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Original Article

The anti-amyloidogenic effect of *Echinacea* drops

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ABSTRACT

The protein-misfolding diseases make the lives of millions bitter. The incidence of currently incurable diseases associated with amyloids is constantly increasing, so it would be important to prevent them. *Echinacea* drops contain several bioactive compounds, including anti-amyloidogenic polyphenolic molecules. Here, we demonstrate the efficient inhibition of amyloid formation of α-chymotrypsin in 55% ethanol at pH 7.0 by *Echinacea* drops. Using turbidity measurements and Congo red binding assay its effectiveness was found to be concentration dependent. Thus, *Echinacea* drops may also be useful in inhibiting the formation of amyloid fibrils.

KEYWORDS: amyloid, chlorogenic acid, Congo red, *Echinacea*, turbidity.

1. INTRODUCTION

There are currently about 50 protein-misfolding diseases that make the lives of millions bitter [1]. Alzheimer's disease, and Parkinson's disease, among others are protein-misfolding, prion-like neurodegenerative diseases [2, 3], which are characterized by the accumulation of protein aggregates in well-ordered amyloids [4]. Since these neurodegenerative diseases are currently incurable, and their incidence is constantly increasing, it would be important to prevent them [5, 6]. Natural products are sources of new medicines [7, 8], as the polyphenols contained in them have antioxidant ability [9]. These phenolic compounds inhibit protein misfolding and aggregation [10, 11]. Phenolic hydroxyls of polyphenolic compounds bind to hydrophobic residues of amyloidogenic proteins, thereby inhibiting amyloid fibril formation [12, 13]. Higher hydrophobicity is known to promote aggregation [14]. Stabilization of native structures and suppression of the fibril growth phase are two essential factors for the inhibitory effect on protein aggregation [15, 16]. Epidemiological studies support that a diet rich in polyphenols reduces the risk of age-related neurodegenerative diseases [17].

Echinacea purpurea (L.) is a well-known herb worldwide. Dietary supplements and extracts of this plant have antiviral, antibacterial, antifungal and antioxidant effects [18]. Among the active ingredients of *Echinacea* spp., various caffeic acid derivatives have been identified, such as caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside and cichoric acid [19]. Both preclinical and clinical studies provide evidence that chlorogenic acid supplementation could protect against neurological degeneration [20]. Using Congo red binding assay it has been shown that chlorogenic acid effectively inhibits α-chymotrypsin amyloid-like fibril formation in a concentration-dependent manner [21]. Chlorogenic acid and caffeic acid are effective inhibitors of Aβ fibrillization [22], showed a significant protective effect against Aβ-induced neuronal death [23], and significantly suppressed the formation of human islet amyloid polypeptide oligomers [24].

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The inhibitory activity of caffeic acid against α-synuclein fibrillation has been demonstrated [25]. It was showed that caffeic acid may be also a preventive agent against the progression of Parkinson's disease [26]. Echinacoside was first isolated from *Echinacea angustifolia* DC, and showed promising potential for treatment of Parkinson's and Alzheimer's diseases [27, 28]. Echinacoside can significantly reduce extracellular accumulation of Aβ [29] and ameliorates the memory impairment [30]. Echinacoside dose dependently inhibited hen egg-white lysozyme aggregation [31]. Cichoric acid inhibits misfolding, aggregation and fibrillation of human islet amyloid polypeptide [32]. Cichoric acid alleviated memory impairment and amyloidogenesis, preventing damage to neurons, suggesting that cichoric acid may be useful in the treatment of Alzheimer's disease [33]. Because *Echinacea* contains many anti-amyloidogenic compounds, it is expected that an extract of it may be an effective amyloid fibrillation inhibitor.

2. MATERIALS AND METHODS

2.1. Materials

Bovine pancreatic α-chymotrypsin (EC 3.4.21.1, lyophilized, triple crystallized) was purchased from Sigma-Aldrich Kft. (Budapest, Hungary). The used Dr. Theiss Echinacea drops were distributed by Naturprodukt Kft. (Törökbálint, Hungary). All other reagents and buffer components used were of analytical grade.

2.2. Amyloid fibrillation of α-chymortypsin

For fibrillation, α-chymotrypsin samples were incubated at 0.15 mg/ml concentration in 55% ethanol/10 mM phosphate buffer at pH 7.0 for 24 h at 24 ^{o}C in the presence and absence of *Echinacea* drops.

2.3. Turbidity measurements

Protein aggregation is characterized by an increase in turbidity over time [34, 35]. Although turbidity measurement is not specific for amyloid fibrils, it can be used to monitor the efficacy of an inhibitory agent on aggregation. The absorbance of the samples incubated for 24 h was measured at 350 nm with a 1 cm long quartz cuvette at a protein concentration of 0.15 mg/ml in the presence of 55% ethanol/10 mM phosphate buffer, pH 7.0, in the presence and absence of various concentrations of *Echinacea* drops. Blank corrections were made on enzyme-free solutions of each sample.

2.4. Congo red binding assay

Fibrillation of α-chymotrypsin in the presence and absence of *Echinacea* drops was also checked by Congo red binding assay monitored spectrophotometrically. If Congo red dye binds to the well-ordered β-sheets of amyloidogenic proteins, then an increase in the absorption intensity and an absorption maximum with a characteristic redshift are observed [36, 37]. 200 μl of the α-chymotrypsin solutions previously incubated at various concentrations of *Echinacea* drops in the presence of 55% ethanol for 24 h and without them was added to 800 μl of a Congo red solution (in 5 mM phosphate buffer and 150 mM sodium chloride, pH 7.0). After 15 minutes of incubation at room temperature, absorbance spectra were recorded between 400-600 nm. Differential spectra were generated by subtraction of the spectra of α-chymotrypsin alone and Congo red alone from the spectrum of α-chymotrypsin in the presence of Congo red. The differential spectrum has a maximum intensity at 540 nm in the presence of amyloid fibrils.

2.5. Statistical analysis

Turbidity measurements were repeated three times. All data are presented as mean ± standard error of the mean (SEM). Experimental data were analyzed by one-way analysis of variance (ANOVA). Significance was defined as $^{***}P < 0.001$ and $^{**}P < 0.01$.

3. RESULTS AND DISCUSSION

In these experiments, α-chymotrypsin was used as a model protein. Amyloid fibrillation of α-chymotrypsin was performed under *in vitro* condition by incubating the protein in 55% ethanol for 24 h at pH 7.0, as previously described [38]. The aggregation inhibitory effectiveness of an inhibitor can be monitored using turbidity measurement [39]. The chlorogenic acid present in *Echinacea* drops inhibited α-chymotrypsin aggregation in a concentration-dependent manner

based on turbidity measured at 350 nm (Figure 1). Chlorogenic acid at concentration of 0.1 mg/ml reduced the aggregate content to 9.4%, while at a concentration of 0.05 mg/ml the reduction was 67.4%, compared to the sample without inhibition.

The tendency of α-chymotrypsin samples to aggregate was observed by turbidity measurements in the presence and absence of various concentrations of *Echinacea* drops to understand its inhibition efficacy. The *Echinacea* drops contained 50% ethanol, which was taken into account in the preparation of each sample. The intensity of turbidity was inversely proportional to *Echinacea* concentrations. We found that the amount of protein aggregation was greatly reduced with increasing concentration of *Echinacea* drops (Figure 2). The five-fold dilution of *Echinacea* drops caused the greatest inhibition, with the amount of aggregates in its presence reduced to 31.9% compared to the sample without inhibition. While at a 50-fold dilution, it reduced the amount of aggregates to only 76.4%.

The presence of a mild solvent causes an increase in the β-sheet conformation in proteins [40], promoting the formation of amyloid fibrils. Our samples contained 55% ethanol, so after one day of incubation they formed amyloid fibrils at a concentration of 0.15 mg/ml α-chymotrypsin. The inhibition of α-chymotrypsin fibrillation by *Echinacea* drops was also confirmed by Congo red binding assay. The addition of Congo red to amyloid fibril-containing samples results in a characteristic redshift in the absorption spectrum as it specifically interacts with them [41] (Figure 3a), making it suitable for monitoring the efficacy of an inhibitory agent [42]. The Congo red differential spectrum typically has a maximum at 540 nm in the presence of amyloid fiblils. A decrease in the maximum value at 540 nm indicates a decrease in the amount of amyloid fibrils. The Congo red differential spectra well reflected the spectral changes. The percentage of inhibition of α-chymotrypsin fibril formation was found to be dependent on the concentration of

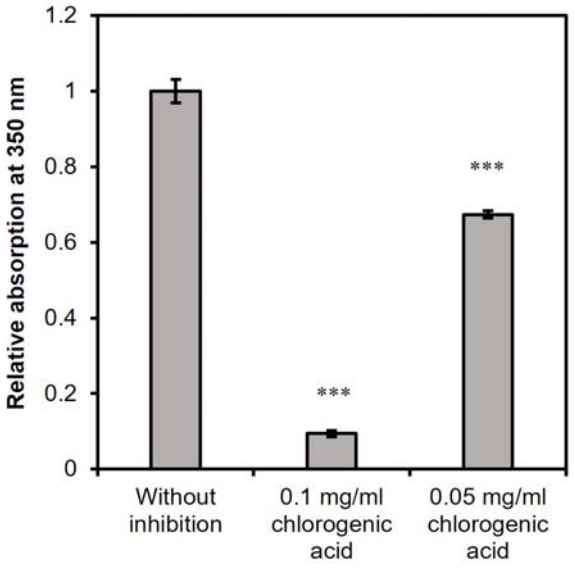


Figure 1. Turbidity measurements in the absence and presence of different concentration of the chlorogenic acid by recording the absorption after 24 h incubation at 350 nm in 55% ethanol at pH 7.0. α-chymotrypsin concentration: 0.15 mg/ml. Each bar represents the average of at least three independent measurements. All data are presented as mean ± standard error of the mean (SEM). Significance was defined as ***$P < 0.001$.

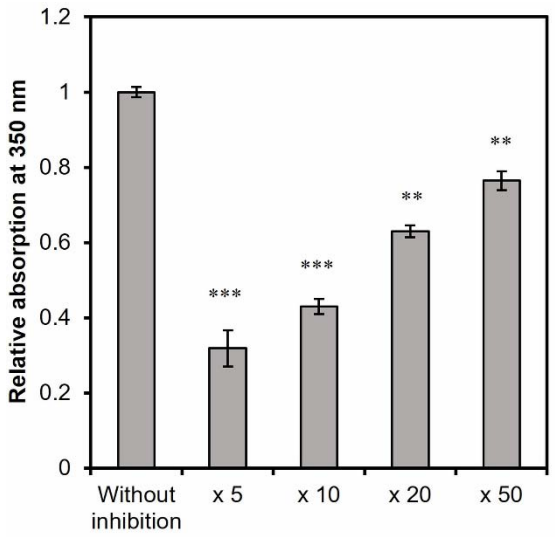
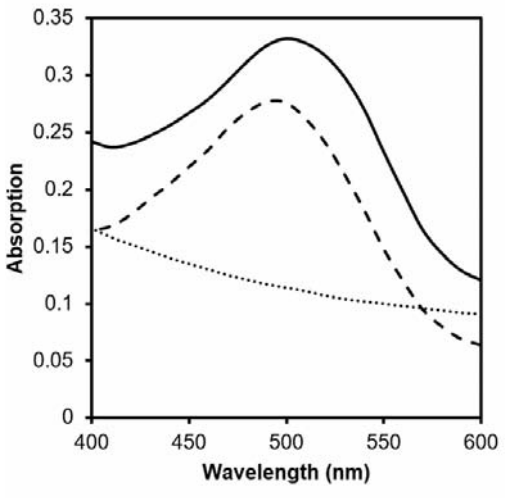
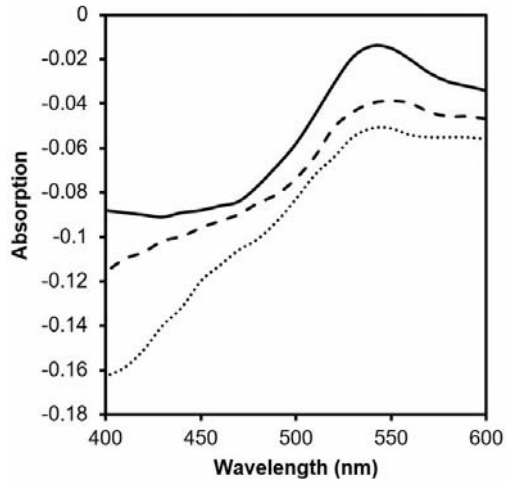


Figure 2. Turbidity measurements in the absence and presence of different concentration of the *Echinacea* drops by recording the absorption after 24 h incubation at 350 nm at 0.15 mg/ml α-chymotrypsin concentration in 55% ethanol at pH 7.0. Each bar represents the average of at least three independent measurements. All data are presented as mean ± standard error of the mean (SEM). Significance was defined as ***$P < 0.001$ and **$P < 0.01$.



(a)



(b)

Figure 3. Congo red visible absorption spectra of α-chymotrypsin in 55% ethanol without *Echinacea* drops (a), α-chymotrypsin + Congo red (solid line), Congo red alone (dashed line), α-chymotrypsin alone (dotted line). Congo red differential spectra (b) of the samples in the absence (solid line) and presence of *Echinacea* drops diluted 20 (dashed line) and 5 times (dotted line).

Echinacea drops as evidenced by the Congo red binding assay (Figure 3b).

Our work revealed that *Echinacea* drops are capable of inhibiting amyloid formation of α-chymotrypsin. Thus, *Echinacea* drops can even be used as a therapeutic agent to treat amyloid-associated diseases.

CONCLUSION

In conclusion, *Echinacea* drops effectively inhibits α-chymotrypsin aggregation in aqueous ethanol at pH 7.0.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

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