The anti-amyloidogenic effect of *Echinacea* drops

Márta Kotormán*, Linda Ágota Trencséni and Afrodité Szarvas
Department of Biochemistry and Molecular Biology, Faculty of Science and Informatics, University of Szeged, Középfásor 52, H-6726 Szeged, Hungary.

**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, cymarin, 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 fibrillation [22], showed a significant protective effect against Aβ-induced neuronal death [23], and significantly suppressed the formation of human islet amyloid polypeptide oligomers [24].

*Corresponding author
kotorman@expbio.bio.u-szeged.hu
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-chymotrypsin

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 °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.
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 fibrils. 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 *Echinacea* drops.

![Figure 1](image1.png)

**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](image2.png)

**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.
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.

REFERENCES
The anti-amyloidogenic effect of *Echinacea* drops