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Inhibition of amyloid fibrillation of apo-carbonic anhydrase by flavonoid compounds

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Flavonoids are polyphenol compounds abundantly found in plants and reported to have an inhibitory effect on amyloid fibrillation. The number and position of hydroxyl groups, as well as the arrangement of flavonoids rings, may influence their inhibitory effects. In this study, we investigate the effect of structural characteristics of flavonoids on amyloid fibril formation. For this purpose, five compounds (i.e., biochanin A, daidzein, quercetin, chrysin and fisetin) were selected that represent a variety in the number and position of their hydroxyl groups. The inhibitory effect of these flavonoids on the amyloid fibril formation of apo-carbonic anhydrase (apo-BCA), as a model protein, was evaluated using thioflavin T and transmission electron microscopy. The results showed that fisetin possessed the most significant inhibitory effect. Interestingly, upon apo-BCA acetylation, none of the tested flavonoids could inhibit the fibrillation process, which indicates that the interactions of these compounds with the amine groups of lysine residues could be somewhat important.

Keywords. Amyloid; carbonic anhydrase; fisetin; flavonoids; lysine acetylation; small molecule

Abbreviations: BCA II, bovine carbonic anhydrase II; ThT, thioflavin T; TEM, transmission electron microscope; DMSO, dimethyl sulfoxide; apo-BCA-Ac, apo-acetylated bovine carbonic anhydrase; EGCG, epigallocatechin gallate

1. Introduction

Amyloid fibrils are among the ordered protein aggregates with long unbranched structures. These fibrils are composed of protofilaments with 2–5 nm diameter. Also, they are twisted around each other such that they can produce fibrils with a diameter of 13 nm (Sunde and Blake 1997; Antzutkin *et al.* 2002). The current interest in the study of amyloid fibrils is especially related to their role in the development of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (Ross and Poirier 2004). Actually, more than 40 types of diseases with distinct clinical features have been reported to be related to protein deposition (Chiti and Dobson 2006). These disorders are related to the aggregation of either unstructured proteins or globular proteins (Fink 1998). Finding solutions to inhibit the aggregation process may prevent or slow down the progress of the diseases. In recent years, the effects of numerous compounds on amyloid fibril formation have been investigated (Doig and Derreumaux 2015). Natural compounds, especially those obtained from plants, have interesting structures and properties that make them attractive in drug discovery studies (Porat *et al.* 2006). It has been reported that apomorphine prevents the formation of amyloid fibrils of α-synuclein (Yarnall *et al.* 2016). Moreover, catechin, resveratrol, rosmarinic acid and tannic acid inhibit fibril formation of Aβ (Feng *et al.* 2009; Ngoungoure *et al.* 2015). Among multiple compounds tested on fibril formation, aromatic compounds, and in particular, phenols and polyphenols (including flavonoids), seem to be very effective (Lashuel *et al.* 2002). Many studies have shown the efficacy of flavonoids as inhibitors of amyloid fibrils (Porat *et al.* 2006). Despite extensive research on the inhibitory effects of polyphenols on amyloid fibrils, their precise molecular mechanism is still unclear and needs more investigation.

Bovine carbonic anhydrase (BCA) is a globular metalloprotein with a single polypeptide chain that consists of 259 amino acid residues and has no disulfide linkages (Saito *et al.* 2004). Indeed, it is a well-characterized protein that has been used as an *in vitro* model in protein folding and

unfolding studies (Bushmarina *et al.* 2001). This protein contains 18 lysine residues in the surface structure and has been widely used as a model in protein surface charge studies (Gitlin *et al.* 2006). Under *in vitro* conditions of low pH and elevated temperature, apo-BCA is converted into amyloid fibrils that bind to amyloid markers such as thioflavin T (ThT) and Congo red with a fibrillar morphology, as observed using transmission electron microscopy (TEM). The acetylated form of apo-carbonic anhydrase (apo-BCA-Ac) also results in the formation of amyloid fibrils at physiological pH (Es-haghi *et al.* 2012). In this study, the potential inhibitory effect of some flavonoids on acetylated and non-acetylated carbonic anhydrase fibrillation is investigated. These compounds, which include biochanin A, daidzein, chrysin, quercetin and fisetin, have never been tested until now under this protein's aggregation process. The structures of these compounds are very similar, with the major differences being the positioning of the B-ring and the number and location of hydroxyl groups on their rings (1A). The structure of the flavonoid compounds tested in this study is presented in figure 1B. It is expected that these compounds have the capacity for hydrogen bonding, aromatic stacking and hydrophobic interaction with polypeptide chains. Therefore, the roles of hydroxyl groups and the position of the B-ring in the inhibition process can be further investigated.

Our results demonstrated that some of these compounds strongly inhibit the amyloid formation of non-acetylated apo-carbonic anhydrase, but they did not affect the fibrillation process of the acetylated form.

2. Materials and methods

2.1 *Materials*

BCA (EC 4.2.1.1) and ThT were purchased from Sigma (St Louis, MO, USA). Dipicolinic acid (DPA) and all the salts and organic solvents were of reagent grade and obtained from Merck (Darmstadt, Germany). All flavonoids were purchased from Sigma-Aldrich, (St. Louis, MO, USA) and were used without further purification.

2.2 *Purification of carbonic anhydrase*

Carbonic anhydrase was obtained from Sigma and was found to have around 7–8% impurities. Further purification was carried out with an affinity chromatography column p-(aminomethyl) benzenesulfonamide equilibrated with 25 mM Tris buffer, pH 8.7, containing 22 mM sodium sulfate. The column was washed five times with equilibrium

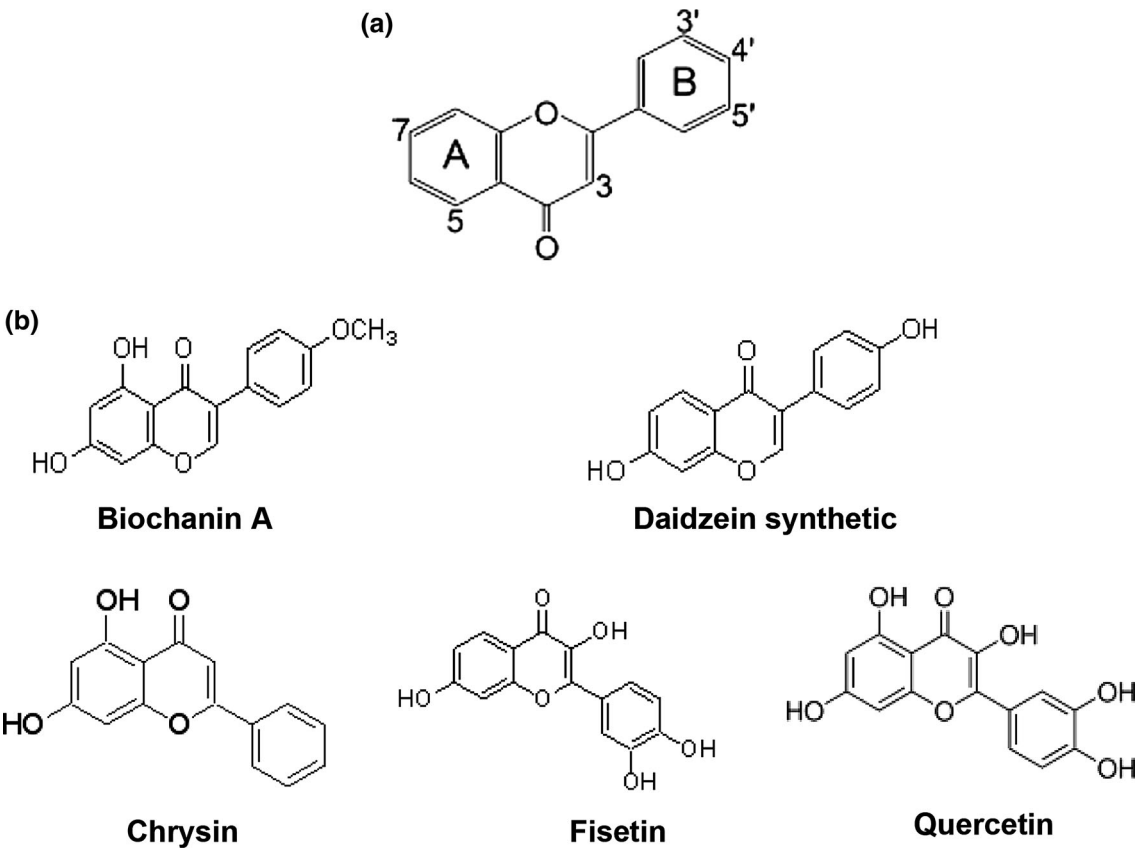

Figure 1. (**A**) General structure of flavonoids. (**B**) Molecular structures of inhibitors tested in this study.

buffer. Protein was eluted using 100 mM sodium acetate buffer containing 500 mM sodium perchlorate at pH 5.6 (Ozensoy *et al.* 2004). The purity of the protein was confirmed using 12% SDS–PAGE gel electrophoresis.

2.3 *Determining protein concentration*

Protein concentration was determined by using 1.83 mg^{-1} mL^{-1} extinction coefficient at 280 nm (Wong and Tanford 1973). Where needed (e.g. low protein content after purification), concentrating was performed using a Centricon centrifugal filter device (cut-off: 3 kD).

2.4 *Apo-BCA preparation and metal content analysis*

Purified protein was dialyzed in 50 mM phosphate buffer (pH 7.5) containing 100 mM DPA (chelator) for 48 h, while the buffer was changed every 12 h. A second dialysis was performed against 10 mM Tris buffer (pH 7.5) for 48 h to remove the chelator. The final solution was centrifuged for 10 min at 10,000 *g* in order to remove any potential aggregates of proteins. Protein concentration was determined and in cases of low protein concentration, centrifugal filtering was used. The zinc content of each apo-protein preparation was determined by inductively coupled plasma-optical emission spectrometry analysis with a standard zinc solution as the blank and the results showed the metal content removed was 92% (Otero-Romaní *et al.* 2009).

2.5 *Acetylation of lysine residues*

Acetylation of lysine residues was performed as described previously. Briefly, small amounts of acetic anhydride were added to the protein solution (100 mM HEPES buffer, pH 8.2) for about 2 h at 10 min intervals. During this process, the pH of the solution was adjusted with 2N NaOH. To de-esterify the other acetylation-prone amino acids (especially tyrosine residues), the reaction mixture was incubated at room temperature and pH 10.5 for 1 h. The acetylated BCA (BCA-Ac) was then dialyzed extensively against 50 mM Tris–HCl buffer (pH 7.5) (Gitlin *et al.* 2006). The extent of modification was verified by fluorescamine assay (Schmitt *et al.* 2005).

2.6 *Amyloid induction*

All amyloid formation processes were performed with the use of 133 μM (4 mg/mL) solutions of apo-BCA in 50 mM glycine buffer, pH 2.4. The samples were incubated at 57°C while being stirred gently (about 150 rpm) using Teflon magnetic bars. To avoid precipitation of the acetylated form at low pH, apo-BCA-Ac amyloid fibrils were obtained by incubation in 50 mM Tris–HCl buffer, pH 7.5.

2.7 *Monitoring amyloid formation*

The formation of freshly prepared amyloid fibrils was monitored by several probes, including fluorescence behavior of ThT over time. All fluorescence experiments were carried out on a Cary Eclipse VARIAN fluorescence spectrophotometer. About 10 μL of apo-BCA samples were added to 590 μL of 25 μM ThT solutions. The excitation wavelength was set at 440 nm and scanning was done between 450 and 600 nm. Excitation and emission slits were 5 and 10 nm, respectively. The wavelength of 485 nm was used as the maximum emission wavelength in all samples. An increase in the emission intensity of ThT is an indication of the formation of amyloid fibrils structures (Krebs *et al.* 2005).

In addition, TEM images were taken from protein samples (5 μL) placed on carbon-covered grids. In this method, after 1 min, the samples are washed with twice-distilled water and stained with 2% uranyl acetate, and TEM images were taken after 2–5 min.

2.8 *Inhibitory assays*

Flavonoids were first prepared with a concentration of 20 mM in dimethyl sulfoxide (DMSO) as the stock solution. Then, different amounts of this stock solution, resulting in 0, 25, 50, 100, 150 and 200 μM final concentration of flavonoids, were added to the protein solution that underwent amyloid formation. In ThT fluorescence spectroscopy tests, 10 μL of a protein solution containing flavonoid was added to 590 μL of 25 μM ThT solution (total volume was 600 μL). Thus, the concentration of protein and flavonoids was diluted 60 times in these solutions. To determine the quenching effect of flavonoids, 3 μM of each flavonoid was freshly added to the protein and ThT fluorescence assay was performed. With this concentration of flavonoids, ThT fluorescence showed a slight decrease for some compounds for which the ThT data were normalized (subtraction from ThT intensity).

2.9 *Statistical analysis*

Statistical analysis was performed using Sigma plot and one-way analysis of variance. All experiments were run in triplicate for each small molecule compared to the control. Data were expressed as means ± standard errors of the mean (SEM), where $n = 3$. Changes were defined as significant (*) if $P < 0.05$.

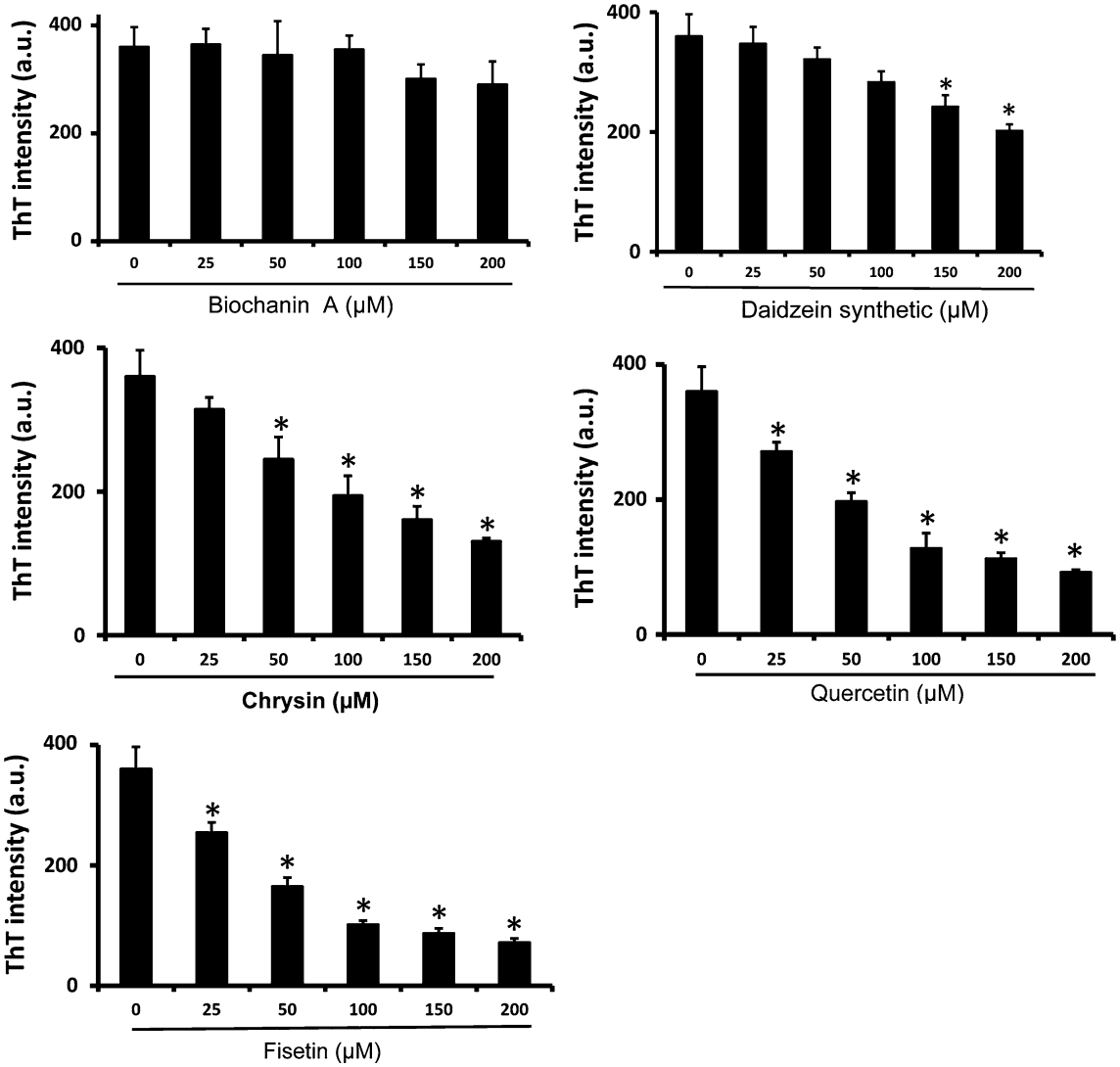

Figure 2. ThT intensity of apo-BCA in the presence of different concentrations of flavonoids; 133 μM Apo-BCA in 50 mM glycine buffer, pH 2.4, and 57°C, was incubated in the presence of different concentrations of flavonoids. The results of three independent experiments after 14 days of incubation have been shown. Changes were considered statistically significant (*) when $P < 0.05$; ($n = 3$).

Table 1. IC50 value of different flavonoids

Small Molecule	IC_{50} (μM)
Fisetin	42.6
Quercetin	57.54
Chrysin	114.8
Daidzein synthetic	>200
Biochanin A	>200

This data was obtained based on figure 2

3. Results

In recent years, many small molecules have been proposed as inhibitors of amyloid fibrils formation. In this regard, aromatic compounds including phenols, polyphenols, flavonoids and quinone, have attracted much attention (Stefani and Rigacci 2013). In this study, the effects of some flavonoids on the fibrillation process of carbonic anhydrase were investigated. Since these compounds generally have low solubility in water, DMSO was used as the solvent. The final concentration of DMSO in the protein solution was 1%. To take into account its effect in the experiments, the same amount was added to the control sample.

3.1 *ThT fluorescence assay of apo-BCA amyloid formation*

In the present study, we incubated 4 mg/mL (133 μM) apo-BCA in 50 mM glycine buffer (pH 2.4) at 57°C in the presence or absence of a flavonoid. In order to monitor amyloid fibril formation, ThT was used as a general probe since a significant increase in fluorescence intensity occurs

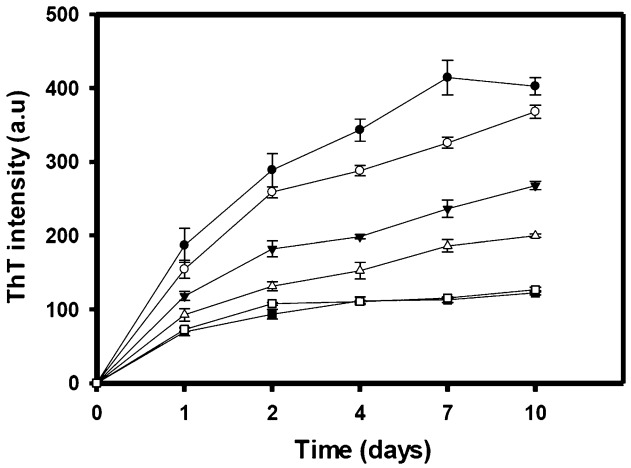

Figure 3. Kinetics of apo-BCA amyloid fibril formation in the presence of 200 μM of flavonoids: control (●), biochanin A (○), daidzein synthetic (▼), chrysin (△) quercetin (□) and fisetin (■).

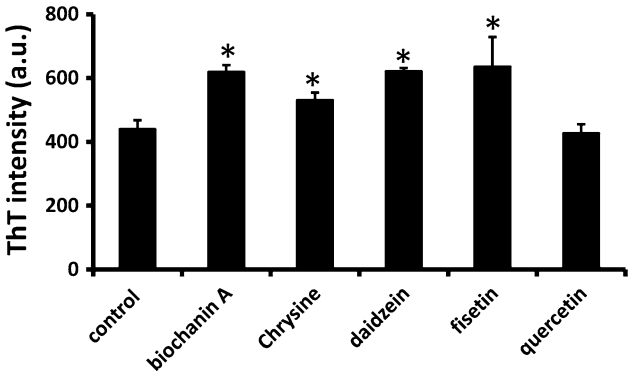

Figure 5. Effect of flavonoids compounds on amyloid fibril formation of acetylated apo-BCA. 400 μM of each compound was added to 133 μM of protein solution in Tris buffer at pH 7.5 and incubated at 57°C. The data provided were obtained after 1 day of incubation. Three replicates of each sample were tested. Changes were considered statistically significant (*) when $P < 0.05$; ($n = 3$).

upon binding of ThT to the β-sheet structures of amyloid fibrils. In the absence of flavonoids, ThT fluorescence intensity increased gradually until it reached a plateau after 14 days of apo-BCA incubation. To evaluate the inhibitory effects of flavonoids, five different concentrations of the compounds (25, 50, 100, 150 and 200 μM) were tested on

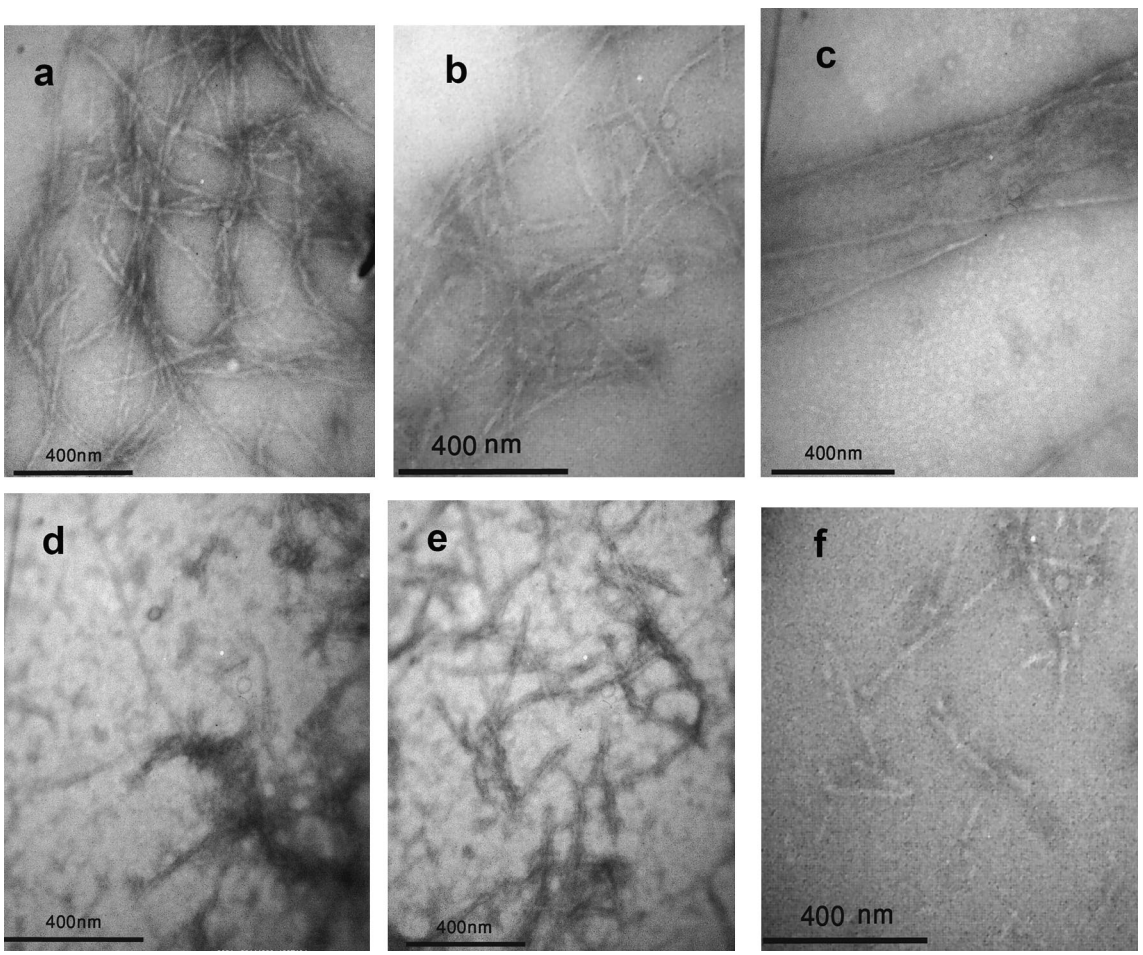

Figure 4. TEM images of 133 μM Apo-BCA in glycine buffer, pH 2.4 and 57°C, incubated with 200 μM of different flavonoids after 14 days of incubation: **A–F**: control, biochanin A, daidzein synthetic, chrysin, quercetin and fisetin.

apo-BCA amyloid formation. As depicted in figure 2, after 14 days of incubation, the presence of all flavonoids led to a reduction in the ThT fluorescence intensity in a dose-dependent manner, in comparison with apo-BCA alone. Fisetin, quercetin and chrysin have the most significant effects in this regard. In the presence of fisetin, quercetin and chrysin, reduction in ThT fluorescence intensity was 83.9, 80.3 and 55.9%, respectively, after 14 days, suggesting a strong inhibitory effect, especially in the case of fisetin.

Moreover, incubation of apo-BCA with daidzein causes a slight decrease in ThT fluorescence intensity that reaches only 40% after 14 days of incubation. In so far as Biochanin A is concerned, the reduction in ThT intensity was not significant with respect to the control, indicating that this compound had no effect on apo-BCA fibrillation. In order to determine the possible quenching effect of small molecules on ThT emission, 3 μM of each compound was added to the protein and ThT fluorescence was measured. In general, at this concentration, there was no quenching and otherwise the corresponding amount was subtracted in our calculations. As shown in figure 2, with increasing concentrations of small molecules, ThT intensity is proportionally reduced. To determine the effective concentration for inhibition, the IC_{50} values for each flavonoid were calculated. As shown in table 1, the IC_{50} values for the best compounds fisetin and quercetin are 42.6 and 57.54 μM.

3.2 *Kinetics of amyloid formation in the presence of flavonoids*

To evaluate the effect of these compounds on the kinetics of amyloid fibril formation, the time dependency of amyloid formation was determined in the presence of each compound at 200 μM. Figure 3 shows that biochanin A and daidzein, which had no (or no significant) effect on amyloid fibrillation in the endpoint test, may actually influence the kinetics of fibrillation by reducing the rate of fibril formation. The other compounds also significantly influenced ThT intensity over time. Overall, in the absence of small molecules, after approximately 7 days, the ThT intensity becomes constant. However, when small molecules are present in the solution, this process continues and the intensity stays constant between 12 to 14 days. According to our previous observations, the process of apo-BCA aggregation has no lag-phase and the enzyme goes directly to a state of amyloid aggregates (Es-haghi *et al*. 2012). Consequently, these compounds affect the amyloidogenesis process and lead to a decrease in fibrils.

3.3 *TEM imaging of samples incubated with flavonoids*

The inhibitory effects of flavonoids on apo-BCA amyloid fibrillation were supported by visualization using TEM. Figure 4A–F present the TEM images of apo-BCA amyloid fibrils aged 14 days, prepared in the presence or absence of flavonoids compounds. In the absence of a flavonoid compound (figure 4A), abundant matured fibrils were observed. They were mainly very long, straight and dense, with diameters of about 16–32 nm. In the presence of biochanin A (figure 4B) and daidzein (figure 4C), the apo-BCA fibrils showed similar morphologies to the control fibrils but the number of fibrils was markedly decreased. In contrast, incubation of apo-BCA with chrysin, quercetin and fisetin resulted in a noticeable reduction of amyloid fibrils and the resultant fibrils had different morphologies; they were short and thin and resemble more protofibrils, with a diameter of about 15 nm (figure 4D–F). The observed length of fibrils formed in the presence of chrysin, quercetin and fisetin was about 130–770 nm, 96–320 nm and 112–464 nm, respectively.

3.4 *Effect of flavonoids on apo-BCA-Ac*

Lysine residues are extremely important charged residues that are usually located on the surface of proteins and may have a crucial role in amyloid fibrillation (Chinisaz *et al.* 2017). Tau acetylation affects their functions due to impaired tau-microtubule interactions and promotes pathological tau aggregation (Cohen *et al.* 2011). It has also been reported that the tau protein located in neurofibrillary lesions is post-translationally modified by lysine methylation (Funk *et al.* 2014). Covalent inhibitors can attack any species in an aggregation pathway but appear to be especially effective on the tau monomer from which the aggregated species are driven (Cisek *et al.* 2014). As an example, oleocanthal, a natural product aldehyde, reacts with epsilon amino groups of lysine residues (Li *et al.* 2009). The effect of flavonoid compounds on the apo-BCA-Ac amyloid fibrils formation was then studied. Acetylated samples were incubated at a concentration of 133 μM in Tris buffer, pH 7.5, in the presence of 200–400 μM of flavonoid compounds. As shown in figure 5, none of the small molecules had an inhibitory effect on acetylated samples and in some instances, ThT intensity has even increased in their presence. The increase in ThT intensity may be due to the salting effect of flavonoids. In a previous study, we found that the mechanism of formation and morphology of the fibrils of apo-BCA-Ac were different from the ones obtained from the native enzyme. The average height of the native fibrils was about 7.8 nm while the average height of acetylated fibrils was about 4.8 nm (Es-haghi *et al.* 2012).

4. Discussion

A common property of protein and polypeptides is the formation of amyloid structures that may be toxic for cells and a potential therapeutic approach in this regard is the inhibition of the fibrillation process. Targeting interactions

between fibrils can be a way to design efficient inhibitory compounds. These interactions are mostly hydrophobic in nature and include π-π stacking between the aromatic rings (Marshall *et al.* 2011). To date, numerous studies have proposed a wide range of various molecules as *in vitro* or *in vivo* inhibitors of amyloid fibrils formation. These compounds may be broadly classified into non-peptides and peptides (Soto *et al.* 1998). Non-peptide inhibitors include various natural and synthesized chemicals, such as polyphenols (e.g. rosmarinic acid) (Ferreira *et al.* 2011), aminonaphthalene compounds (e.g., Congo red) (Pratim Bose *et al.* 2010), bactericidal semi-synthetic antibiotics (rifamycin and its derivatives) (Li *et al.* 2004), surfactants such as di-C6-PC and di-C7-PC (Wang *et al.* 2005), and other compounds such as nicotine, melatonin and trehalose (Torok *et al.* 2008). Polyphenols are a large group of natural and synthetic small molecules containing one or more aromatic phenolic rings. There are various natural polyphenols found in plants that are classified into three groups: phenolic acids (e.g., benzoic acid), flavonoids (including flavonols) and non-flavonoid polyphenols (such as curcumin) (Tsao 2010). For example, curcumin inhibits the formation of Aβ amyloid fibrils *in vitro* and *in vivo* and is reported to reduce the number of amyloid plaques in transgenic mice with Alzheimer's disease (Yang *et al.* 2005).

In order to study the effect of flavonoids on the formation of apo-BCA fibrils, five different flavonoids (i.e., biochanin A, daidzein, chrysin, quercetin and fisetin) were selected. As shown in figure 1b, these flavonoids differ in the number of positions of hydroxyl groups as well as the positioning of the B-ring, which is similar in biochanin A and daidzein, while the remaining compounds have also a similar B-ring position. Among these compounds, fisetin shows the most significant inhibitory effect on fibril formation. Fisetin is found in a number of foods and possesses various biological effects including protection of nerve cells from death caused by oxidative stress and differentiation of nerve cells (Ishige *et al.* 2001). Fisetin has four hydroxyl groups on its rings, while quercetin, which has one more hydroxyl group than fisetin (in position 7), has a slightly lower effect. Compared to fisetin, chrysin lacks hydroxyl groups at the 3′ and 5′ positions, instead it bears a hydroxyl group in position 7 and presents a lower inhibitory effect than fisetin and quercetin. In addition to a number of hydroxyl groups, the position of the B-ring may also play a role in the inhibitory effects of the compounds. It is to be noted that this ring could actually rotate around the bond between rings B and C. The resulting overall conformation of the molecule may become important in this regard. Biochanin A and daidzein have different B-ring positions and a lower inhibitory effect compared to fisetin. The positioning of the hydroxyl groups may also affect their inhibitory strength. Quercetin has five hydroxyl groups while fisetin with four hydroxyl groups is a better inhibitor. Therefore, hydroxyl groups may be either essential or non-essential in the final effective conformation taken by the molecule. In a previous report, it was shown that hydroxyl groups in positions 3 and 7 of ring A had no role in the inhibition of Aβ amyloid formation, while hydroxyl groups in the 3′ and 4′ positions were necessary (Akaishi *et al.* 2008). Both the lack of an inhibitory effect on Aβ fibrillation and an increase in the Aβ amyloid fibrils have been reported for chrysin (Kim *et al.* 2005). Besides, chrysin is able to prevent the amyloid formation of apomyoglobin (Azami-Movahed *et al.* 2013). According to our data, chrysin had a lower inhibitory effect compared to those of quercetin and fisetin. Differences in the mechanism of aggregates formation or difference in the fibrillar structure of these proteins could be the cause of this discrepancy. The inability of flavonoids to inhibit the acetylated samples may be due to the structural differences between native apo-BCA fibrils and the acetylated form. Amyloid fibrils that are formed by apo-BCA from the native structure are thick in appearance and consist of more than one protofibril, while acetylated apo-BCA fibrils as the representative of single protofilament have a thin appearance (Es-haghi *et al.* 2012). In addition, in acetylated apo-BCA, the amine groups of lysine residues are shielded by acetyl groups. In this regard, if the interaction with the amine groups of unmodified lysine is a reason for the effectiveness of the flavonoids, modification of lysine residues will make apo-BCA-Ac less susceptible to the effect of these compounds. The small molecule may even form covalent bonds with lysine residues (Hong *et al.* 2008). It has been reported that the inhibition of amyloid fibrils of the lysozyme by phenolic compounds like catechol and hydroquinone is performed via quinone intermediates that are a prerequisite for these compounds to exert their effect (Feng *et al.* 2012). Covalently bound semiquinone or quinone metabolites have also been reported in the inhibition process of transthyretin (Ferreira *et al.* 2009), α-synuclein (Bieschke *et al.* 2010) and merozoite surface protein amyloid formation by epigallocatechin gallate (Chandrashekaran *et al.* 2010). An oxidative form of polyphenols, with a possibly higher affinity toward the growing ends of fibrils, may be a more potent inhibitor of the amyloid formation compared to the reductive form (Hirohata *et al.* 2007). Finally, quinone or quinonoid substances could also be involved in the inhibition of amyloid fibril formation by polyphenols. The flavonoid compounds tested in this study have three-cyclic structures with differences in their hydroxyl groups. These hydroxyl groups can form hydrogen bonds and also react or interact with functional groups of proteins. Since the flavonoid compounds tested in this study showed no inhibitory effect on the apo-BCA-Ac, we suggest that these compounds could interact or react with the lysine residue. A number of studies have shown that several polyphenols exert their effects on the nucleation phase of amyloid formation, which results in the inhibition of larger oligomers and finally fibrils production. The interaction of these compounds may thus occur with the fibrils and not with the monomeric form.

In conclusion, the proposed inhibitory mechanism is based on covalent or non-covalent interactions between these compounds and aromatic or hydrophobic components of β-sheets in amyloid fibrils, as well as possible H-bond donors and acceptors. In this regard, modifying promising flavonoids with other functional groups may lead to better effects on the inhibition of amyloid structures. On the other hand, the amyloid structures of various proteins show different responses toward polyphenolic compounds and the specificity of these interactions should be taken into account when designing potential therapeutic molecules. Finally, many proteins that undergo amyloid formation become chemically modified by oxidation (Wong *et al.* 2010), acetylation, nitration (Kummer *et al.* 2011) or deamidation (Nilsson and Dobson 2003). However, these modifications may affect the efficacy of small anti-amyloidogenic molecules due to a lack of inhibitory activity of polyphenols on apo-BCA-Ac.

The results of the present study provide further evidence with regard to the anti-amyloidogenic potential of polyphenols and suggest some points to be taken into account for the design of better phenolic-based inhibitory agents.

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