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Monitoring Fluorescence Emission Behaviors of Dietary Polyphenols in Serum Albumin Environment

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Content	Page No.
1. Experimental section	1-3
1.1. General Information	1
1.2. UV-Vis absorption spectroscopy	1
1.3. Fluorescence spectroscopy	1-2
1.4. Molecular docking	3
2. Supplementary scheme: Scheme S1	4
3. Supplementary figures: Fig. S1-S9	5-11
4. Supplementary tables: Table S1-S2	12-13

1. Experimental Section

1.1. General Information

Albumin from human serum (A1653), morin hydrate (M4008) and kaempferol (60010) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Daidzein (B22877) was obtained from Alfa Aesar. All the other analytical grade chemicals were procured from HiMedia Laboratories and SRL, India. The reagents were used as received without further purification. Due to the low aqueous solubility of the polyphenols, their stock solutions were prepared in ethanol. The stock concentration of the HSA was prepared in 20 mM phosphate buffer (PB) of pH 7.4 whose concentration was ascertained spectrometrically using the molar absorptivity coefficient (ϵ_{280}) as $35,500 \text{ M}^{-1} \text{ cm}^{-1}$.^{1,2} All the wet lab experiments were carried out using 20 mM PB of pH 7.4. The final concentration of ethanol used was less than 3% (v/v) in order to nullify the effect on protein structure.

The experiments were carried out in accordance with the rules and guidelines of National Institute of Technology Meghalaya. No studies were conducted related to living systems e.g. human or animal. The protein used in the study, human serum albumin was procured directly from Sigma-Aldrich (product code: A1653). The funding agency has been acknowledged in the main manuscript and guidelines are followed properly.

1.2. UV-Vis absorption spectroscopy

The UV-Vis absorption experiments were carried out on a PerkinElmer Lambda 35 spectrometer instrument using 1 cm² quartz cuvette using 20 mM PB of pH 7.4 as the reference. For this experiment, 20 μM polyphenol (DZ/ MOR/ KMP) and their 1:1 complex with HSA were incubated for 2 h in PB prior to the recording of the spectra. The spectra were recorded in the range of 200-600 nm.

1.3. Fluorescence spectroscopy

The steady state fluorescence measurements (emission and excitation) were conducted on Jobin Yvon Fluoromax-4 spectrofluorometer (Horiba, Japan) equipt with a temperature controller (Newport, Model 350 B, California, USA) and a quartz cuvette of 1 cm path length. The fluorescence measurements were recorded on exciting the samples at an appropriate wavelength, subsequently recording the emissions, and appropriate blanks were subtracted from respective measurements. The excitation and emission slit widths were fixed at 5/5 nm for all the experiments.

Monitoring the fluorescence emission of the polyphenols in the presence of HSA: For this experiment, 20 μM MOR/KMP in 3 mL PB were titrated successively with HSA (0-35.0 μM) upon exciting the samples at 390 nm and 380 nm, respectively, whereas as DZ is highly

fluorescent, hence a 5 μM , 3 mL solution was titrated with HSA using an excitation wavelength of 340 nm. For the anionic form of KMP, the sample was excited at 415 nm.

Monitoring the excitation fluorescence profiles of the polyphenols in the presence of HSA: For this experiment, 20 μM of the polyphenols and their 1:1 complex with HSA were scanned from 275-520 nm upon setting the appropriate emission maximum (λ_{em}) of the respective polyphenols. The λ_{em} was fixed at 469 nm (DZ), 512 nm (MOR), 535 nm (KMP), and 495 (an anionic form of KMP).

Steady state fluorescence anisotropy: The steady state fluorescence emission anisotropy measurements were carried out in an Agilent Cary Eclipse Fluorescence Spectrophotometer (Varian) fitted with automated polarizer accessory with a fixed slit width of 5/5 nm. For this experiment, 20 μM of polyphenols (in 3 mL solution) were successively titrated with an increasing concentration of HSA. Excitation and emission wavelength maxima of the polyphenols were fixed in the instrument. For DZ and MOR, $\lambda_{\text{ex}}/\lambda_{\text{em}}$ were fixed at 340/469 nm and 390/512 nm, respectively. For, KMP and its anionic form, $\lambda_{\text{ex}}/\lambda_{\text{em}}$ were fixed at 380/535 nm and 415/495, respectively. The fluorescence anisotropy (r) values were determined using the following equation (eqn. 1).³

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I is the fluorescence intensity. VV indicates the excitation and emission polarizers are aligned vertically and VH indicates the excitation polarizer is vertically aligned, and emission polarizer is horizontally aligned. G sensitivity factor of the detection systems, having the following relation (eqn. 2).³

$$G = \frac{I_{HV}}{I_{HH}} \quad (2)$$

Determination of the binding constant (K_b): Fluorometric emission titration experiments were carried out to determine the binding constant (K_b). For this experiment, a 3 μM HSA solution (3 mL) was successively titrated with the respective polyphenols (DZ/MOR/KMP, 0-16.4 μM) at 25 $^\circ\text{C}$. To correct/minimize the inner filter effect (IFE) resulting due to absorption of the ligands, the polyphenols were used in the micromolar range and subsequently corrected by employing the equation and procedure described by Lakowicz.³ The observed emission profiles were analyzed and fitted to the following equation (eqn. 3) to obtain the binding constant (K_b) value.⁴⁻⁶

$$\log \frac{F_0 - F_{\text{cor}}}{F_{\text{cor}}} = n \log K_b - n \log \left[\frac{1}{\left\{ [Q] - \frac{[P_t](F_0 - F_{\text{cor}})}{F_0} \right\}} \right] \quad (3)$$

where F_0 denotes the fluorescence intensity of HSA in the absence of polyphenol and F_{cor} represent the corrected fluorescence intensity in the presence of polyphenols. $[Q]$ and $[P_t]$ represent the quencher (polyphenol) and total protein concentration, respectively.

Warfarin displacement studies: For this experiment, a 1: 1 complex of HSA and warfarin were incubated for 2 h and then titrated successively with polyphenols (0-16.4 μ M) using an excitation wavelength of 308 nm.

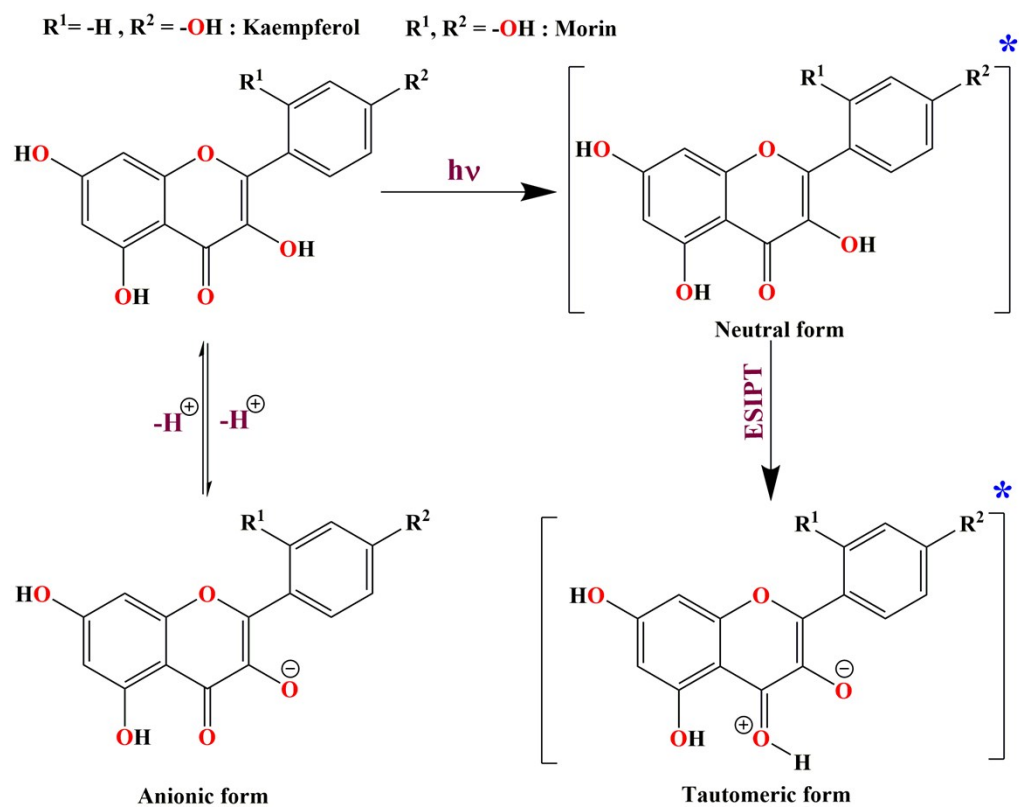
1.4. Molecular docking studies

The molecular docking studies were carried out in Achilles blind docking server (<https://bio-hpc.ucam.edu/achilles/>) that utilizes Vina_vision (http://bio-hpc.eu/software/vina_vision/), a customized version of Autodock Vina. The 3D crystallographic structure of HSA (PDB ID:1AO6) was downloaded from Protein Data Bank,⁷ while the crystallographic coordinates of DZ, MOR and KMP were exported from ChemSpider (<http://www.chemspider.com>) as a .mol file which has been further optimized using Gaussian 09 using B3LYP/6-311G (d,p) and converted to .pdb files. The results were downloaded from the Achilles blind docking server and the docked poses viewed with the help of PyMOL viewer.⁸ The non-covalent forces involved in the interaction process were analyzed with the help of Protein-Ligand Interaction Profiler (PLIP),⁹ while the electrostatic surface potential of the HSA– polyphenol complexes was generated by solving the Poisson– Boltzmann method implemented in the APBS electrostatics (<http://www.poissonboltzmann.org/>) plugin of PyMOL. The accessible surface area calculation was carried out using the NACCESS program.¹⁰

References

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2. Supplementary Scheme



Scheme S1: Schematic representation of ESIP process.

3. Supplementary figures

Fig. S1

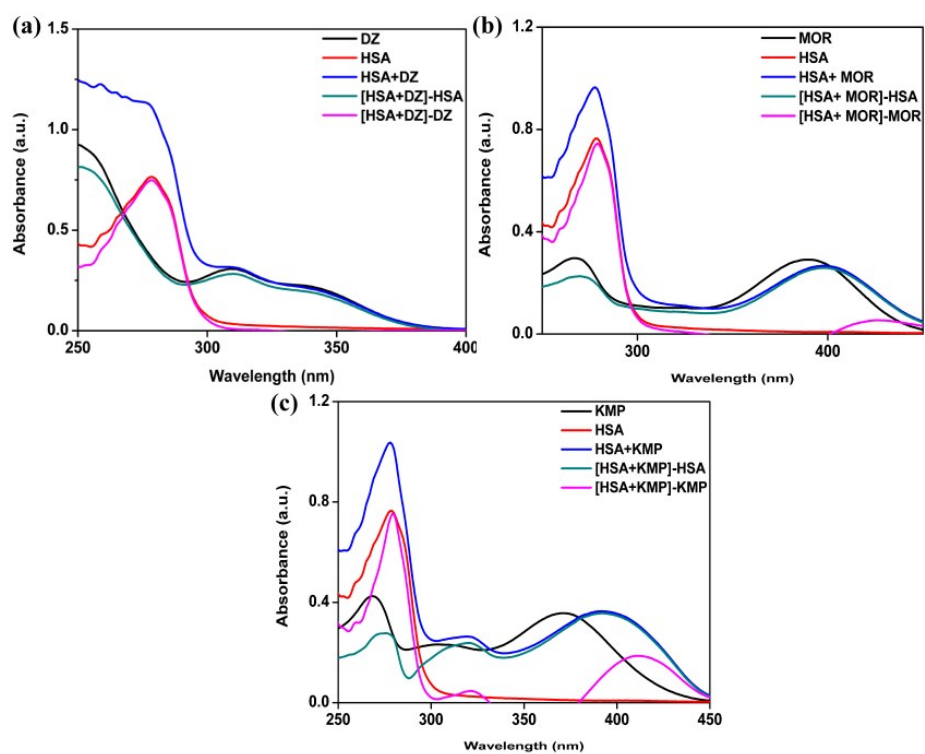


Fig. S1: UV-Vis spectra of (a) DZ, (b) MOR, and (c) KMP in the native and HSA bound states along with the difference spectra in pH 7.4. [HSA]=[Polyphenols]= 20 μ M

Fig. S2

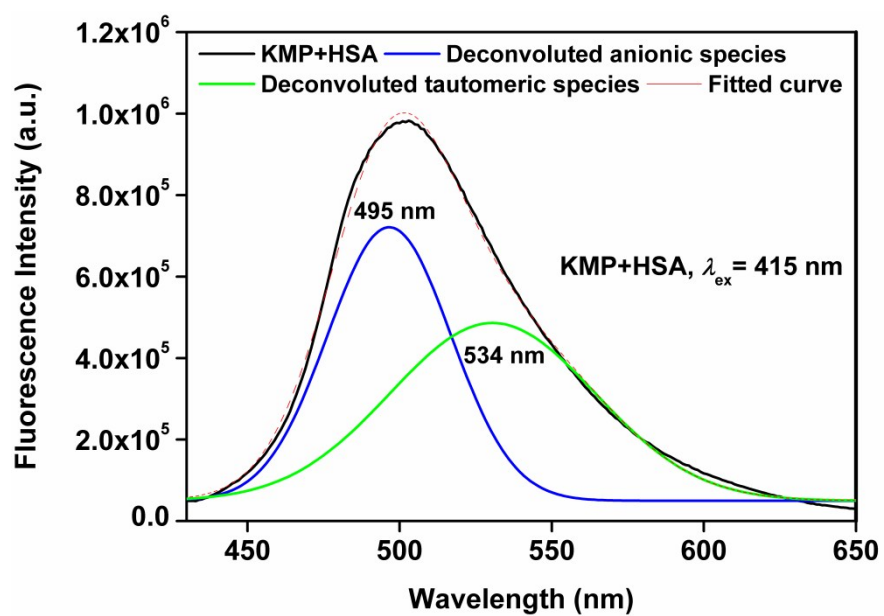


Fig. S2: Deconvoluted spectrum of KMP in the presence of HSA upon excitation at 415 nm.

Fig. S3

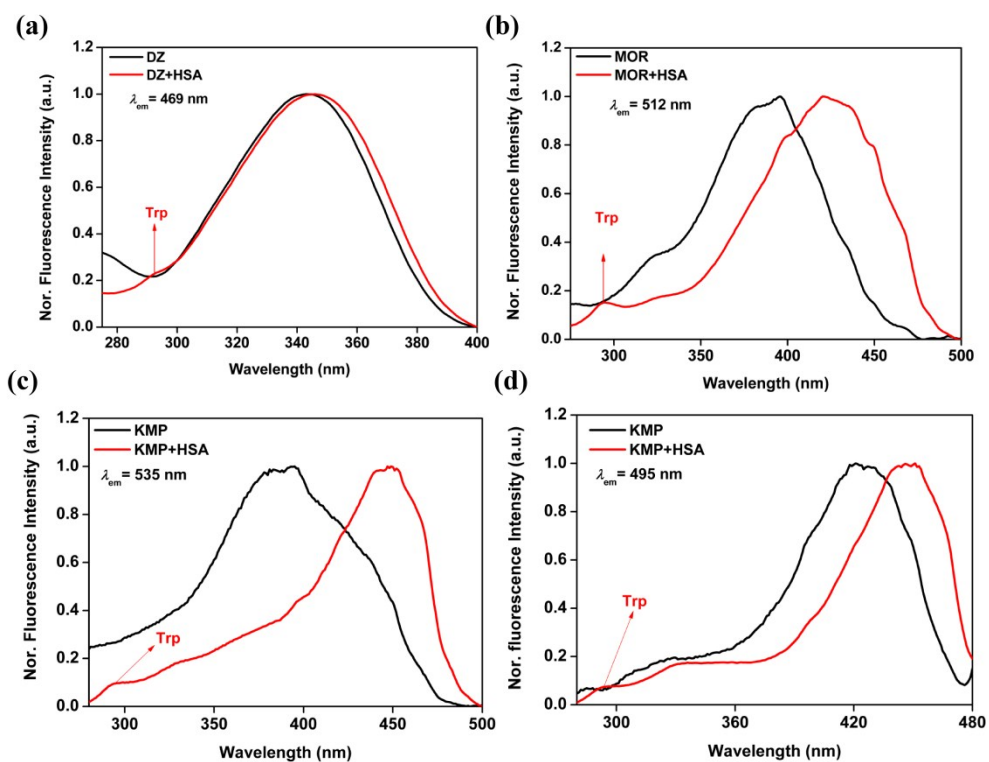


Fig. S3: Normalized excitation spectra of (a) DZ, (b) MOR, and (c) KMP in the native and HSA bound states. (d) The normalized excitation spectrum of anionic form of KMP at $\lambda_{em} = 495$ nm.

Fig. S4

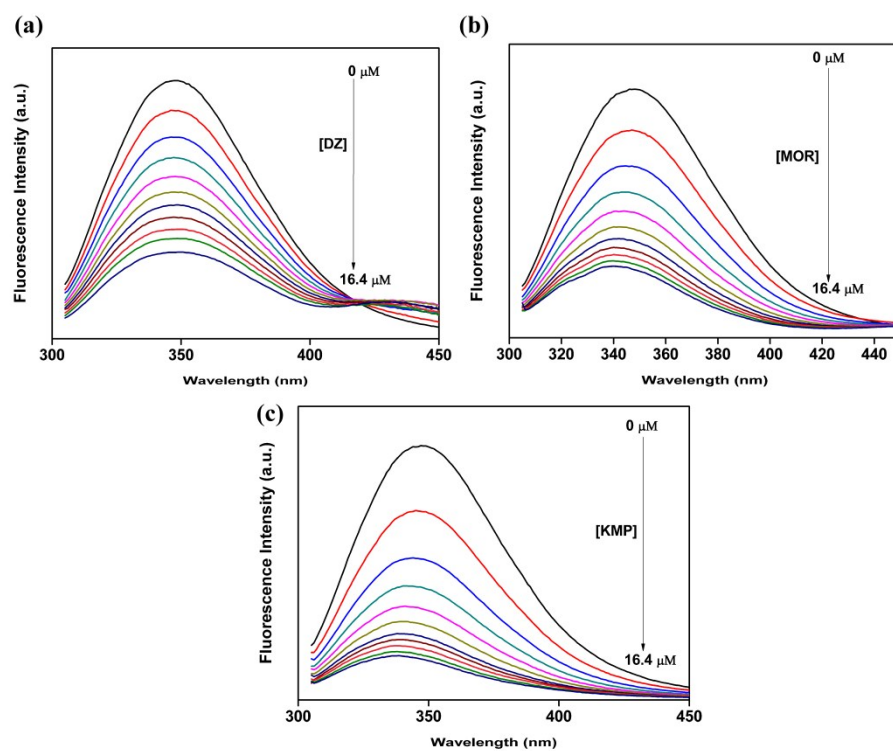


Fig. S4: Modulation in the intrinsic fluorescence profile of HSA upon addition of (a) DZ, (b) MOR, and (c) KMP respectively. $\lambda_{\text{ex}} = 295 \text{ nm}$.

Fig. S5

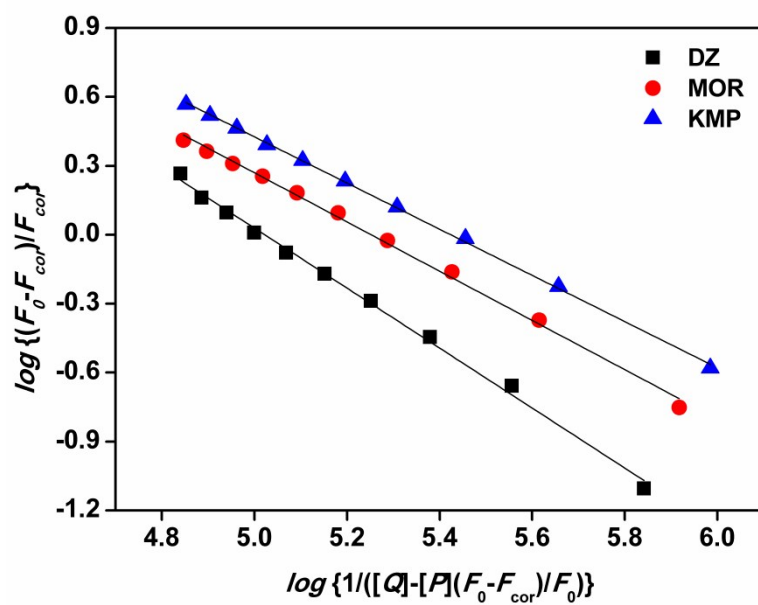


Fig. S5. Double log plot for the determination of the binding constant (K_b) at 25 °C.

Fig. S6

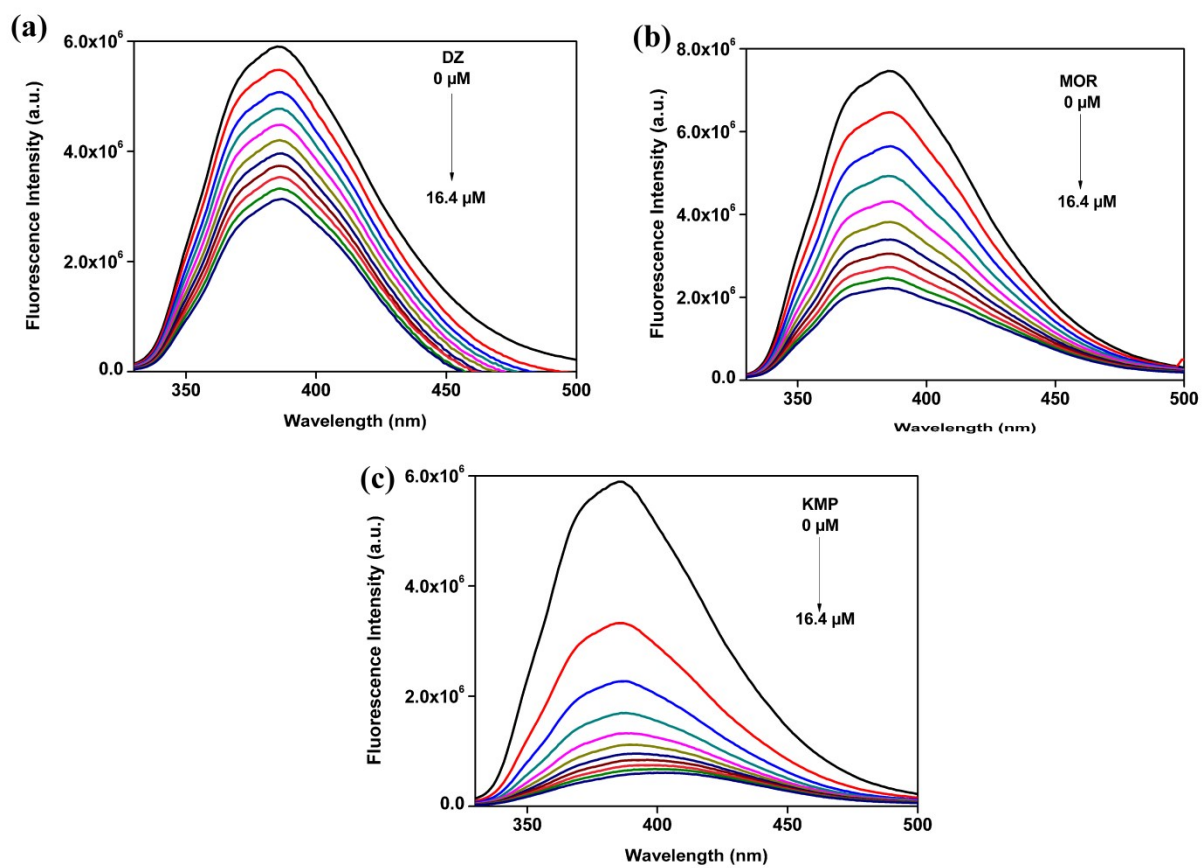


Fig. S6. Site marker displacement emission profile of HSA-warfarin complex upon addition of (a) DZ, (b) MOR, and (c) KMP, respectively in pH 7.4. $\lambda_{\text{ex}} = 308 \text{ nm}$

Fig. S7

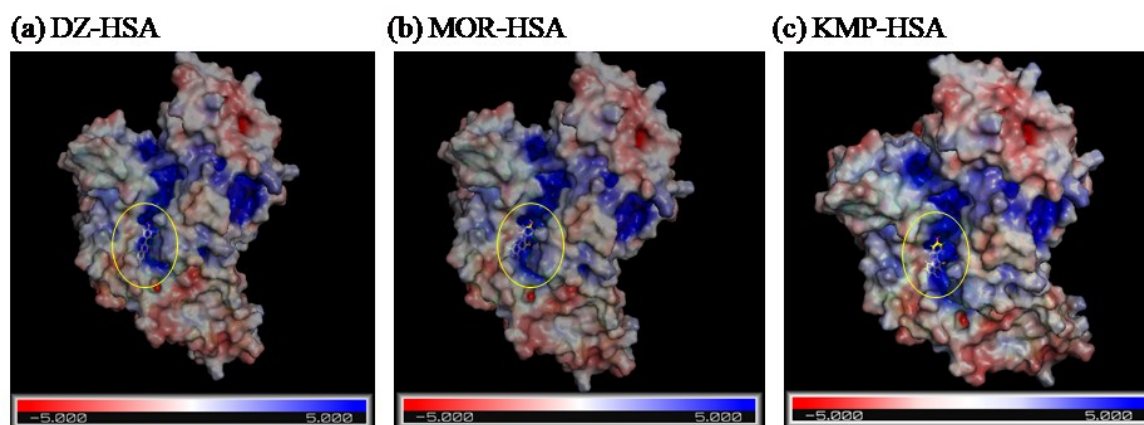


Fig. S7. Electrostatic surface potential of HSA-polyphenol complexes. Bound polyphenol is shown in yellow-colored stick representation (circled). Blue and red colors depict the most positive and negative electrostatic potentials, respectively.

4. Supplementary tables

Table S1. Binding parameters for the interaction of polyphenols with HSA calculated at 25 °C.

System	K_b (10^5, M^{-1})	n	R^2
DZ-HSA	1.058±0.131	1.301	0.996
MOR-HSA	1.778±0.113	1.071	0.996
KMP-HSA	3.539±0.047	1.024	0.998

Table S2. Non-covalent forces involved in the interaction of the polyphenols with HSA from docking studies.

System	Hydrogen Bonds	Hydrophobic interactions	π-Cation interactions	π-stacking interactions
DZ-HSA	Tyr150 Arg222 Arg257 Arg193	Tyr150 Glu153 Leu238 Leu260 Ala291	Lys195	-
MOR-HSA	Ser192 Gln196 (2) Lys199 Arg222 (2) Arg257 (2) Glu292	Glu153 Lys199 Leu238	Lys195	Ty150
KMP-HSA	Tyr150 Ser192 Lys199 Arg257 (2) Ala291	Tyr150 Glu153 Lys199 Leu260	Lys195	-

*(): Digits within the parenthesis represents the number of hydrogen bonds with that residue.