experiments have

2   suggested that the fluorescence emission spectrum wavelength and the synchronous

3   fluorescence spectrum wavelength of HSA in the absence and presence of drugs show

4   distinct differences and sharp changes, which provide relative information on the

5   configuration of the protein [52]. The three-dimensional spectra and contour maps of HSA

6   and HSA-TQ complexes are presented in Fig. 7 and supplementary Fig. S3 and the

7   obtain values are listed in supplementary Table T-1. In the figure of three-dimensional

8   spectra, two characteristic fluorescence peaks of HSA (peak 1 and peak 2) were clearly

9   observed, while peak 3 and peak 4 represents Rayleigh scattering peak ($\lambda_{ex} = \lambda_{em}$) and

10   second-order scattering peak ($\lambda_{em} = 2\lambda_{ex}$) [29, 52], respectively. The peak 1 represents

11   fluorescence arising mainly from tryptophan and tyrosine (negligible contribution of

12   phenylalanine fluorescence) when the protein is excited at 280 nm. On the other hand,

13   peak 2 is the characteristic fluorescence peak representing polypeptide backbone

14   structure. In our study, the decrease in the intensities of peaks 1 and 2 clearly indicated

15   that the fluorescence of HSA was quenched as a result of TQ binding and the

16   conformation of the protein was also altered as a result of it.

17   **Molecular docking**

18   Main aim of the study was to presumptive binding site of TQ in HSA as reported earlier

19   by G. Lupidi *et al*. [13]. We used X-ray structure of HSA with high resolution (PDB ID:

20   1AO6, res. 2.5 Å) as a template and the prediction of binding mode of TQ was done

21   using AutoDock software package. To comprise the subdomain IIA and IIIA of HSA,

22   the two regions of interest used for molecular docking were defined to determine the

23   binding residues and their positions and their energy score was compared. The docking

1 results showed that TQ binds to HSA within the binding pocket of subdomain IIA with

2 an estimated docking energy of about -5.83 kcal mol^{-1}, while for the second binding site

3 subdomain IIIA the free energy of binding was found to be -5.62 kcal mol^{-1}.

4 Therefore we can argue that TQ has a better binding preference for the drug binding

5 site I (subdomain IIA) of HSA. This result is in agreement with earlier published data,

6 as it is reported that quinone-related derivatives bind to subdomain IIA of HSA. The

7 binding of TQ to subdomain IIA of HSA was also confirmed by the drug-displacement

8 experiments [54, 55]. This binding site for TQ at subdomain IIA was located

9 predominantly in a hydrophobic cleft walled by the amino acid residues Tyr150,

10 Leu199, Trp214, Leu219, Arg222, Leu238, Arg257, Leu260, Ala261, Ile264, Ser287,

11 Ile290, and Ala291 (as shown in Fig.8A). One of the oxygen from TQ was interacting

12 with Tyr150 through hydrogen bonding, and its methyl group was found to interact

13 with Trp214 through hydrophobic interactions (Fig. 8B).

14 **Esterase-like activity of HSA-isoforms in presence of TQ**

15 HSA has an esterase-like activity for the deglucuronidation of acyl-glucuronide of

16 fenoprofen, etodolac, ketoprofen and gemfibrozil [56-59]. The double-reciprocal plots for

17 substrates were characterized by a family of linear, nonparallel lines that converged to

18 the left of the y-axis (Supplementary Fig. S4). In presence of TQ, the K_m increased at

19 pH 7.4 from 6.66×10^{-6} M to 9.37×10^{-6} M. The higher value of K_m obtained for the

20 HSA incubated at pH 7.4 in the presence of TQ showed a lower substrate affinity for

21 the substrate. The incubated enzyme besides showing higher K_m for substrate also

22 showed a higher k_{cat} value yielding overall a decrement in catalytic efficiency (k_{cat}/K_m)

23 relative to the HSA in the absence of TQ. Altogether, our results indicated that addition

1   of TQ alters both K_m and V_{max} values of HSA. Increase in K_m and V_{max} indicated that

2   the esterase activity of HSA was inhibited in a competitive manner because TQ was

3   directly competing with the substrate for a fixed number of active sites on enzymes.

4   The large increase in K_m upon the binding of TQ indicated   changes in the tertiary

5   structure of HSA that might lead to steric effects resulting from limitation of the

6   accessibility of substrate to the active site . The catalytic efficiency value, which is the

7   ratio of k_{cat} over K_m was also different for free HSA and HSA-TQ complex. As shown

8   in Table 5, the catalytic efficiency of HSA-TQ was lower than the free enzyme

9   indicating that the enzyme was poorer on the substrate in the presence of TQ.

10   We have obtained opposite pattern of K_m and V_{max} at pH 9.0, decrease in K_m from

11   8.55×10^{-5} M to 4.09×10^{-5} M   and V_{max} 6.70×10^{-7} to 5.12×10^{-7} M/min revealed that

12   HSA in the presence of TQ at pH 9.0 had an improved affinity and tighter substrate

13   binding capability as compared to that at pH 7.4 (Table 5). The second order rate

14   constant k_{cat}/K_m ratio indicates the catalytic efficiency and kinetic perfection of the

15   enzyme in transforming substrates. The higher the k_{cat}/K_m ratio, the better the enzyme

16   works on that substrate. A comparison of k_{cat}/K_m ratio for the same enzyme with

17   substrates in different conditions is widely used as a measure of enzyme effectiveness.

18   TQ induced the catalytic activation of HSA at pH 9.0 and allowed the reaction to

19   approach the limit of maximum diffusion just like in an ideal enzyme (acetyl

20   cholinesterase) where every interaction with substrate yields a product and for these

21   enzymes, from the diffusion theory, the value of k_{cat}/K_m ranges 6×10^{9}- 6×10^{10} M^{-1} min^{-}

22   ^{1}. Our enzyme kinetics results suggested that the TQ acts as an activator of the esterase

23   activity of HSA in alkaline conditions. The k_{cat} values were strikingly dependent on pH

1   and showed that the susceptibility of the active sites to nucleophilic attack increases

2   with pH. It has been reported that pH dependent conformational changes ('N'↔'B'

3   transition) occur in albumin when going from neutral to slightly alkaline pH [25].

4   Therefore, alteration in nucleophilic attack in active sites and in the affinity to *p*-NPA

5   could be due, totally, or partly, to changes in the tertiary structure of albumin, which

6   convoy the pH-dependent N-B transition. From all of our experiments dealing with the

7   secondary and tertiary structure of HSA in the presence of TQ molecules, it becomes

8   apparent that upon interaction between TQ and HSA, the affinity of the protein for its

9   substrate is enhanced in alkaline condition.

10 **Antioxidant or Radical scavenging activity of HSA in presence of TQ**

11 TQ   has several important functional properties, out of them we have evaluated the

12 free radical scavenging activity because of its deleterious job in the food and biological

13 systems [60]. Some of its properties were previously demonstrated by several

14 pharmacological studies as membrane lipid peroxidation, reduction of eicosanoid

15 generation [61], anti-inflammatory and analgesic [2, 62], protection of body organs against

16 oxidative damage induced by various type free radical generating agents [63-65]. Oral

17 intake of TQ is capable of protecting numerous organs against oxidative damage

18 induced by free radical-generating agents including doxorubicin-induced cardiotoxicity

19 [66, 67]. TQ act as scavenger of superoxide, hydroxyl radical and singlet molecular oxygen

20 [68]. HSA itself have a very good antioxidant activity that plays an important role in

21 human health. In alkaline condition, antioxidant property of HSA increases due to its

22 conformational change and the activation of antioxidant activity is also thiol-dependent

23 [69]. The carboxyl group modification of HSA causes approximately 40-fold increase in

1   the antioxidant activity. These chemical modification studies indicate that the addition

2   to functional cysteine(s) or cationic amino acid residues such as arginine, histidine and

3   lysine involve in antioxidant reactions. These results recommend that the

4   activation of thiol-dependent antioxidant activity of HSA at alkaline pH is due to

5   the conformational change which was favorable for the functional cysteine(s)-mediated

6   catalysis. HSA shows specific antioxidant property is also due to its multiple ligand-

7   binding and free radical-trapping properties and are directly connected to the

8   conformational change in structure and the redox state of molecule[70, 71]. Currently

9   various methods are used to described the antioxidant activity of plant derived phenolic

10   compounds. These chemical assays are based on the ability to scavenge the free radical

11   by various radical generating system and method for decolorization. $ABTS^{\bullet+}$ radical

12   scavenging method is most appropriate format for decolorization assay and very

13   common spectrophotometric procedure to determine the antioxidant capacity of plant

14   derived components due to its sensitive, sample, rapid and reproducible procedure [72].

15   Biochemical assay are based on the scavenging ability of synthetic free radicals which

16   are generated by different radical-generating systems. Free radicals are generating prior

17   to reactions that involve in the production of blue/green $ABTS^{\bullet+}$ chromophores that was

18   formed due to reaction between ABTS and potassium persulphate.

19       $ABTS^{\bullet} + AH \rightarrow ABTS^{+} + A^{\bullet}$

20   The extent of inhibition of the absorbance of the $ABTS^{\bullet+}$ is plotted as a function of

21   concentration in order to determine the TEAC, that can be assessed as a function of

22   time. The dose-response curve obtained by analysis of a range of concentrations of

23   antioxidant compounds, was plotted as the percentage inhibition of the absorbance of

the ABTS$^{\bullet+}$ solution as a function of concentration of antioxidant (Fig. 9A). Trolox and

BHA were used as standard reference compounds (Fig. 9B). To calculate the TEAC,

the gradient of the plot of the percentage inhibition of absorbance vs. concentration plot

for the antioxidant in question is divided by the gradient of the plot for Trolox and

BHA. The scavenging capability of ABTS$^+$ radical was calculated using the following

equation:

$$ABTS^{o+} \; scavenging \; effect \, (\%) = \left(1 - \frac{As}{Ac} \right) \times 100$$

(19)

where, Ac is the initial concentration of the ABTS$^+$ and As is absorbance of the

remaining concentration of ABTS$^+$ in the presence of TQ [73].

Thymoquinone   **Dihydro thymoquinone**
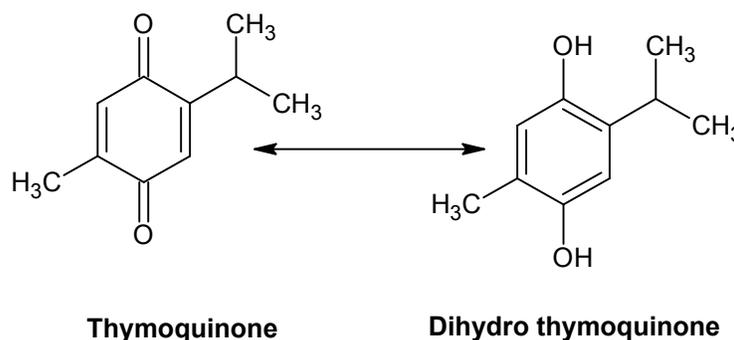
This gives the TEAC at the specific time point and the calculated results for the

flavonoids are given in supplementary Table T-2. All the tested compounds exhibited

admirable radical cation scavenging activity. As seen in Fig. 9C, TQ had effective

radical scavenging activity in a concentration-dependent manner (16.42-164.2 µg/ml).

TQ undergoes reduction process and gets converted into its more anti-oxidative form

i.e. dihydrothymoquinone (DHTQ). TQ and DHTQ inhibited non-enzymatic process in

liver that was also dose dependent [67]. There was a significant increase in the overall

1 concentration of ABTS$^{\bullet+}$ due to the scavenging capacity of TQ concentrations. Also,

2 the scavenging effect of TQ and standards, on the ABTS$^{\bullet+}$ decreased in that order: at

3 pH 7.4 HSA-TQ > HSA (100 µM) > TQ (2500 µM) > trolox (100 µM), which were

4 81.39%, 50.37%, 47.45% and 43.76% , and at pH 9.0 HSA-TQ > HSA (100 µM) > TQ

5 (2500 µM) > trolox (100 µM), which were 100%, 99.97%, 96.19% and 88.39% at the

6 concentration of 100 µL/ml respectively.

7

8 **Conclusion**

9 In the present study, we evaluated the binding properties of 'N' and 'B' isoform of

10 HSA (at pH 7.4 and pH 9.0, respectively) with TQ, an important constituent of *Nigella*

11 *sativa*. The binding affinity and thermodynamics parameters were higher for 'N'

12 isoform as compared to 'B' isoform of HSA. The interaction of TQ with HSA was

13 favored by H-bonding and hydrophobic interactions. The molecular size and thermal

14 stability of HSA were increased in the presence of TQ. We found that the esterase

15 activity of HSA is enhanced in 'N' isoform in the presence of TQ as compared to 'B'

16 isoform, while the antioxidant activity is quite significant in 'B' isoform. The overall

17 antioxidant activity of HSA is enhanced in the presence of TQ. Thus, the phenolic

18 compound TQ which is a component of *Nigella sativa* has a great potential to bind

19 HSA and induces its free radical scavenging activity. This study provides insight into

20 HSA-TQ interaction, which is of great importance in understanding the chemico-

21 biological interactions for drug-designing, pharmacy and biochemistry.

22

23

8

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Table 1: Binding and thermodynamic parameters of HSA and TQ at different temperatures obtained from fluorescence quenching experiments and ITC.

pH/isoform	Temp. (°C)	K_{sv} $\times 10^4$ (M^{-1})	k_q $\times 10^{12}$ ($M^{-1}s^{-1}$)	n	K_b $\times 10^4$ (M^{-1})	$\Delta G°$ (kcal mol^{-1})	$\Delta H°$ (kcal mol^{-1})	$T\Delta S°$ (kcal mol^{-1} K^{-1})	Dominating forces Involve (inferred)
pH 7.4/N	15	2.45	4.23	1.01	3.08	-5.90		0.80	H-bonding, Hydrophobic interactions
	25	2.11	3.65	1.00	2.30	-5.93	-5.10	0.83	
	37	1.15	1.98	1.02	1.63	-5.97		0.87	
	45	0.85	1.47	0.99	0.82	-5.99		0.89	
NaCl (0.15 M)	37	0.98	1.69	1.01	1.24	-5.80			
pH 9.0/B	15	0.55	0.09	0.98	0.44	-4.79		1.08	
	25	0.35	0.06	0.99	0.34	-4.82	-3.71	1.11	
	37	0.27	0.04	1.01	0.28	-4.87		1.16	
	45	0.08	0.01	1.08	0.27	-4.90		1.19	
Values obtain by ITC pH 7.4/N	37			1.01± 0.14		-127.55	-63.50 ± 2.48	-63.55*	H-bonding, Conformational changes
Values obtain by ITC pH 9.0/B	37			0.58± 0.07		-75.01	-37.05 ± 1.58	-37.51*	

K_{SV} = Stern-Volmer constant; k_q = bimolecular rate constant;

n = stoichiometry of binding;

K_b = binding constant;

$\Delta G°$ = change in free energy;

$\Delta H°$ = change in enthalpy;

$T\Delta S°$= change in entropy;

*kcal/mol/deg.

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10 **Table 2:** Secondary structural analysis to determine the percent structural change in the isoforms

11 of HSA and HSA-TQ complexed.

pH/isoform	HSA(μM)	TQ (μM)	% increment in α-helix	T_m (^{o}C)
pH 7.4/N	02	00	0	68.18
	02	10	4	72.35
	02	50	9	72.66
pH 9.0/B	02	00	0	72.81
	02	10	9	74.24
	02	50	18	75.02

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7   **Table 3:** Characteristics of hydrodynamic radii (R_h) and polydispersity (P_d) distribution of

8   HSA solution in absence and presence of TQ at pH 7.4 and pH 9.0.

pH/isoform	HSA:TQ	R_h (nm)	Change in R_h (%)	P_d (%)
pH 7.4/N	1:00	4.0	100	16.0
	1:05	4.2	105	16.2
	1:25	4.4	110	19.6
pH 9.0/B	1:00	3.6	100	17.4
	1:05	3.8	106	13.9
	1:25	4.0	111	17.6

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Table 4: FRET data obtained from spectral overlap of HSA emission and TQ absorption at 37

14   °C.

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Variables	pH 7.4	pH 9.0
F	142.862	200.044
Fo	205.504	232.628
E_{FRET}	0.3048	0.1400
J ($cm^3 M^{-1}$)	3.24×10^{-14}	2.702×10^{-15}
Ro (nm)	2.981	1.970
r (nm)	3.42	2.66

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6 **Table 5:** Kinetic parameters for the hydrolysis of *p*-nitrophenyl acetate by HSA.

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pH/isoform	System	RA (%)	V_{max} (M/min)	K_m (M)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
pH 7.4/N	HSA	100	4.27×10^{-8}	6.66×10^{-6}	0.854×10^{-2}	1.28×10^{3}
	HSA+TQ	127	5.42×10^{-8}	9.37×10^{-6}	1.048×10^{-2}	1.11×10^{3}
pH 9.0/B	HSA	100	6.70×10^{-7}	8.55×10^{-5}	13.41×10^{-2}	1.56×10^{3}
	HSA + TQ	113	5.12×10^{-7}	4.09×10^{-5}	10.24×10^{-2}	2.50×10^{3}

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1   **Figure captions**

2   **Figure 1:** UV-visible absorption spectra of HSA (6 μM) in absence and presence (00 μM -30 μM)

3   of TQ at (A) pH 7.4; (B) pH 9.0, respectively.

4   **Figure 2:** (A-I and B-I) the Stern-Volmer plots for the HSA-TQ interaction of N isoform pH 7.4

5   and B isoform pH 9.0 at 15, 25, 37, 45 °C and in presence of 0.15 N NaCl at 37°C. (A-II and B-II)

6   Plot of log $[(F_o/F)-1]$ *vs* log[Q] for the determination of binding constants and binding

7   stoichiometry for HSA-TQ interaction at pH 7.4 at 15, 25, 37, 45 °C and in presence of 0.15 N

8   NaCl at 37 °C at pH 7.4.

9   **Figure 3:** Isothermal titration calorimetric profile of HSA in presence of TQ at pH 7.4 (A) and pH

10   9.0 (B) at 37 °C. Titration of TQ with HSA shows calorimetric response as successive titrations of

11   TQ to the sample cell.

12   **Figure 4:** Far-UV CD spectra of HSA (2 μM) in absence and presence of TQ at pH 7.4 (4-IA) and

13   pH 9.0(4-IB). Far-UV CD thermal unfolding spectra of HSA (2 μM) in the absence and presence

14   of TQ in molar ratio of 1:05 and 1:25 at pH 7.4 (4-IIA) and pH 9.0 (4-IIB), respectively.

15   **Figure 5:** Dynamic light scattering of HSA-TQ complex. (5-I) Determination of hydrodynamic

16   radii (R_h) of HSA in the absence and presence of TQ (HSA:TQ molar ratio 1:0, 1:5 and 1:25); A-I,

17   A-II, A-III and B-I, B-II, B-III for pH 7.4 and pH 9.0 respectively. (5-II)   TQ concentration

18   dependent changes in hydrodynamic radii of HSA at both pH, shows that hydrodynamic radii

19   increases on increasing the concentration of TQ at both pH.

20   **Figure 6:** Tryptophan fluorescence resonance energy transfer. Spectral overlap of the fluorescence

21   emission of HSA $(\lambda_{ex} = 295$ nm) and absorption spectra of TQ [HSA = TQ = 2 μM] at (A) pH 7.4;

22   (B) pH 9.0, respectively.

1   **Figure 7:** Three-dimensional fluorescence spectra of HSA (2μM) in absence (A and B) and

2   presence (A-I and B-I) of TQ at pH 7.4 and pH 9.0, respectively. [HSA: TQ =1:1].

3   **Figure 8:** Molecular docking results (A) TQ represented as ball and stick model and HSA in

4   ribbon, (B) Hydrogen bonding between Tyr150 and Trp214 with TQ in the region of binding

5   pocket.

6   **Figure 9:** The effects of concentration of the antioxidant on the inhibition of the ABTS•$^+$ by trolox

7   and BHA at pH 7.4 and pH 9.0. Absorbance of ABTS radical scavenging activity of different

8   concentrations of HSA, TQ and reference antioxidants as trolox and BHA (ABTS$^+$: 2,20-

9   azinobis(3-ethylbenzthiazoline-6-sulfonic acid) at pH 7.4 (9-IA), pH 9.0 (9-IB). Percent inhibition

10 of standard reference compounds trolox and BHA (9-II) as well as in presence of phenolic

11 compound TQ and protein at particular concentrations at pH 7.4 (9-IIIA) and pH 9.0 (9-IIIB),

12 respectively.

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4 **Illustrations**
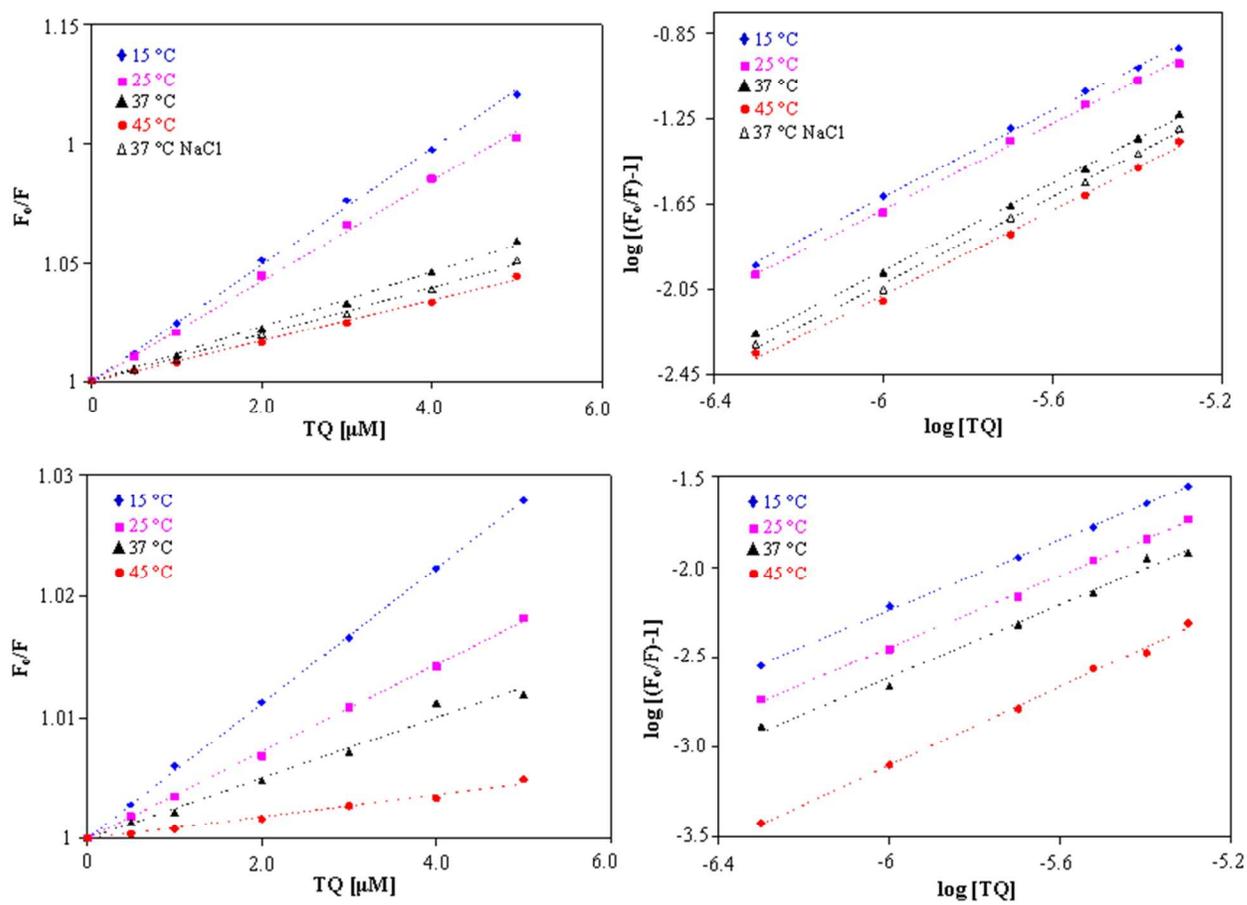
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14 Figure 1

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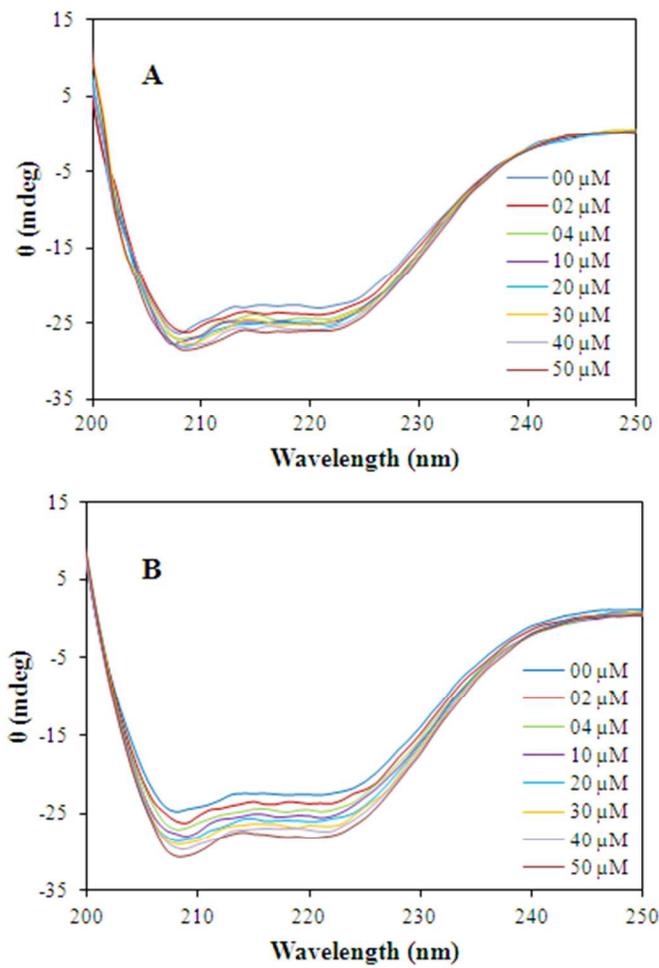
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Figure 3

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Figure 4-I

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Figure 4-II

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Figure 5-I

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Figure 5-II

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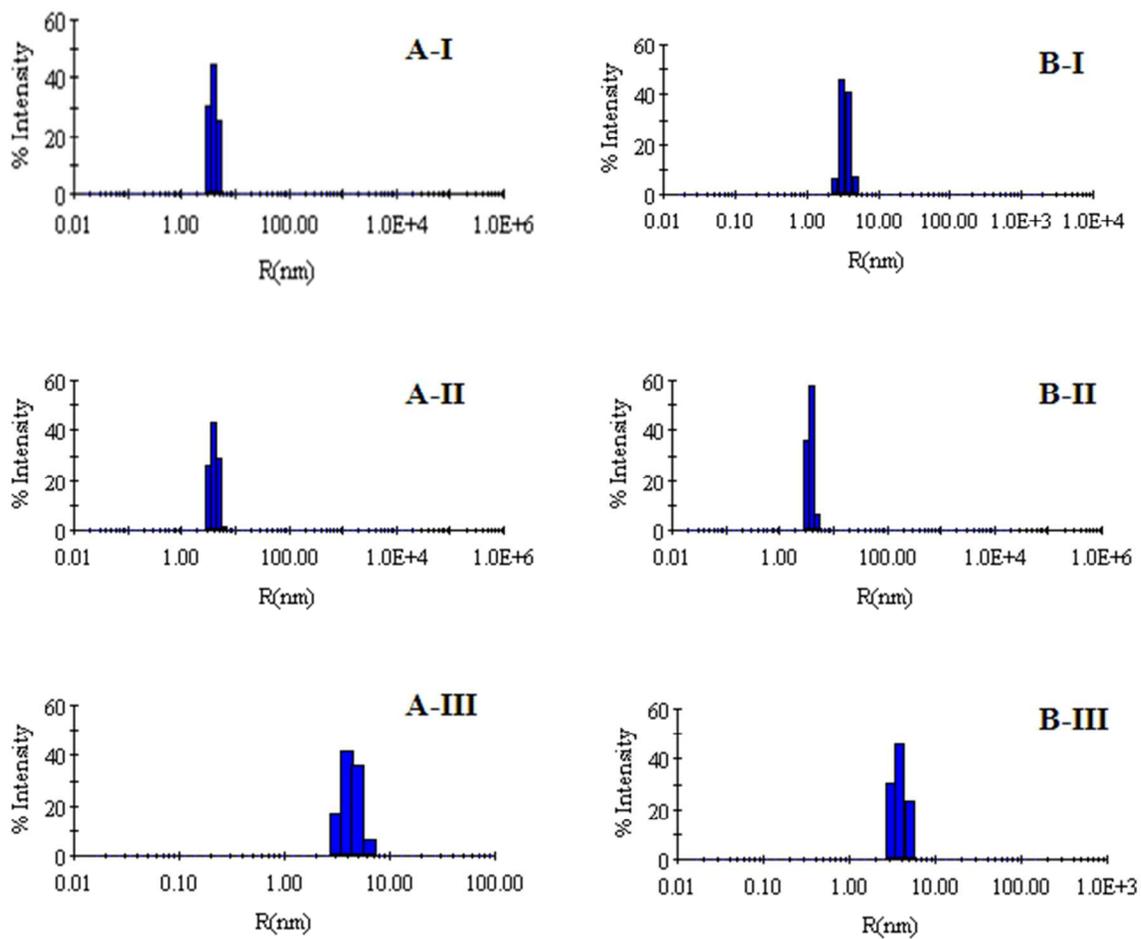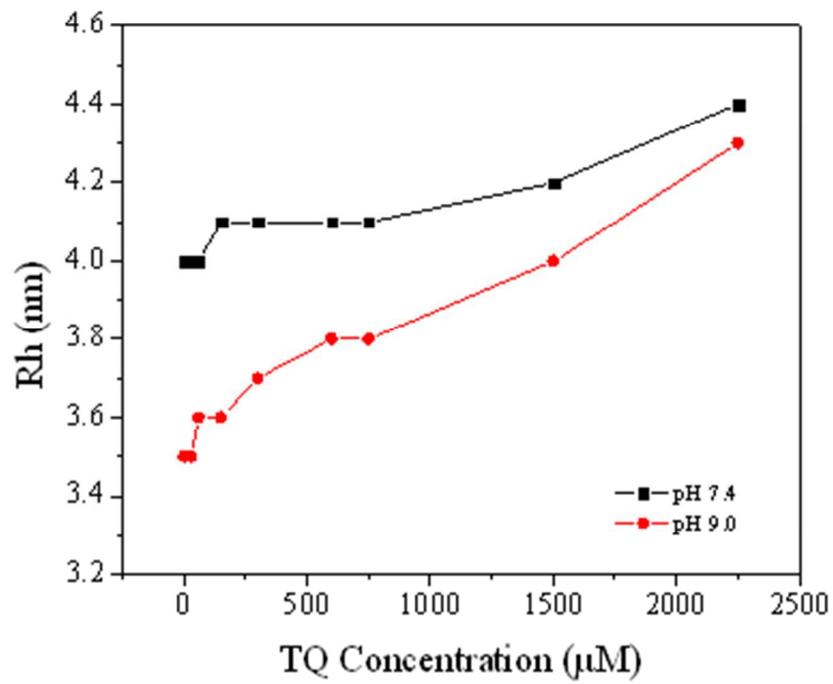
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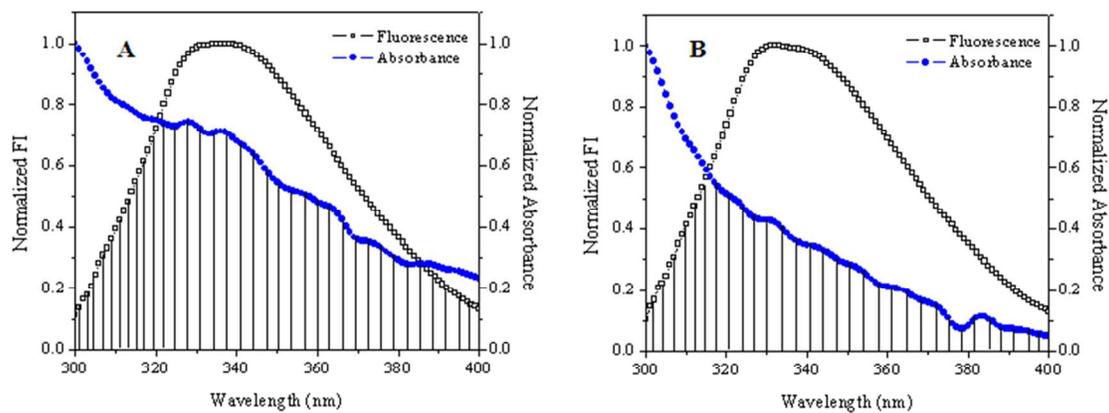
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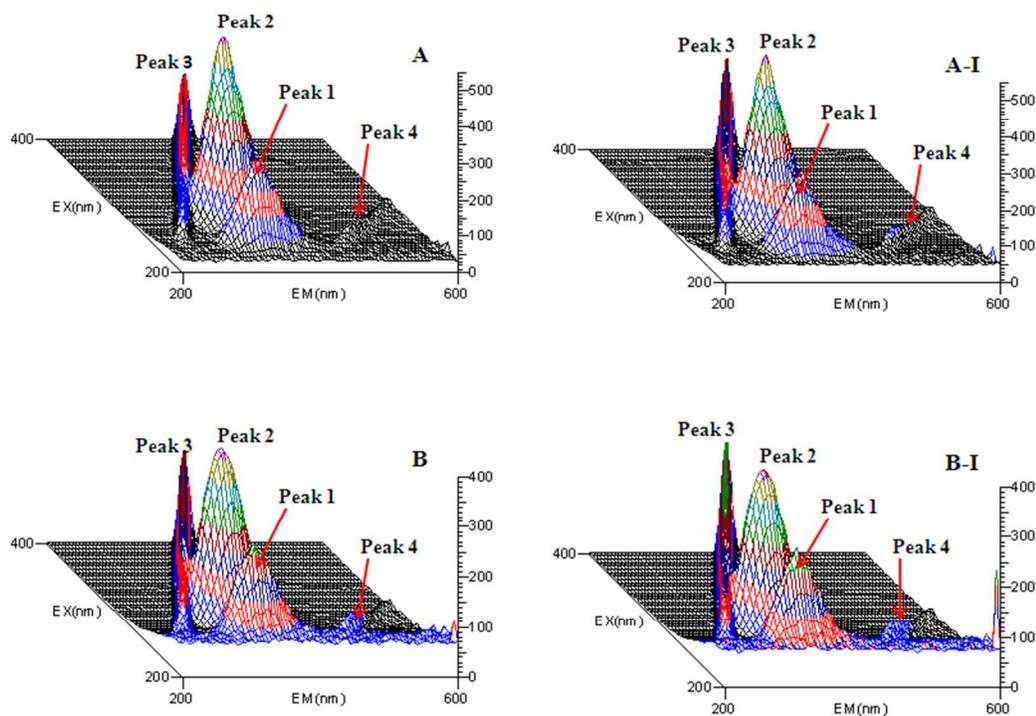
Figure 6

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Figure 7

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Figure 8

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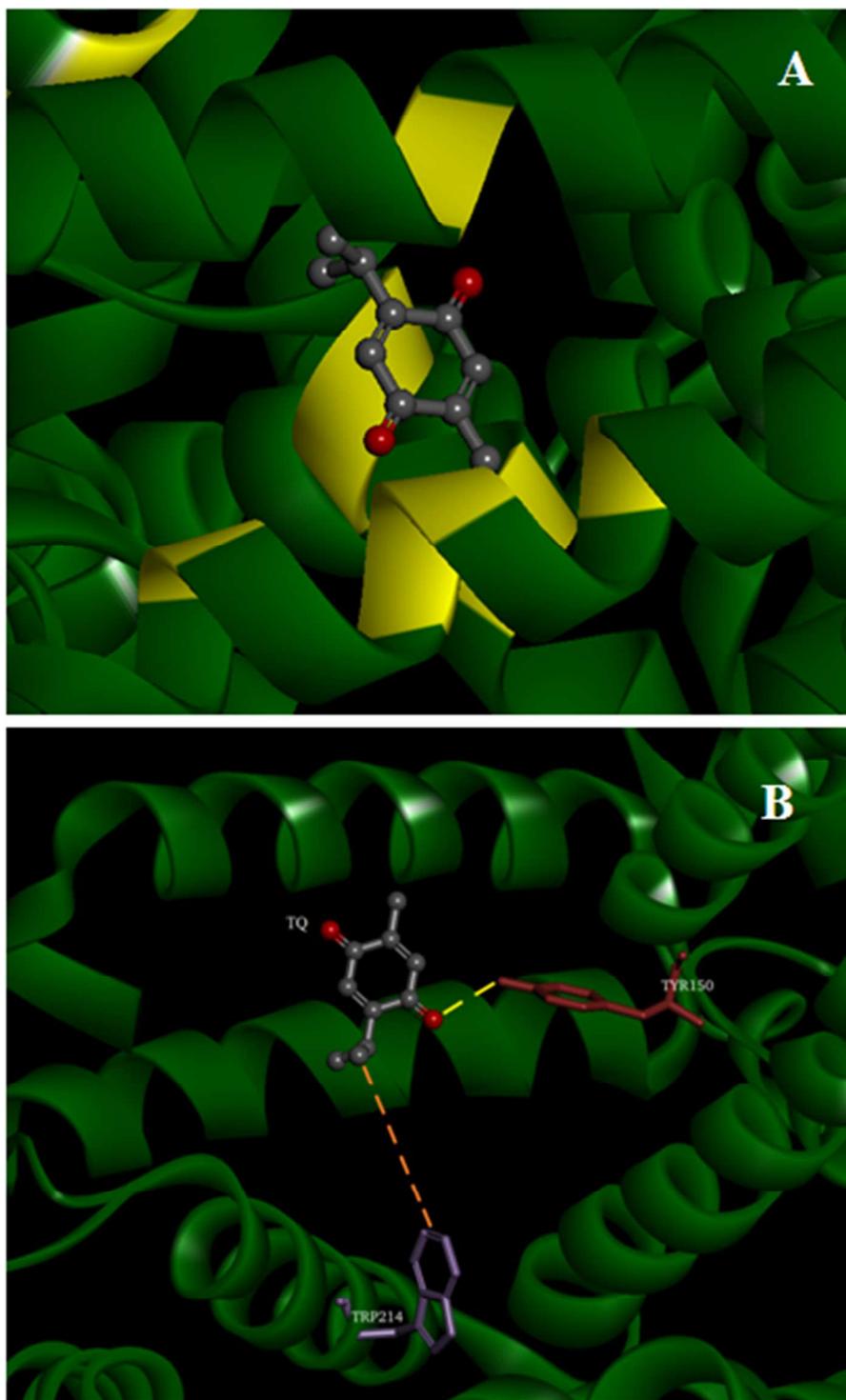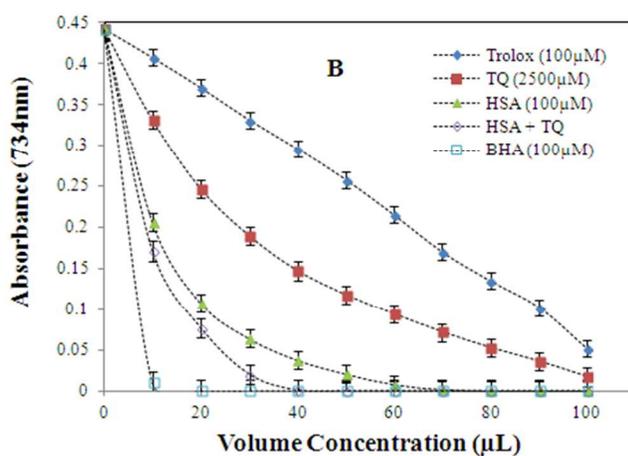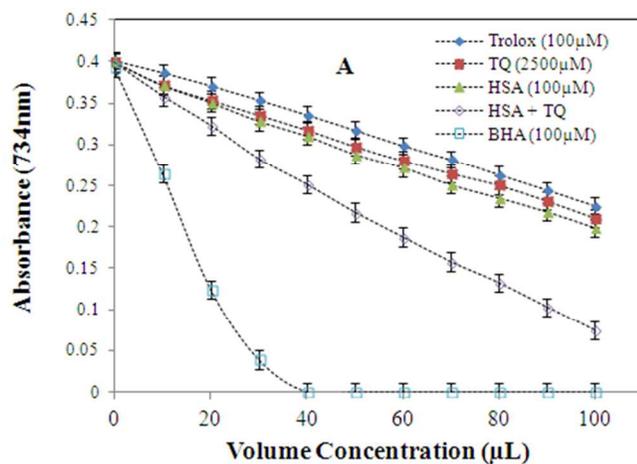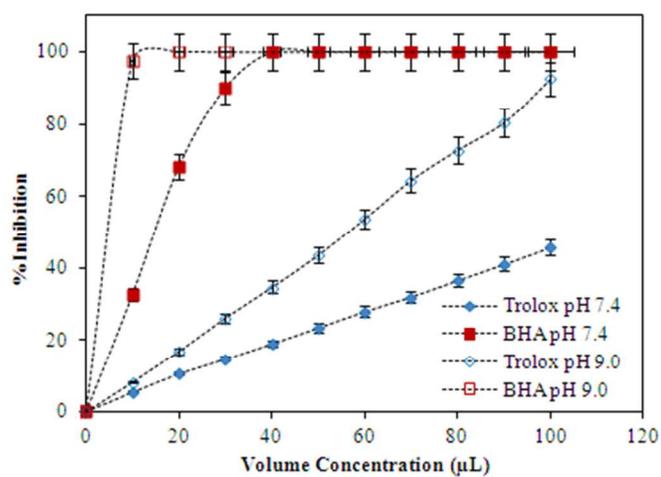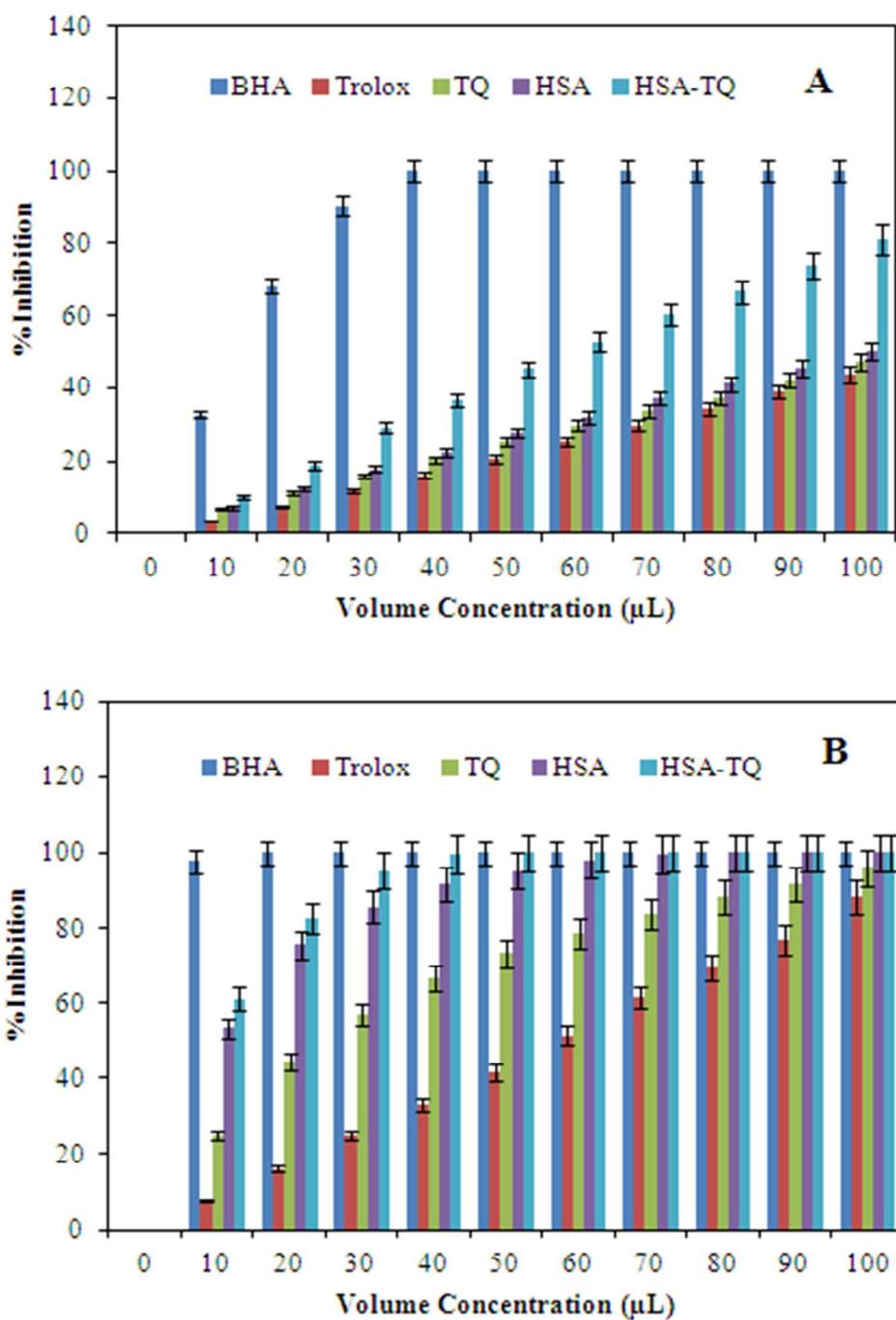
1   **Supporting Information description**

2   **Figure S-1:** Fluorescence emission spectra of HSA (2 μM) in presence of TQ (0-25 μM) at (A) pH

3   7.4; (B) pH 9.0.

4   **Figure S-2:** (A) Van't Hoff plot; and (B) Thermodynamic signatures for HSA-TQ interaction in N

5   and B isoforms at 15, 25 and 37 °C.

6   **Figure S-3:** The contour of three-dimensional fluorescence spectra of HSA (2 μM) in absence (A

7   and B) and presence (A-I and B-I) of TQ at pH 7.4 and pH 9.0, respectively. [HSA: TQ =1:1].

8   **Figure S-4:** Enzyme kinetics for HSA. The Michaelis-Menton equation based activity against

9   substrate concentrations at a fixed TQ (5μM) and HSA (5μM) concentration of HSA and HSA-

10   TQ complex (1:1) at (A) pH 7.4, (B) pH 9.0, respectively.

11   **Table T-1:** Three-dimensional fluorescence spectra characteristics of HSA-TQ interaction at pH

12   7.4 and 9.0

13   **Table T-2:** Comparison between the antioxidant activity of TQ with ABTS$^{\bullet+}$ at specific

14   concentration (aliquot of 100 μL), and at particular time-points.

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