Supporting Information

Lysozyme-luteolin binding: Molecular insights into the complexation process and the inhibitory effects of luteolin towards protein modification

Sourav Das[a], Somdev Pahari[b], Sharat Sarmah[a], Mostofa Ataur Rohman[c], Debojit Paul[d], Madhurima Jana[b,*], Atanu Singha Roy[a,*]

[a]Department of Chemistry, National Institute of Technology, Meghalaya, Shillong 793003, India

[b]Molecular Simulation Laboratory, Department of Chemistry, National Institute of Technology, Rourkela 769008, India

[c]Centre for Advanced Studies, Department of Chemistry, North-Eastern Hill University, Shillong 793022, India

[d]Department of Chemistry, Indian Institute of Technology Guwahati, Guwahati 781039, India

***Corresponding Author:** Atanu Singha Roy
Tel.: +91 364 2501294
Fax: +91 364 2501113
Email: singharoyatanu@gmail.com; asroy86@nitm.ac.in

***Corresponding Author:** Madhurima Jana
Tel.: +91 661-2462663
Fax: +91 661-2462651
Email: janam@nitrkl.ac.in

Detailed Methodologies

S1. Preparation of the samples

S1.1. Preparation of HEWL and luteolin solutions

HEWL crystals have been dissolved in 20 mM Tris-HCl buffered solution (pH 7.4), and its concentration was determined spectrophotometrically using the molar absorption coefficient, ε_{280}= 37646 M^{-1} cm^{-1}.[1] Luteolin was dissolved in ethanol and the experiments were performed in such a way that the concentration of ethanol was maintained below 3 % (v/v) in all cases.

S1.2. Preparation of samples for HEWL glycation studies

HEWL (1 mg/mL) samples have been incubated in 0.1 M phosphate buffer (pH 7.4) containing 1 mM sodium azide at 37 °C for 21 days in the absence and presence of 0.1 M D-ribose and luteolin (150 μM). The native HEWL incubated without D-ribose or luteolin for 21 days under the same condition as the control. At different intervals of time aliquots have been taken from the reaction mixtures for fluorescence studies. The Trp fluorescence and the formation of an AGE, malondialdehyde have been monitored by using excitation wavelengths of 295 nm and 370 nm, respectively.

S1.3. Preparation of samples for HEWL amyloid fibrillation studies

The amyloid fibrillation process has been carried out by dissolving HEWL (2 mg/mL) in 50 mM Glycine-HCl buffer (pH= 2.20) in the absence or presence of luteolin (100 μM and 200 μM).[2] Sodium azide (1 mM) was added to these solutions in order to avoid any bacterial growth. The solutions were incubated at ~60 °C under continuous stirring at 200 rpm for 72 h the solutions, and vortexed at regular intervals to homogenize the samples. The kinetics of fibrillation has been monitored using ThT fluorescence assay. For this, 130 μM aliquots of samples have been taken out at different intervals of time and diluted with buffer in 3 mL

containing 10 μM of ThT. The samples were excited at 440 nm and the emission maximum was monitored at 485 nm.

S2. Experimental techniques

S2.1. Steady state and excited state fluorescence measurements.

A Cary Eclipse spectrofluorimeter (Agilent Technologies) fitted with a PCB 1500 water Peltier system from and a Fluoromax-4 Jobin Yvon (Horiba Scientific, Japan) equipped with Newport temperature controller (Model 350 B, California, USA) has been used to carry out the steady state emission fluorescence studies in 1 cm path length quartz cells. Temperature dependent intrinsic fluorescence studies have been carried out on the Cary Eclipse spectrofluorimeter. A 3 μM HEWL solution was titrated successively with luteolin solution (0-16.4 μM) at multiple temperatures (293, 300, 307 and 313 K) by selective excitation of Trp at 295 nm. The emission spectra have been collected in the region 305-450 nm using a slit width of 10/5 nm (excitation/emission) along with a slow scan speed.

Three dimensional (3D) fluorescence measurements have been performed on the same instrument at 300 K. For this purpose, HEWL (3 μM) and its 1:4 complex with luteolin were scanned in the emission wavelength range of 250-500 nm with 1 nm interval. Simultaneously, the excitation wavelength was fixed at 200-380 nm with 5 nm increments and a slow scan speed was maintained. Synchronous fluorescence (SFS) measurements have been carried out on the Fluoromax-4 instrument using a slit width of 5/5 nm at 300 K and SFS spectra were collected with fixed offsets $\Delta\lambda$= 15 nm (for Tyr) and 60 nm (for Trp) respectively.

The red edge excitation shift (REES) effect has also been executed on Fluoromax-4 instrument by exciting 3 μM HEWL and its 1:5 complex with luteolin at 295 nm and 305 nm

respectively keeping a slit width of 5/5 nm. Thermal melting studies have been performed for HEWL and its 1:5 luteolin complex by monitoring Trp fluorescence emission (λ_{ex}= 295 nm) in the temperature range of 298-363 K with an interval of 5 K, and a reverse process was applied for cooling the sample to 298 K. The experiment was performed in the Fluoromax-4 unit with slit width of 5/5 nm.

Excited state lifetime measurements for HEWL and HEWL-luteolin complexes have been carried out on a Pico Master Time-correlated single photon counting (TCSPC) lifetime instrument (PM-3) from Photon Technology International (PTI), USA. An excitation wavelength of 295 nm has been used to excite the samples using a LED source and the emission profiles have been collected at a magic angle of 54.7°. The instrument response function (IRF) has been determined by a colloidal suspension of coffee whitener. The emission data have been collected at 340 nm for all the samples.

S2.2. UV-vis absorption measurements

The UV-vis measurements have been performed on a PerkinElmer Lambda 365 spectrophotometer using a quartz cuvette of 1 cm path length in the range of 250-500 nm. To investigate the ground state complexation, a 20 μM HEWL and it's 1: 1 luteolin complex have been scanned in the range of 250-500 nm in 20 mM Tris HCl buffer of pH 7.4.

The enzymatic assay has also been carried out in the same instrument by monitoring the change in absorbance at 450 nm (ΔA_{450}) over a time period of 400 s. A solution of 0.15 mg/mL *M.lytus* has been prepared in Tris HCl buffer and the solutions containing 10 μM HEWL in the absence and presence of different molar ratios of luteolin (1:0, 1:5 and 1:10) have also been incubated. Prior to the recording of enzyme kinetics, the *M.lytus* solutions have been mixed with HEWL solutions separately and placed in the cuvette holder for measurements.

S2.3. Circular Dichroism studies

The far UV CD spectra have been recorded on a Jasco 815 CD spectrophotometer using a 0.1 cm quartz cuvette in the range of 190-240 nm. The CD spectra have been collected using a scan rate of 100 nm/min and response time of 4 s. In this experiment, solutions containing HEWL (20 μM) and its 1:2 luteolin complex with luteolin were scanned in between 190-240 nm. The percentages of different secondary structural components have been estimated using an online server, DICHROWEB. The far UV CD spectra for the glycation and fibrillation processes have been measured on a JASCO-J1500 spectrometer with data interval of 0.5 nm using a 0.1 cm quartz cuvette and a scan speed of 100 nm/min.

S2.4. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of native HEWL and its luteolin complex have been collected on a PerkinElmer Spectrum Two instrument fitted with a single reflection diamond universal attenuated total reflectance (UATR) accessory having 0.5 cm^{-1} spectral resolution. A solution of HEWL (5 mg mL^{-1}) has been prepared in Tris HCl buffer (pH 7.4) and its concentration determined spectrophotometrically, based on its concentration a 1:2 complex of HEWL-luteolin has also been prepared and incubated for 2 h prior to the recording of a spectrum. A 256 scan interferogram was used along with a 4 cm^{-1} resolution obtaining the individual spectrum and each of them corrected by subtracting the corresponding blank.

The method reported by Byler and Susi has been utilized to assess the secondary structural components of native and luteolin bound HEWL.[3,4] The more conformationally sensitive amide I band region (1600–1700 cm^{-1}) has been analyzed for obtaining the secondary structural components of HEWL with the help of Savitzky-Golay 15-point smoothing function. The Fourier self-deconvolution along with second derivative methods has been used to resolve the major peaks of the smoothed spectra. The percentages of α-helix, β-sheet, turn

and coil have been estimated using Gaussian curve fitting procedure in the region 1600-1700 cm^{-1}.

Reference:

(1) Pace, C. N.; Vajdos, F.; Fee, L.; Grimsley, G.; Gray, T. How to Measure and Predict the Molar Absorption Coefficient of a Protein. *Protein Sci.* **1995**, *4* (11), 2411–2423.

(2) He, J.; Wang, Y.; Chang, A. K.; Xu, L.; Wang, N.; Chong, X.; Li, H.; Zhang, B.; Jones, G. W.; Song, Y. Myricetin Prevents Fibrillogenesis of Hen Egg White Lysozyme. **2014**, *62* (39), 9442–9449.

(3) Byler, D. M.; Susi, H. Examination of the Secondary Structure of Proteins by Deconvolved FTIR Spectra. *Biopolymers* **1986**, *25* (3), 469–487.

(4) Susi, H.; Byler, D. M.; Purcell, J. M. Estimation of β-Structure Content of Proteins by Means of Deconvolved FTIR Spectra. *J. Biochem. Biophys. Methods* **1985**, *11* (4–5), 235–240.

Fig. S1

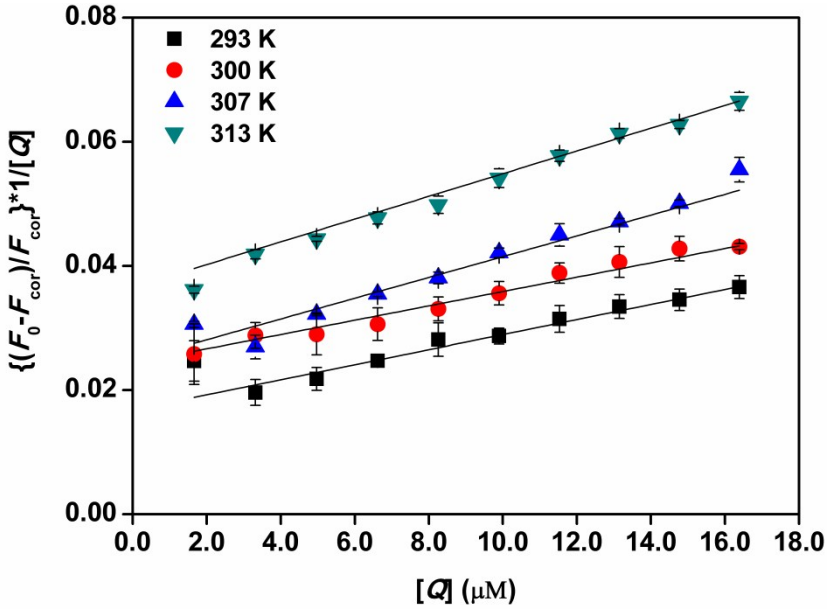

Fig. S1: Regression plot for the interaction of luteolin with HEWL

Fig. S2

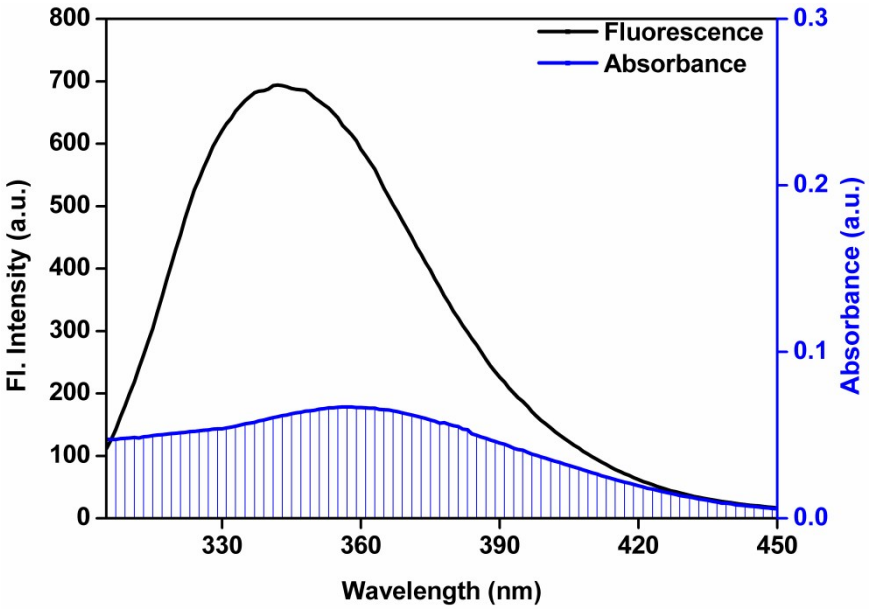

Fig. S2: Spectral overlap of the HEWL emission spectrum with the absorption profile of luteolin. [HEWL] = [Luteolin]= 3 μM

Fig. S3

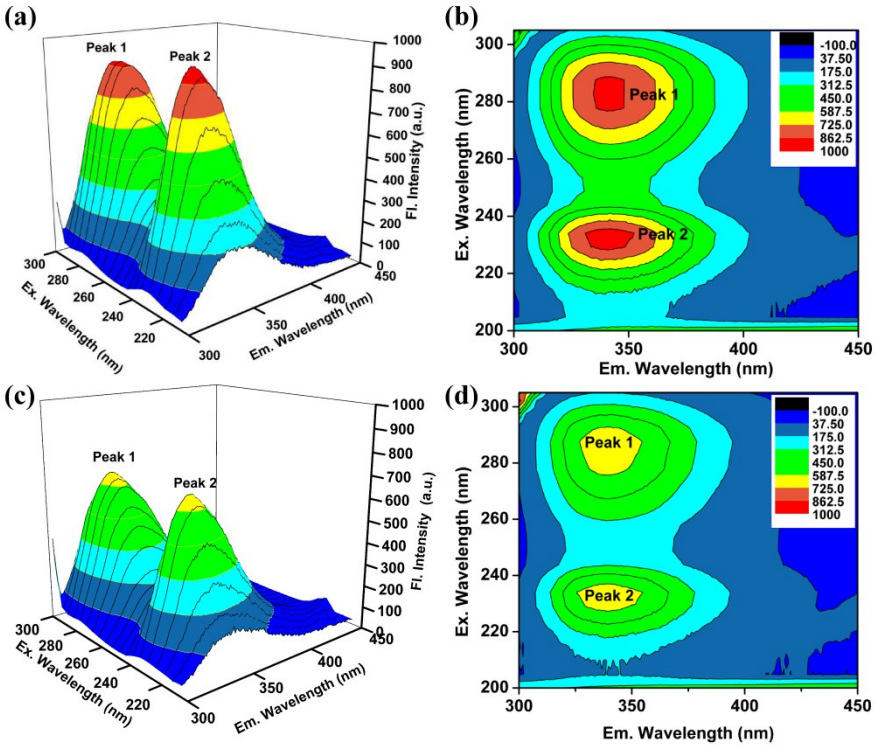

Fig. S3 3D fluorescence spectral profile for (a) HEWL (3 µM), and (c) HEWL-luteolin complex (3:12 µM/ µM) in 20 mM Tris-HCl buffer of pH 7.4. (b) and (d) illustrates the corresponding 2D contour plots of HEWL and HEWL-luteolin complex respectively.

Fig. S4

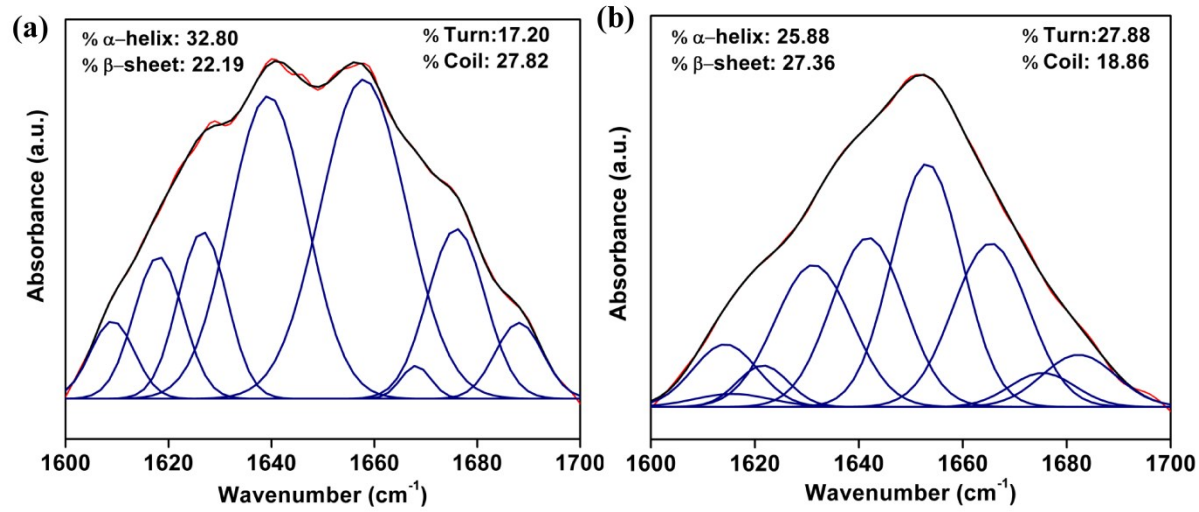

Fig. S4: Deconvulated FTIR curve fitted amide I peak (1600-1700 cm^{-1}) of (a) HEWL, and (b) HEWL-luteolin complex in the molar ratio of 1:2 in Tris HCl Buffer of pH 7.4.

Fig. S5

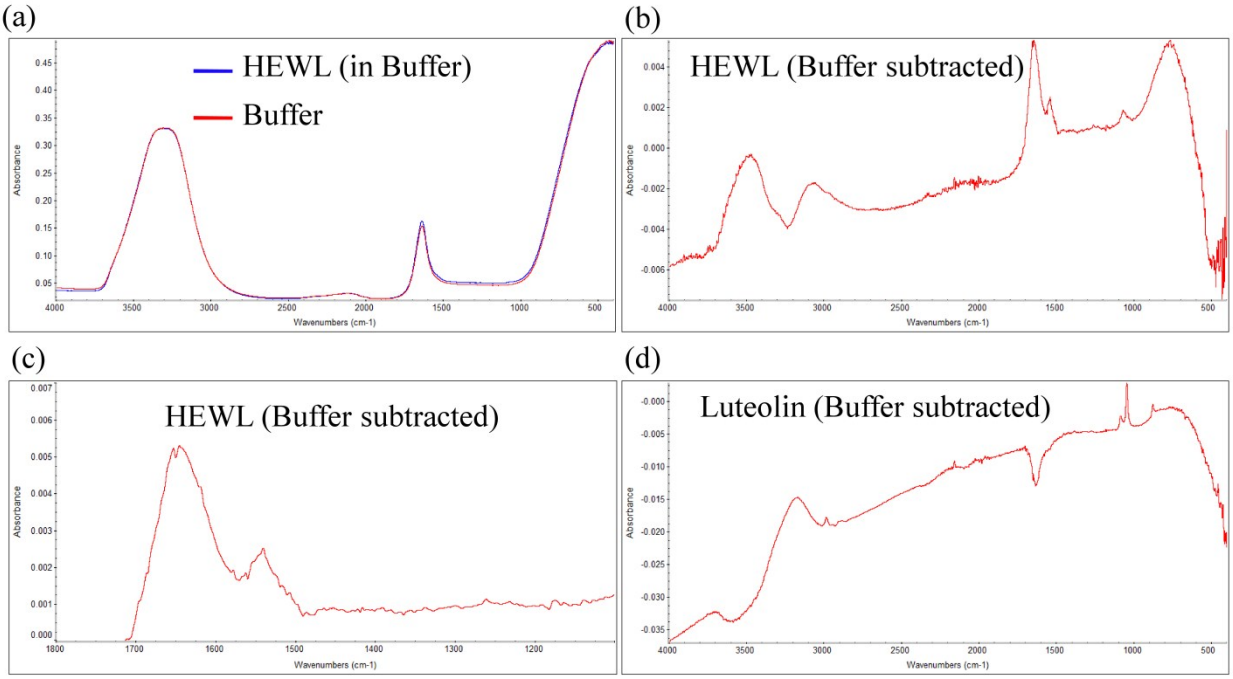

Fig. S5: FTIR spectra of (a) native HEWL and Tris HCL buffer of pH 7.4. (b) HEWL after subtracting the buffer spectrum in the region of 4000-400 cm^{-1}. (c) HEWL spectrum in the expanded region of 1800-1400 cm^{-1}. (d) Luteolin spectrum after buffer subtraction.

Fig. S6

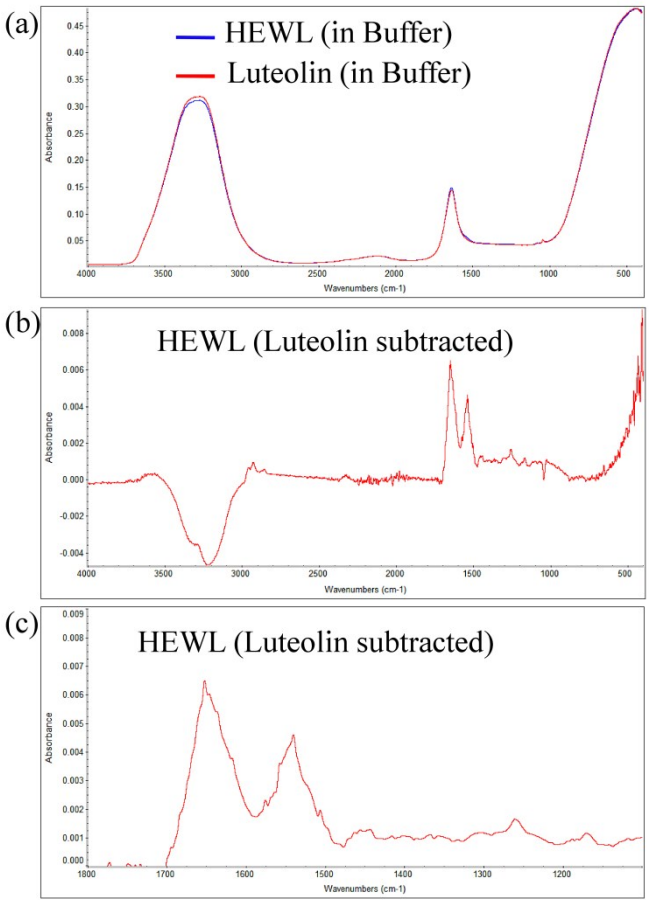

Fig S6: FTIR spectra of (a) native HEWL and luteolin in 20 mM Tris HCl buffer, (b) HEWL after subtracting the luteolin spectrum in the region of 4000-400 cm^{-1}, and (c) HEWL (luteolin subtracted) in the expanded region of 2000-1400 cm^{-1}.

Fig. S7

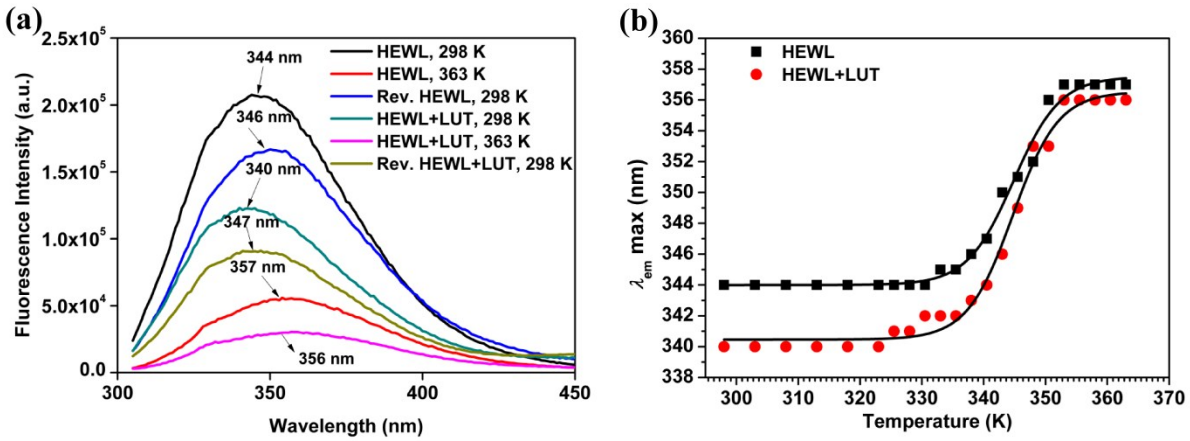

Fig. S7: (a) Variation in the emission profiles of native and unfolded HEWL (at 298 K and 363 K respectively) and its complex with luteolin (molar ratio 1:5) along with its reversibility spectra. (b) Thermal melting profile of HEWL and its complexes with luteolin respectively. [HEWL]=3 μM, λ_{ex}=295 nm. *LUT=luteolin.

Fig. S8

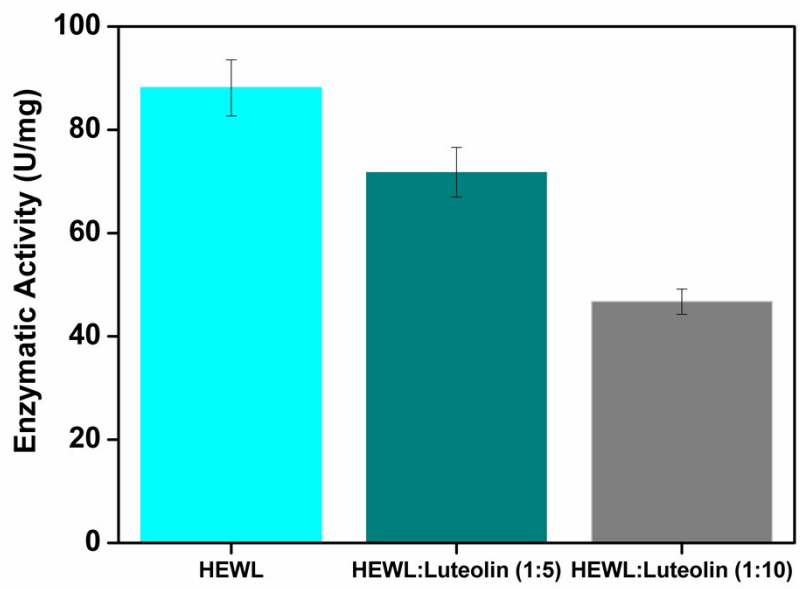

Fig. S8: Influence of luteolin on the enzymatic activity of HEWL in 20 mM Tris-HCl buffer of pH 7.4. [HEWL]= 10 µM, [*M. lytus*]= 0.15 mg mL^{-1}.

Fig. S9

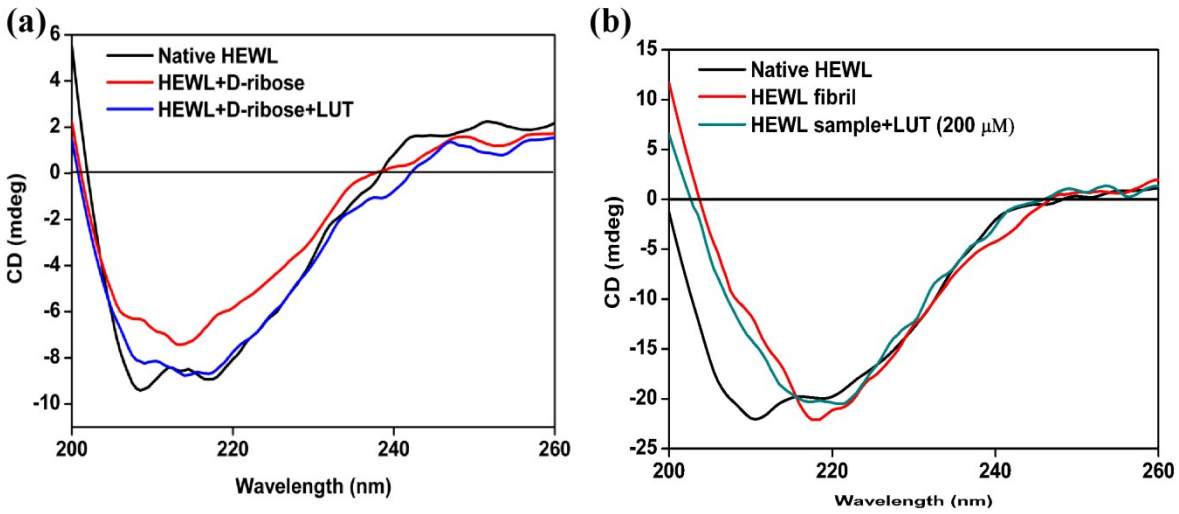

Fig. S9: (a) Far UV CD spectra native HEWL, glycated HEWL (D-ribose mediated) and influence of luteolin on the glycation property of HEWL. (b) Far UV CD spectra of native HEWL, HEWL fibril and the influence of luteolin on the fibrillation property of HEWL.*LUT= luteolin

Note: For Glycation, 100 µL of the sample was used from the 1 mg/ mL stock solution.

For Fibril, 100 µL of the sample was used from the 2 mg/ mL stock solution.

Fig. S10

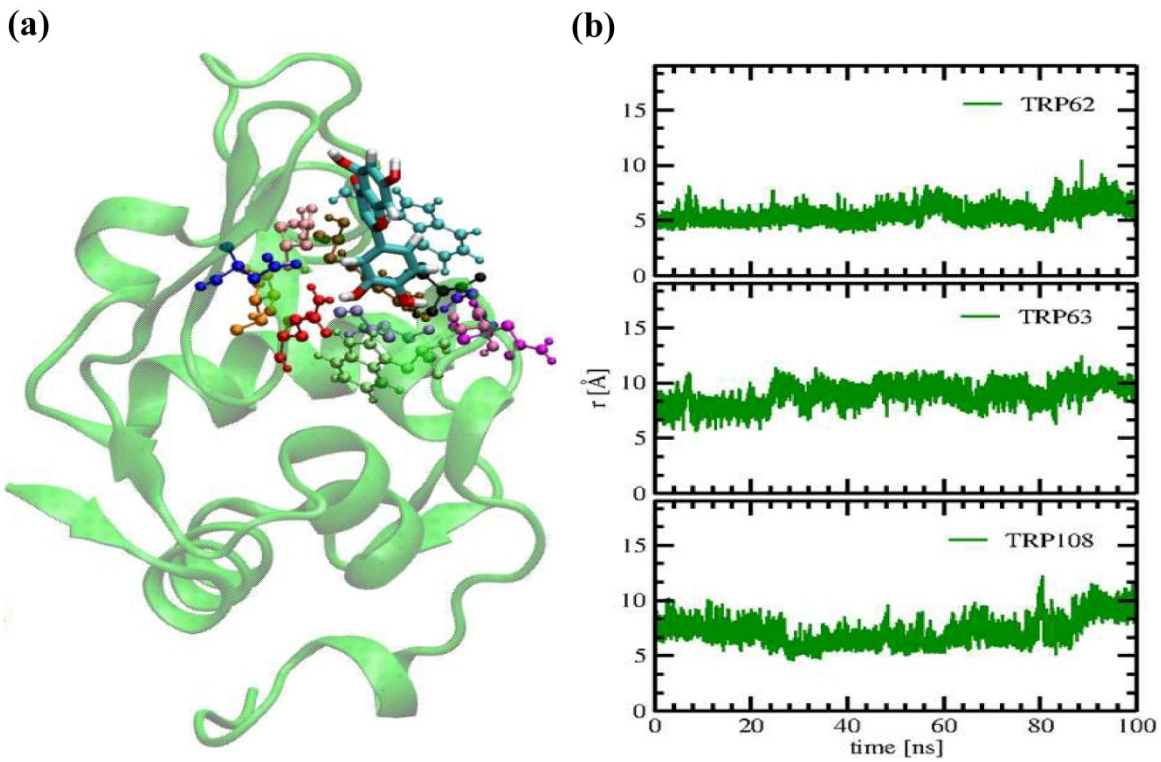

Fig. S10: (a) Spatial position of the residues present in the active cleft region of the protein (time frame = 0 ns, residue sequence as stated above blue, red, orange, pink, cyan, ochre, ice blue, black, violet, magenta, mauve, lime). (b) Distance of Trp62, Trp63, and Trp108, as a function of time.

Table S1: Stern Volmer parameters for the interactions of luteolin with HEWL at different temperatures.

Temp. (K)	K_{SV} (10^4, M^{-1})	k_q (10^{13}, $M^{-1}s^{-1}$)
293	2.54±0.19	1.55
300	3.12±0.24	1.91
307	3.54±0.04	2.17
313	4.54±0.01	2.78

*±standard error

Table S2: Characteristic 3D fluorescence spectral parameters associated with native HEWL and its complex with luteolin.

System	Peak 1 (nm)		Stokes shift $\Delta\lambda$ (nm)	Intensity (a.u.)	Peak 2 (nm)		Stokes shift $\Delta\lambda$ (nm)	Intensity (a.u.)
	λ_{ex}	λ_{em}			λ_{ex}	λ_{em}		
HEWL	280	342	62	894	235	339	104	938
HEWL-luteolin	285	338	53	672	235	338	103	681

Table S3: Docking summary of HEWL with luteolin by the Autodock 4.2 program generating different ligand conformations with the help of Lamarckian GA.

Rank	Binding energy (kcal mol^{-1})	K_i (µM)	K_b (10^5, M^{-1})	Cluster RMS	Ref. RMS
1	-7.43	3.55	2.55	0.00	36.14
2	-7.42	3.65	2.51	0.10	36.14
3	-7.40	3.74	2.42	0.15	36.12
4	-7.39	3.82	2.38	0.19	36.16
5	-7.34	4.20	2.19	0.39	36.17
6	-7.31	4.35	2.08	0.99	36.02
7	-7.22	5.13	1.79	0.96	36.06
8	-7.14	5.81	1.56	0.91	36.10
9	-7.00	7.44	1.24	0.86	36.26
10	-6.92	8.50	1.08	1.60	35.62

*K_b calculated at 300 K

Table S4: The percentage probability of formation of hydrogen bond numbers between HEWL and luteolin, as obtained from the simulation of HEWL-luteolin complex

Number of hydrogen bonds	Percentage probability
0	24.38
1	43.75
2	25.90
3	5.30
4	0.62
5	0.05