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# A quercetin-based flavanoid (rutin) reverses amyloid fibrillation in β-lactoglobulin at pH 2.0 and 358 K

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ABSTRACT

β-lactoglobulin (BLG) is a well characterized milk protein and a model for folding and aggregation studies. Rutin is a quercetin based-flavanoid and a famous dietary supplement. It is a potential protector from coronary heart disease, cancers, and inflammatory bowel disease. In this study, amyloid fibrillation is reported in BLG at pH 2.0 and temperature 358 K. It is inhibited to some extent by rutin with a rate of 99.3 $h^{-1}$ $M^{-1}$. Amyloid fibrillation started taking place after 10 h of incubation and completed near 40 h at a rate of $16.6 \times 10^{-3}$ $h^{-1}$, with a plateau during 40–108 h. Disruption of tertiary structure of BLG and increased solvent accessibility of hydrophobic core seem to trigger intermolecular assembly. Increase in 7% β-sheet structure at the cost of 10% α-helical structures and the electron micrograph of BLG fibrils at 108 h further support the formation of amyloid. Although it could not block amyloidosis completely, and even the time required to reach plateau remains the same, a decrease of growth rate from $16.6 \times 10^{-3}$ to $13.5 \times 10^{-3}$ $h^{-1}$ was observed in the presence of 30.0 μM rutin. Rutin seems to block solvent accessibility of the hydrophobic core of BLG. A decrease in the fibril population was observed in electron micrographs, with the increase in rutin concentration. All evidences indicate reversal of fibrillation in BLG in the presence of rutin.


## 1. Introduction

Human amyloidosis is accompanied by modification in protein structure from a normal folded form to non-productive folded structure, i.e., amyloid fibril. Amyloid fibrils are directly involved in several neuronal diseases such as Parkinson's, Alzheimer's, systemic amyloidosis, Creutzfeldt-Jakob diseases and Type II diabetes [1,2]. In addition to neurodegenerative diseases, amyloid fibrils are also deposited in several other tissues and organs and cause other non-neurogenic pathologies [3,4]. Both proteins (pathogenic and non-pathogenic) differ in their native structure due to differences in amino acids sequence but fibril structures of almost all proteins exhibit similar ultra-structure and possess similar biochemical properties [5]. Amyloid fibrils are thermodynamically more stable compared to normal protein oligomer and inhibition or disaggregation of amyloid fibrils is a challenging problem in the biomedical sciences [6]. Amyloid-related diseases affect millions of people every year; therefore- there is a huge demand to discover drugs for their treatment. The complete treatment for both prevent and dissolve of fibrils structures into monomer or smaller non-toxic species. Currently, many researchers are involved in dissolution and inhibition of amyloid fibrils by small molecules and drugs [7,8]. However, several small molecules, particularly melatonin, curcumin, vitamin D, glycosaminoglycans, quercetin and apomorphine derivatives have shown anti-amyloidogenic activity against numerous proteins [9–11]. Various polyphenols like curcumin, myricetin, epigallocatechin gallate and resveratrol have now been shown to inhibit the amyloid fibrillation pathways [12,13]. Detailed insight in small molecules-induced amyloid fibril inhibition is still lacking. Therefore, it will be interesting to see the effects of polyphenols in amyloid fibril inhibition.

Rutin ($C_{27}H_{30}O_{16}$; MW 610.5 $g \cdot mol^{-1}$) is a polyphenol with a wide range of biological activities. Rutin is comprised of quercetin and the disaccharide rutinose i.e., rhamnose and glucose (Fig. 1A). Rutin is present in various plants (buckwheat seeds), fruits (citrus fruits) and vegetables [14,15]. Rutin is an important flavonoid in foods and has several pharmacological properties such as anti-carcinogenic, cytoprotective,

*Abbreviations:* BLG, β-lactoglobulin; ThT, Thioflavin-T; ANS, 8-anilino-1-naphthalene sulfonic acid.

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antithrombic, and cardio protective activities [16]. Rutin scavenges free radicals and lessens stress of cells. Rutin can also inhibit Aβ aggregation, minimize the production of ROS, MDA, NO, iNOS, prevent mitochondrial damage, and reduce cytotoxicity [17]. From an in vitro study was found that rutin protects neuronal cells, damaged by amylin aggregation [18]. Rutin and quercetin weaken and reverse the Aβ25–35 fibrillogenesis under in vitro [19]. Herein, we have examined the inhibitory effects of rutin on β-lactoglobulin (BLG) fibrillation.

BLG is a whey protein found in cow milk that belongs to the lipocalin superfamily (Fig. 1B). The lipocalin family has a common central β-barrel calyx, assembled from eight antiparallel β-sheets that provide suitable sites for hydrophobic ligands binding [20]. BLG possesses two disulfide bridges and one free thiol group. In the native state, BLG is predominantly a β-sheet protein containing nine β-strands and three α-helices. The core of the protein is formed by a flattened β-barrel (a calyx) composed of eight-antiparallel β-strands (A-H). For non-polar ligands two sites have been postulated; one inside the calyx and the other at the dimer interface, on the outer surface of the protein between the α-helix and the β-barrel [21].

The true function of BLG is unknown, but it has been suggested that it is involved in the transport of retinol, fatty acids, cholesterol and vitamin D. BLG exists in six pH-dependent states. At physiological pH, bovine BLG is a dimer with each monomer consisting of 162 amino acids and MW of 18,350. The pK of rutin is 4.3, so at pH 2.0, rutin will be cationic [22]. Similarly, the pI of BLG~5.3, thus it will also be cationic at pH 2.0. Bovine BLG is dimeric over the pH range of 3.5–7.5 [23]. Below pH 3.5, the dimer dissociates into monomer due to electrostatic repulsion, but native conformation remains intact. Between pH 2.0 and 9.0, no change in native like β-barrel conformation has been observed. Monomers of BLG are more stable than native state [24].

BLG forms gel at certain conditions such as high temperature and high hydrostatic pressure and in the presence of denaturants [25,26]. BLG gel is utilized to enhance the quality of the food and its products [27]. Interestingly, gelation is also found in other proteins, which leads to amyloid fibril formation [28]. BLG transforms into an amyloid-like structure composed of networks of long filaments with diameter around 4–10 nm [29]. Generally, amyloid fibrils are formed in slightly mild conditions, while BLG forms amyloid fibrils at extreme conditions: high temperature (>75 °C), extremely acidic pH or in the presence of high urea concentrations [30,31]. BLG is thoroughly used in food industries because of its fibril-forming property. BLG fibrils can increase the viscosity and microcapsulation in food products [32]. Interestingly, it shows an unusual but important intermediate with only alpha helices, despite the fact that the native structure is β-sheeted. Evolution has probably selected the helical intermediate to avoid aggregation during the folding process [33].

The docking study of rutin binding on BLG has been done by our group with Schrodinger suite (Desmond, Schrödinger, LLC, New York, NY, 2017). The docking results showed that the docking complex of rutin with BLG was stabilized by six hydrogen bonds at Site 1 and seven hydrogen bonds at Site 2. The thirteen hydrophobic residues are involved in hydrophobic interaction at Site 1 while at Site 2 only nine hydrophobic residues are involved in the hydrophobic interaction [34].

In the current work, we have induced amyloid fibrils in BLG at 358 K and deciphered the amyloid fibril inhibitory action of rutin at the same temperature at pH 2.0. We have characterized the inhibitory action of rutin by utilizing several biophysical techniques. The inhibitory mechanism may provide better insight into the flavanoids used in amyloid fibrillation inhibition. This study can also be utilized in the area of amyloid biology and food industry.

## 2. Materials and methods

### 2.1. Materials

BLG from bovine (lot # SLBP8394 V), Thioflavin-T, Glycine–HCl, 8-Anilino-1-naphthalenesulfonic acid (ANS) and Tris-HCL were procured from Sigma Chemicals Co. (St. Louis, MO, USA). Rutin was purchased from BDH biochemical. Other chemicals used in this study are of analytical grade. Milli-Q water was used throughout the study.

### 2.2. pH measurement

pH was measured of every solution by using a Mettler Toledo Seven Easy pH meter (model S20) that was regularly calibrated with provided standard buffers. All buffer solutions used in this study were filtered through a 0.45 μm syringe filter.

### 2.3. Preparation of BLG and rutin stock solutions

BLG was dissolved in 20.0 mM Tris-HCl buffer, pH 7.4, and filtered through a 0.45-micron syringe filter. After filtration, the concentration of BLG was calculated by spectrophotometer using molar extinction coefficient 17,600 $M^{-1}$ $cm^{-1}$ [35]. The BLG stock concentration was taken at 272.0 μM in 20.0 mM glycine-HCl buffer (pH 2.0). Rutin (5.0 mM) was dissolved in 99% ethanol and further diluted in working buffer (20.0 mM glycine-HCl, pH 2.0). The absorbance maxima of rutin were at 260 nm and 358 nm (Suppl Fig. S4). The concentration of rutin was calculated by spectrophotometer using molar extinction coefficient $\varepsilon = 18{,}800 \pm 900$ $M^{-1}$ $cm^{-1}$ at 358 nm [36].

### 2.4. Method of amyloid induction in BLG protein

Amyloid fibril was induced in BLG protein according to published reports [37]. Briefly, BLG (272.0 μM) was incubated at pH 2.0 at 358 K for 108.0 h in a thermomixer at 400 rpm.

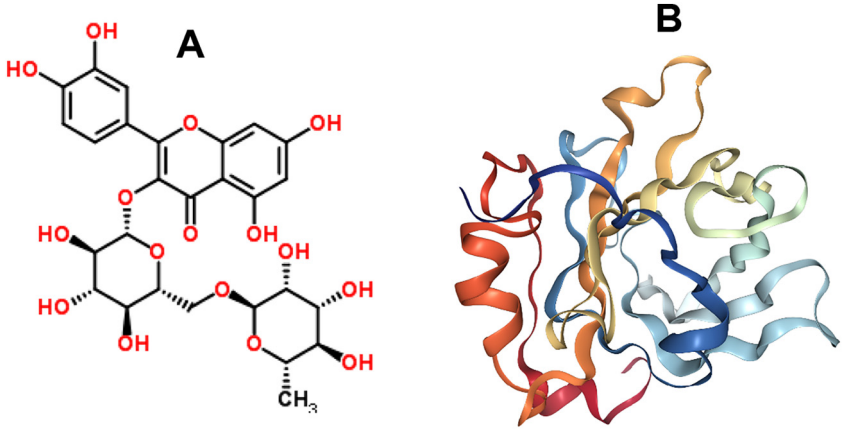


**Fig. 1.** (1A) Rutin [Chemspider ID: 4444362] and [1B] β-lactoglobulin [PDB1CJ5].

### 2.5. Thioflavin T (ThT) binding assay

ThT stock was made by dissolving in Milli-Q water and filtering through a 0.45-μm syringe filter. The concentration of ThT was calculated using a molar extinction coefficient $\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$ at 412 nm. The BLG was dissolved in 20.0 mM glycine-HCl buffer pH 2.0 and the stock concentration of BLG was taken at 272.0 μM. The BLG (272.0 μM) was mixed with and without different concentrations of rutin (10.0, 20.0 and 30.0 μM) at pH 2.0 and incubate at 358 K for 108 h in a thermomixer at constant stirring at 400 rpm. In all, 40.0 μl were picked up from BLG (272.0 μM) stock and mixed into 1.0 ml of respective buffer. Now the BLG working concentrations was 10.88 μM. The ThT (10.88 μM) was added to every sample (which contained 10.88 μM BLG and different concentrations of rutin and was incubated for 0.5 h in the dark. The ThT spectra were scanned in the wavelength range of 450–600 nm after excitation at 440 nm. The excitation and emission slit width were set at 5.0 nm. The ThT fluorescence intensity at 482 nm of samples of BLG alone and with different concentrations (10.0, 20.0 and 30.0 μM) of rutin was plotted against time in hours.

### 2.6. Aggregation growth rate determination

ThT fluorescence of BLG against time was a logistic growth rate as the resources (amount of protein) is limited. Logistic (sigmoidal or S-curve) functions are non-linear. It has shown an initial exponential growth until the inflection point and exponential decay until stagnation. To make it simple, an exponential graph has been plotted between 10 and 90% of saturation (to avoid non-linear section of the plot) and fulfilling the linear section of the exponential graph. Thus, the solution is given by:

$$f_{(t)} = f_{(o)} * e^{r*t}$$

where, the initial fluorescence is $f_{(0)}$, time is t, fluorescence at time t is $f_{(t)}$ and r is the rate of aggregation. The aggregation rates in the absence and presence of 10.0, 20.0 and 30.0 μM rutin are reported in Table 1.

### 2.7. Intrinsic fluorescence measurements

Intrinsic fluorescence was measured on Cary Eclipse fluorescence spectrofluorometer at room temperature. The BLG was incubated with rutin (10.0, 20.0 and 30.0 μM) at pH 2.0 at 358 K for 108 h. The intrinsic fluorescence was measured of the samples containing BLG (10.88 μM) and different concentrations of rutin by excitation at 295 nm and emission was recorded in the wavelength range of 300–400 nm. The excitation and emission slit widths were fixed at 5.0 nm.

### 2.8. ANS fluorescence measurements

ANS binding assay was performed on Cary Eclipse fluorescence spectrofluorometer at room temperature. ANS stock was made by dissolving in Milli-Q water and filtering through a 0.45-micron syringe filter. The concentration of ANS was calculated using extinction coefficient 5000 $M^{-1}\ cm^{-1}$ by taking optical density at 350 nm. ANS (50.0 μM) was mixed with the protein samples (incubated at different hours at 355 K in the absence and presence of rutin in 20.0 mM glycine-HCl buffer pH 2.0). The ANS incubated samples were kept for 0.5 h at room temperature in the dark. The ANS incubated samples were excited at 380 nm and emission spectra were recorded in the wavelength range of 400–650 nm. The excitation and emission slits were kept constant at 5.0 nm.

In order to minimize inner filter effects, corrections to the fluorescence. First, the background solvent emission spectrum was corrected using following equation [38–40]

$$F = \left(1-10^{-Aexc}\right)/2.303 * Aexc$$

when, Aexc is the absorbance of the sample in the same solvent at the excitation wavelength. The corrected background spectrum was then subtracted from the sample emission spectrum to obtain the spectrum F0 ($\tilde{\nu}$). Then, the following correction factor was used to correct the primary and secondary inner filter effects:

$$Fcorr\ (\tilde{\nu})4 = F0\ (\tilde{\nu}) * 10^{(Aex*lex+Aem*lem)}$$

where Fcorr ($\tilde{\nu}$) is the corrected fluorescence intensity, Aex is the absorbance at excitation wavelength, lex is the penetration depth of the light in the sample, Aem is the absorbance over the emission wavelength and lem is the emission pathlength. According to the previous study lex and lem considered 0.44 cm and 0.05 cm respectively [41,42].

### 2.9. Far-UV CD measurements

The far-UV CD was performed on an Applied Photophysics, ChirascanPlus, UK spectropolarimeter attached with Peltier. The far-UV CD experiments were carried out on samples with BLG (10.88 μM) incubated at 358 K at different time intervals (20.0, 40.0 and 68.0 h) in the absence and presence of 10.0, 20.0 and 30.0 μM of rutin. The far-UV CD spectra were scanned in the wavelength range of 200–250 nm. The cuvette path length of 0.1 cm was used. Each sample was scanned three times and average spectra were taken.

### 2.10. Transmission electron microscopy (TEM)

TEM images were captured by a JEOL transmission electron microscope operating at an accelerating voltage of 200 kV. The amyloid fibril formation was assessed by applying 10.0 μl of BLG (10.88 μM) incubated for 108 h at 358 K alone and with 10.0 and 30.0 μM of rutin on 200-mesh copper-coated grid. Excess fluid was removed after 2 min and the grids were then negatively stained with 2% (w/v) uranyl acetate and keep in a desiccator for complete dryness. Before taking the images, the samples were incubated overnight.

## 3. Results

ThT fluorescence and ANS binding based evaluation of SDS-induced aggregation in lysozyme and its inhibition by rutin have been studied [43]. Similar studies may be useful for understanding the effect of rutin on BLG or other food protein aggregation.

### 3.1. ThT fluorescence of BLG in the presence of rutin

ThT is a benzothiazol dye that acts as an indicator of amyloid fibrillation in protein [44]. ThT binds specifically to the cross-β structure of amyloid. Fig. 2A shows the ThT fluorescence of BLG in the absence and presence of rutin. In all, 5.0 $mg \cdot ml^{-1}$ BLG stock were used for all studies, i.e. equivalent to 272.0 μM. The protein was left to aggregate at 358 K for 108 h and the fluorescence was recorded at 0 to 108th hours, in the absence and presence of 10.0, 20.0 and 30.0 μM rutin. The ThT fluorescence spectra at 20.0, 40.0 and 68.0th hour without and with rutin are shown in Fig. 2B, C, and D, respectively. The peaks of emission spectra were at 485 nm, with extensively increased intensity being a characteristic feature of ThT that binds to amyloids [45]. In Fig. 2A, the intensity of BLG-

**Table 1**
Decreasing aggregation rates of β-lactoglobulin, as observed by ThT binding, in the absence and presence of increasing concentration of rutin at 358 K.

| S. No. | Conditions | Aggregation rate (in $10^{-3}\ h^{-1}$) | $R^2$ |
|---|---|---|---|
| 1 | BLG | 16.6 ± 1.0 | 0.94 |
| 2 | BLG + 10 μM rutin | 15.7 ± 0.9 | 0.94 |
| 3 | BLG + 20 μM rutin | 15.0 ± 0.3 | 0.98 |
| 4 | BLG + 30 μM rutin | 13.5 ± 0.1 | 0.99 |
| 5. | Rate of Inhibition | 99.3 ± 2.9 $h^{-1}\ M^{-1}$ | 0.97 |

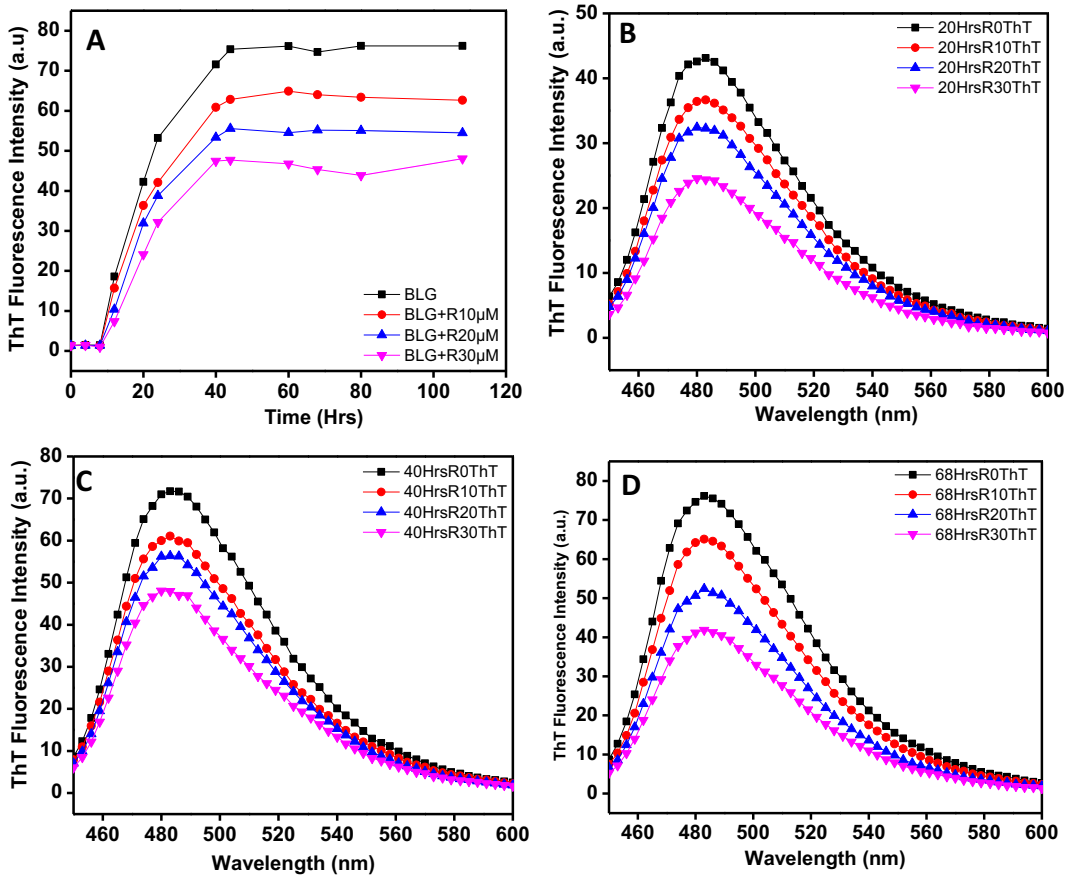


**Fig. 2.** (A) ThT fluorescence kinetics measurements by emission at 485 nm of BLG incubated at 358 K for 108 h in absence (-■-), and in the presence of rutin 10.0 μM (-●-), 20.0 μM (-▲-) and 30.0 μM (-▼-) at pH 2.0. The ThT (10.0 μM) fluorescence spectra were plotted at different rutin concentrations i.e., 0.0 μM (-■-), 10.0 μM (-●-), 20.0 μM (-▲-) and 30.0 μM (-▼-) at different time intervals 20 (B), 40 (C) and 68 h (D). BLG working concentration was taken 10.88 μM. ThT (10.0 μM) was added to all the samples.

ThT complex with 0.0, 10.0, 20.0 and 30.0 μM rutin at 485 nm has been plotted against time in hours. No aggregation has been observed up to 10 h, and then ThT fluorescence increased exponentially till 40 h. No further increment in fluorescence was observed till 108 h. Although the behaviour of emissions was found to be similar irrespective of the concentration of rutin, the extent of emissions is clearly reciprocal to the rutin concentration. In between 40 and 108 h, the emissions of BLG-ThT complex were around 76.0, 63.0, 55.0 and 48.0 AU in the presence 0.0, 10.0, 20.0 and 30.0 μM rutin respectively. A lag phase of almost 10 h was recorded in BLG at 358 K, and the lag phase was not altered in the presence of different concentration of rutin while fluorescence intensity was almost at baseline. The ThT kinetics results suggest that BLG alone and with different concentrations of rutin at 358 K form amyloid-like fibrils [46]. To confirm if rutin had any effect on ThT fluorescence, control experiments have been done [Suppl. Fig. S1]. Rutin itself did not show any significant ThT fluorescence. The drop of the calculated rate of aggregation of BLG from $16.6 \times 10^{-3}$ to $13.5 \times 10^{-3}$ $Hr^{-1}$ in the presence of 0.0 to 30.0 μM rutin shows the antagonistic effect on BLG amyloidosis (Table 1). The rate of rutin inhibition was found to be 99.3 $h^{-1}$ $M^{-1}$. Interestingly, the lag phase of aggregation is not affected by the presence of rutin. Such a situation may be possible when the inhibitor disintegrates the aggregated protein rather than inhibiting aggregates form forming. Another control was also taken as a [Suppl. Fig. S2]. The chances of competition between rutin and ThT was overruled by doing experiments in Suppl. Fig. S2. The aggregated samples incubated with ThT and rutin was further titrated with different concentrations of rutin and measured the ThT fluorescence. From the results, it was seen that the ThT fluorescence was not changed in the titration of rutin which signified that the completion was not taking place between rutin and ThT. From the test and control experiment, it is now confirmed that the decrement in ThT fluorescence is not because of competition between ThT and rutin binding. The decrees in ThT fluorescence intensity was due to amyloid fibrillation inhibition by rutin.

We further evaluated whether the rutin itself showing any ThT fluorescence, we have further checked the ThT fluorescence of rutin after excitation at 440 nm. There is no significant fluorescence was recorded in rutin alone shown in Suppl. Fig. S3.

### 3.2. Intrinsic fluorescence of BLG with rutin

Proteins are having aromatic amino acid, specifically tryptophan, show intrinsic fluorescence. Tryptophan is excited at 295 nm and maximum fluoresces come around 340 nm. BLG contains two tryptophan residues Trp-19 and Trp-61 on the A and C strand respectively. Trp-19 is buried in the hydrophobic core, but Trp-61 remains exposed. Any stereo-specific change in protein; like polarity of its environment, could reflect in the alteration of intrinsic fluorescence spectroscopy of proteins. The intrinsic fluorescence spectra of BLG at 358 K at different incubation time in the absence and presence of rutin was shown in Fig. 3. Fig. 3A, B, C and D are showing emission spectra of BLG (10.88 μM) in the presence of 0.0, 10.0, 20.0 and 30.0 μM rutin at 0, 20, 40 and 68 h respectively. In all, the fluorescence spectrum of BLG at 0 h was taken as a control. Fig. 3A shows BLG emission maximum at 335 nm after excitation at 295 nm at 358 K. Presence of rutin had no effect BLG emission. Nevertheless, after 20 h (Fig. 3B), BLG emission got red shifted from 335 to 342 nm. Further, with increase in rutin fluorescence get quenched from 120.0 to 80.0 AU. Red shift of emission spectrum is an indication of movement of intrinsic fluorophores (or the core of the protein) towards more polar microenvironment. It could happen due to weakening of tertiary structures.

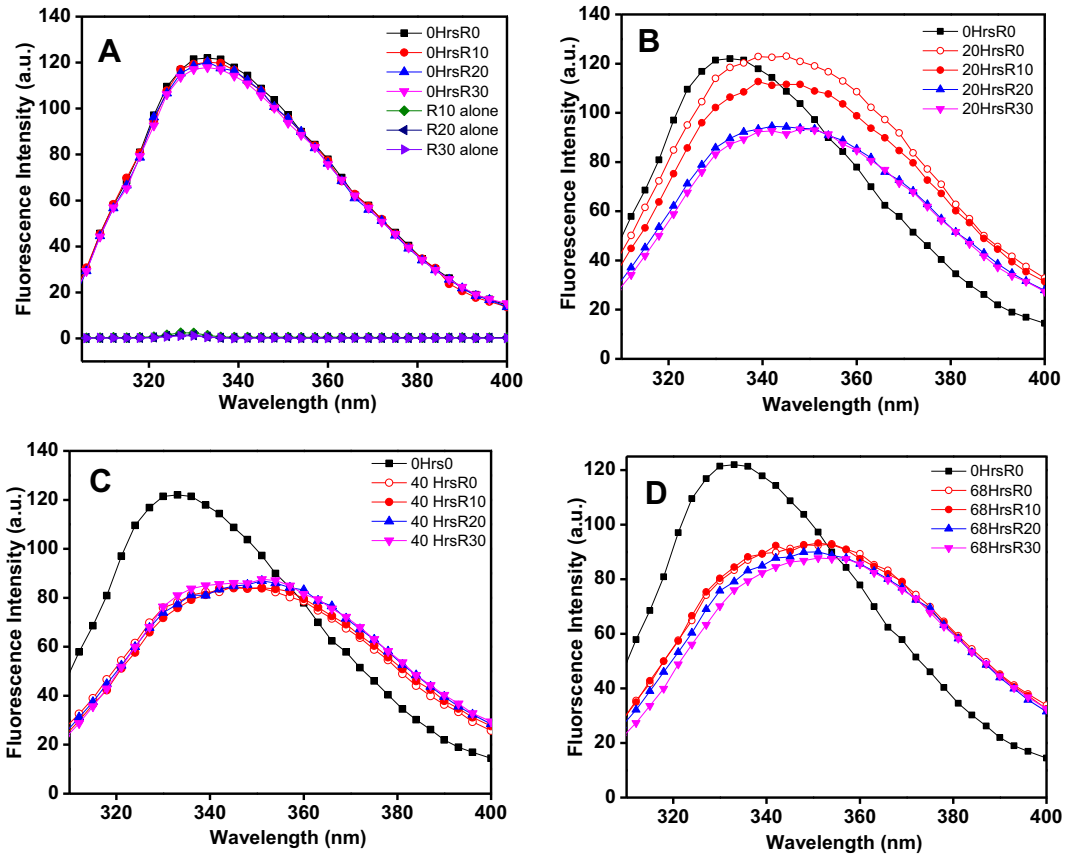


**Fig. 3.** Intrinsic fluorescence spectra of BLG (10.88 μM) was plotted in the presence of different rutin concentrations, 0.0 μM (-■-) at 0 h, 0.0 (-○-), 10.0 (-●-), 20.0 (-▲-) and 30.0 μM (-▼-) at different time intervals i.e., 0 (A), 20 (B), 40 (C) and 68 h (D) at 358 K. BLG concentration was taken 10.88 μM and excitation wavelength was 295 nm. The control samples; 10.0 (-♦-), 20.0 (-◄-) and 30.0 μM (-►-) rutin were excited at 295 nm.

In Fig. 3C, one can observe further red shift (up to 350 nm) after 40.0 h, but unlike 3B, no quenching was observed in the presence of 10.0, 20.0 or 30.0 μM rutin. The results after 68 h (Fig. 3D) were also similar to Fig. 3C. The figures clearly showed that the effect of rutin could only be observed after a long incubation at 358 K, when the tertiary structure seems get unfolded, as observed by red shift in emission. But at 40 h and above, rutin may not have any further effect on the alteration in tertiary structure. Above 40 h incubation, the effect of rutin on the tertiary structure of the protein seems nullified at 358 K and pH 2.0. At 353 K, Trp-61 only supposed to contribute to trpyptophanyl fluorescence signal and that to much lower than the room temperature, due to thermal denaturation [47]. Further, Rutin binds to BLG at site-1 and site-2 by hydrogen bonding and hydrophobic interaction; far off any involvement either trp-19 or trp-61 [48]. Assimilating the previous and current results we can say, the tryptophanyl signal studies in the said conditions are unlikely to represent any change in structure due to rutin interaction.

We have added a bar diagram of emission at 330 nm for intrinsic fluorescence (average of 3 observations) in Suppl. Fig. S6. From the earlier published reports it was established that the rutin binds to BLG at both site 1 and 2 through hydrogen and hydrophobic interaction. The effect of time is very evident. Further, the little effect of rutin in this range (except 20th hour) along with the clear effect of rutin in BLG-ThT fluorescence intensities (Fig. 2A) supports that rutin has an inhibitory effect on amyloid formation.

### 3.3. ANS fluorescence of BLG with rutin

ANS is an extrinsic fluorophore that binds to the hydrophobic core or patches of protein and fluoresce [49]. Fig. 4, shows emission spectra of BLG-ANS complex at ~505 nm, in the absence and presence of 10.0, 20.0 and 30.0 μM rutin at 40 (Fig. 4A), 108 h (Fig. 4B) at 358 K, after excitation at 380 nm. BLG at 0 h was kept as control because protein is presumably at pH 7.0, i.e., native state. At 40 and 108 h, BLG-ANS complex emission intensity increases several folds, an indication of exposure of protein core due unfolding of tertiary structure. But, interestingly, in the presence of rutin emission intensity starts decreasing. The intensity decreases to almost half in the presence of only 10.0 μM rutin. In the presence of 30.0 μM rutin, the emission diminishes to the level of native BLG. This could be explained by only by three possibilities:

A. Rutin binds to ANS and will not let ANS bind to the unfolded hydrophobic patches of BLG.
B. Rutin competes with ANS to bind at the hydrophobic pockets of BLG.
C. Rutin reverses the effect of ANS, meaning, the rutin-BLG complex inhibits the unfolding of protein the core at 358 K. Thus no significant number of hydrophobic patches is made available to ANS to bind.

No change in ANS fluorescence has been observed in the presence of increasing concentrations of rutin (Fig. 4C), which refutes possibility A. The effect of rutin on the intrinsic fluorescence of BLG is a testimony to the direct binding of rutin on the binding sites of BLG. With an increase in the incubation time, the protein core shifted towards polar solvent, but except for the 20th hour, no effect of rutin in any concentration has been observed (Fig. 3). This refutes possibility B that there could be any direct binding of rutin to BLG at observed concentrations. For a similar reason we can predict that there may be no competitive binding of rutin and ThT towards BLG. There could be a possibility that at 20 Hrs different folding species of BLG may have arisen before the advent of aggregation represented by different fluorescence spectrum. In contrary, even if we consider that some specific conformation has been built up

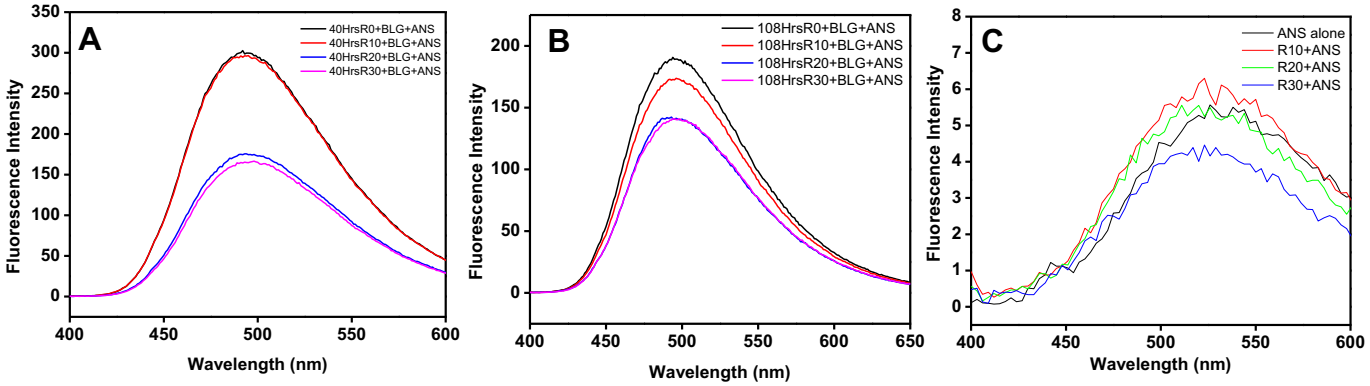


**Fig. 4.** ANS fluorescence spectra of BLG (10.88 μM) incubated at 358 K in absence (-■-) and presence of various concentrations of rutin 0.0 (—), 10.0 (—), 20.0 (—) and 30.0 μM (—) at 40 (A) and 108 (B) hours. All ANS concentration was taken 50.0 μM. (C) The emission spectra of samples containing ANS alone (—) 10.0 (—), 20.0 (—) and 30.0 μM (—) rutin with 50.0 μM of ANS were scanned after excitation at 385 nm. SD ± 5 AU.

in BLG at the 20th hour of incubation that may favour binding of rutin to BLG, such conformation may have disappeared with increases in incubation time and hence with increase in amyloidosis. Therefore, it seems only possibility C is plausible.

Another control was also taken to confirm the amyloid fibrillation was inhibited by rutin. The ANS fluorescence was decreased just because of amyloid fibrillation inhibition not to competitive binding of rutin and ANS. As confirmed by [Suppl. Fig. S5], the ANS fluorescence intensity was not changed in the titration of rutin.

To further check the above mentioned possibilities, helps of far UV-CD and electron microscopy ware taken.

### 3.4. Far UV-CD of BLG with rutin

Far UV-CD studies are done to understand the secondary structures of a protein. BLG is a β-sheet protein, but it passes through an α-helical intermediate during folding [50]. During amyloid formation, proteins form cross β structure irrespective of native structure, and so its secondary structure becomes pivotal to demarcate amyloid formation [51]. On thermal denaturation, BLG shows a two-stage transition: first, dissociation into monomers (which also gained by pH denaturation below 3.5), and second, formation of a molten globule state, called R-state, with a loss of α-helix and equivalent gain of β-sheet structures (Tm = 351 K) [52].

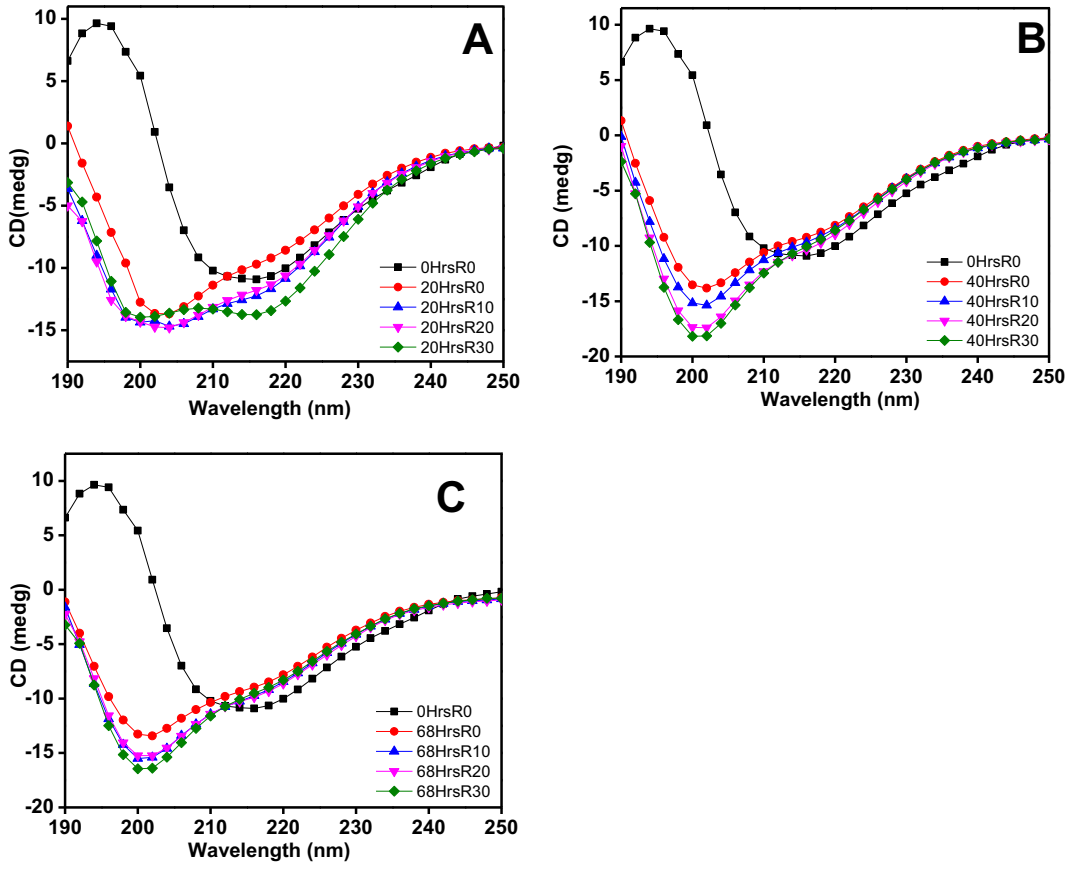


**Fig. 5.** Far-UV CD spectra of BLG (10.88 μM) in the absence (-■-) 0 h at 20, 40 and 68 h hours (-●-) and in the presence of different concentrations of rutin, 10.0 (-▲-), 20.0 (-▼-) and 30.0 μM (-◆-) at different intervals (20 (Panel A), 40 (Panel B) and 68 h (Panel C)).

**Table 2**
Percent secondary structure calculated at different conditions by K2D2 method. All calculations are an average of three scans with a minimum $R^2$ value of 0.95.

| S. No. | Conditions | Alpha-helix (%) | Beta-sheet (%) |
|---|---|---|---|
| 1 | 0Hrs BLG at pH 7.4 | 19.7 ± 0.9 | 31.5 ± 1.6 |
| 2 | 20Hrs BLG | 16.3 ± 0.8 | 34.7 ± 1.7 |
| 3 | 20Hrs BLG+ Rutin 30 μM | 13.8 ± 0.7 | 34.6 ± 1.7 |
| 4 | 68HrsBLG | 9.6 ± 0.5 | 38.7 ± 1.9 |
| 5 | 68Hrs BLG + Rutin 30 μM | 13.8 ± 0.7 | 34.6 ± 1.7 |

Fig. 5 depicts the far UV-CD of BLG at 358 K in the absence and presence of 10.0, 20.0 and 30.0 μM of rutin after 20 (5A), 40 (5B) and 68 h, (5C) of incubation. The spectrum of BLG at pH 2.0 and 358 K, in the absence of rutin at 0 h is taken as control in all sections of Fig. 5.

At pH 2.0, BLG shows a positive ellipticity at 200 nm and a minimum at 215 nm. This is characteristic of β-sheet proteins in general, and matches earlier reports of BLG [52]. The spectrum remains unchanged even after 0–10 h of incubation that corresponds to the lag phase of aggregation, observed by ThT assay in Fig. 2A. In the range of far UV-CD, the spectrum of rutin falls on baseline (figure not shown). The changes that occurred after 20 h incubation in BLG spectrum in the presence of 0.0 to 30.0 μM rutin were significantly different from the spectra after 40 and 68 h incubation. In the presence of rutin, instead of one minimum at 215 nm, a couple of minima have emerged at 200 nm and 218 nm respectively, which seems to be an α-helical protein-like feature. Thus it could be a β-sheet to α-helix transition, along with tryptophanyl fluorescence quenching, shown in the Fig. 3B.

After 40 and 68 h of incubation at 358 K, the spectrum has changed significantly. The minimum at 215 nm has been shifted to 200 nm (blue shift), with a shallow dip near 215 nm. The presence of rutin did not change the shape of the far UV CD spectrum, but ellipticity at 200 nm kept decreasing from −14 to −19 mdeg, with increases in rutin concentration from 0.0 to 30.0 μM.

The quantitative analyses of secondary structure of BLG at different conditions have been done with K2D2 [53] and depicted in Table 2. The calculated α-helix and β-sheet of native BLG were found to be 19.7 and 31.5%, respectively, which matches well with previous reports [53]. With incubation of 20.0 h at 358 K, there is a nominal increase in β-sheet (~3.0%) structure at the cost of equal percent α-helix. But on incubation of 40 to 68 h, β-sheet has increased ~7.0% (31.5% to 38.7%) at the cost of 10.0% α-helix.

Interestingly, the presence of rutin acted antagonistically. After 20 h incubation, a 3% increase in α-helix took place without any net change in β-sheet. But, after 40 h and 68 h incubation, a 4% increase in α-helix took place at the cost of 4% β-sheet with 10.0–30.0 μM rutin.

The BLG secondary structure in the presence of rutin seems to acquire α-helical structure when recorded after 20 h of incubation. β-sheet to α-helix transitions may have risen due to the formation of intra-molecular hydrogen bonds at the cost of inter-molecular hydrogen bonds. This may have happened either due to withdrawal of protein-water hydrogen bonding, or enhanced hydrophobic interaction at the protein core that favours formation of α-helices, or both. Because the transition seems clear even at BLG: rutin stochiometric ratio of 1, it is more likely a sign of increased hydrophobic interactions in the inner core of the protein.

By observing ThT assay, tryptophanyl fluorescence, ANS binding and CD studies, we could speculate a process of the interference of rutin in BLG amyloidosis. The process of BLG amyloidosis and inhibition by rutin has been explained by a scheme (Fig. 7). At pH 2.0, BLG forms monomers due to high cationic repulsion, but with a temperature of 358 K, BLG intermolecular interaction has enhanced in the form of non-local hydrogen bonding, that may have registered as increased β-

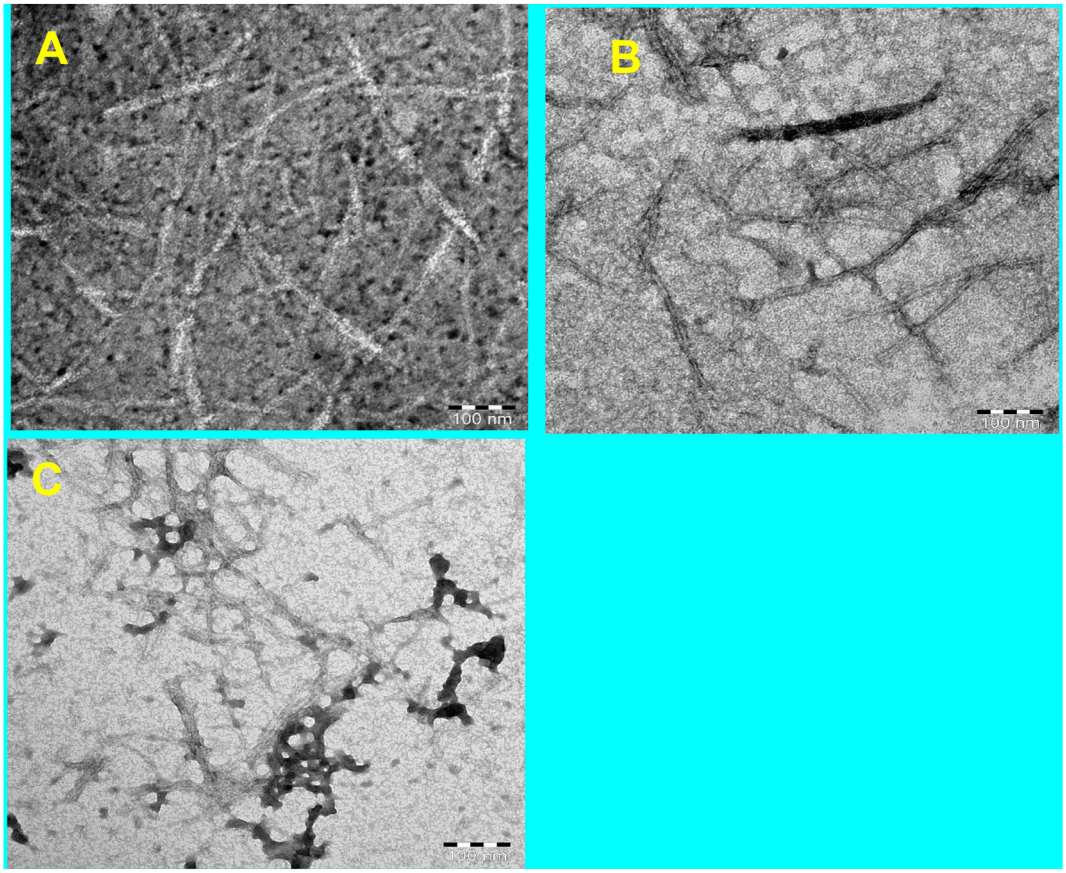


**Fig. 6.** Transmission Electron Microscopic images of BLG (10.88 μM) without rutin (A) and BLG was treated with 10 (B) 30.0 μM (C) rutin at 358 K for 108 h.

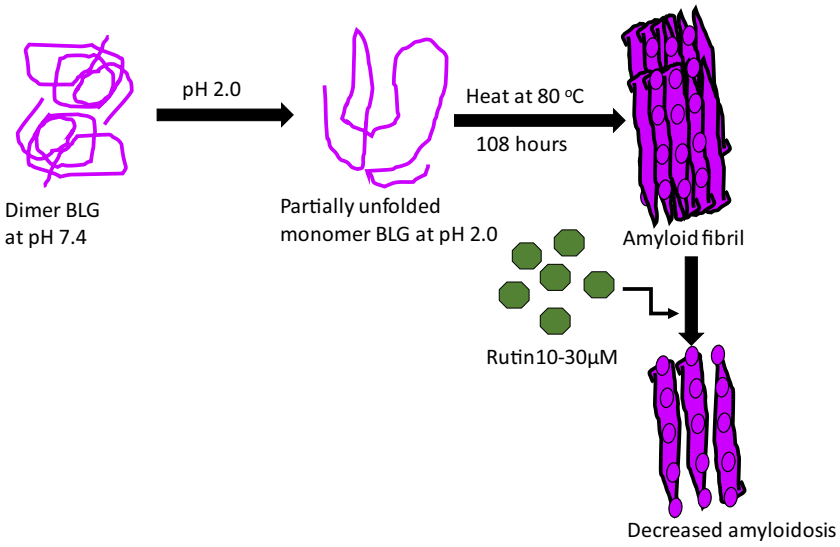


**Fig. 7.** Schematic presentation of BLG amyloidosis and its inhibition by rutin.

sheet structure. But the presence of rutin in the first phase (upto 20 h of incubation) of aggregation seems to play antagonistically; to form intramolecular hydrogen bonding in the form of β sheet -α helix transition. We can assume that rutin may have catalysed local hydrogen bonding in the protein core by holding hydrogen bonds with 10 hydroxyl (—OH) and 2 carbonyl (>C═O) groups. This also supported by the quenching of tryptophanyl fluorescence with rutin at 20 h incubation. This could have happened due to movement of the protein core towards a more hydrophobic environment, a sign of α-helix induction. But beyond 20 h incubation, with higher concentration of aggregates, the effect of rutin may have been subdued by a higher concentration of non-local hydrogen bond formation in amyloidosis.

### *3.5. Electron micrograph of BLG with rutin*

BLG-ThT complex fluorescence confirmed the formation of amyloid, but to understand its morphology, we need microscopic observation. TEM is a well-known technique to study amyloid formation. Several amyloid proteins have been characterized by TEM image [54,55]. Fig. 6A, B, and C show BLG at 358 K after 108 h in the absence and presence of 0.0, 10.0 and 30.0 μM rutin, respectively. It is clear, while amyloid fibrils were abundant in the absence of rutin (Fig. 6A), but it keeps on decreasing with increases in rutin concentration. In fact, only 30.0 μM rutin were enough to diminish significant amounts of fibrils (Fig. 6C).

## 4. Conclusion

Rutin, a well-known amyloid formation inhibitor, has been found to affect BLG amyloid formation at pH 2.0 and 358 K. The ThT fluorescence confirms formation of amyloid and inhibition by rutin. A significant decrease in fibril formation was also observed with TEM. While far UV-CD shows the recovery of lost α-helix at the cost of β-sheet in the presence of rutin, with ANS fluorescence it could be observed how the presence of rutin helps in withdrawal of hydrophobic patches. Therefore, rutin could be considered an inhibitor of BLG amyloidosis at pH 2.0 monomeric state.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2019.02.004.

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