The anti-amyloidogenic effect of natural product extracts on amyloid-like fibril formation of trypsin in aqueous organic solvents

Ph.D. Thesis

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List of abbreviations

Aβ	β-Amyloid
Aβ40	β-Amyloid peptide of 40 residues in length
Aβ42	β-Amyloid peptide of 42 residues in length
AD	Alzheimer's disease
AFM	atomic force microscopy
APP	amyloid-precursor protein
BAEE	*N*-benzoyl-L-arginine ethyl ester
BBB	blood-brain barrier
CA	caffeic acid
CGA	chlorogenic acid
CR	Congo red
ECD	electronic circular dichroism
EGCG	epigallocatechin-3-gallate
EM	electron microscopy
FDA	Food and Drug Administration
FTIR	fourier-transformed infrared
GA	gallic acid
GC	gallocatechin
hIAPP	human amylin or human islet amyloid polypeptide
MCI	mild cognitive impairment
MD	molecular dynamics
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFT	neurofibrillary tangle
NMR	nuclear magnetic resonance

$(PAP)_{248-286}$	phosphatase-cleaved amyloid precursor peptide
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SSNMR	solid-state nuclear magnetic resonance
tau 2N4R	isoform of human tau containing 4 microtubule binding domains and 2 amino terminus inserts
TEM	transmission electron microscopy
ThT	thioflavin T
UV-Vis	ultraviolet-visible (light)

1. Introduction

One of most remarkable phenomena of nature is the ability of proteins to fold from linear polypeptide chains into functional structures serving as molecular machines that are indispensable for living organisms (Dill *et al*., 2008). Proteins are essential in many areas of life. They play a remarkable role as structural material. They act as structural scaffolds, catalyze biochemical reactions, regulate cell cycles, carry out transport and storage in living cells, and are essential elements of many signal transduction pathways (Ardito *et al*., 2017).

There are about 24,500 protein-encoding genes in the human genome (Clamp *et al*., 2007). Proteins consist of amino acid residues linked by peptide bonds. Protein folding is a rather complicated process; the details of it have not been fully understood yet. A failure at folding may lead to illness (Raimondi *et al*., 2017). Sometimes misfolding results in harmful aggregation. Molecular chaperones prevent incorrect folding and aggregation (Hendrick and Hartl, 1995). The misfolding of protein and their aberrant assembly into unbranched amyloid fibrils have been paid great attention (Siddiqi *et al*., 2017). The structural knowledge of protein aggregation will be significantly expanded in the next decades (Hu, 2017). Protein misfolding and aggregation cause a lot of illnesses (Khan *et al*., 2017). Amyloid fibrils with an ordered characteristic cross-beta structure appear in numerous neurodegenerative diseases (Dobson, 2017). At present, it is known that the appearance of amyloid fibrils is an indicator of various central nervous system neurological disorders and neurodevelopmental diseases affecting the brain and peripheral tissues (Stefani and Rigacci, 2013; Kim *et al*., 2013). Amyloid is a generic structural form of proteins and peptides and most polypeptides can form amyloid-like fibrils under suitable conditions (Chiti *et al*., 1999). The propensity of non-disease associated proteins to form pathological amyloid fibrils depends on their amino acid sequence (Siddiqi *et al*.,

2017). The core structure of the amyloid fibrils is stabilised by hydrogen bonding between the atoms of the polypeptide backbone (Dobson, 2003). Whereas various proteins are responsible for each disease, it is true for each that they have β-sheet-rich secondary structures and fibrillar aggregates in common (Šneideris *et al*., 2015). The formation of insoluble amyloid fibrils is associated with various incurable medical conditions, such as Alzheimer's, Huntington's and Parkinson's disease, transmissible spongiform encephalopathy, cerebellar ataxia, primary and secondary systemic amyloidosis and type II diabetes mellitus (Stefani, 2004; Bieschke, 2013).

The search for therapeutic agents that inhibit amyloid formation is a major challenge today (Young *et al*., 2015; Ma *et al*., 2018). For many chronic illnesses, prevention is more effective than treatment. Functional foods contain more biologically active ingredients, that have received remarkable attention, not only in traditional but in modern medicine. Natural phenolic compounds, which make up a long family of plant substances, are one of the most actively investigated categories of potential anti-amyloid drugs (Stefani and Rigacci, 2013; Shariatizi *et al*., 2015). There are currently more than 8,000 plant polyphenols and among them there are more than 4,000 flavonoids (Karunaweera *et al*., 2015). Human beings have been using various spices for more than 2,000 years, even so studying their biological activities has only begun today. Spices can prevent neurodegenerative processes (Mirmosayyeb *et al*., 2017).

1.1. The amyloid state of proteins

Amyloids were defined more than 150 years ago when tissue deposits of extracellular filaments were observed in the body's different organs. These microscopically visible deposits were found on different organs in many seemingly unrelated diseases, both systemic and localized in the body (Sipe and Cohen, 2000; Haass and Selkoe, 2007).

Although many diseases involve amyloid formation of distinct aggregation-prone proteins or peptides, the ability to form amyloid is not limited to these disease-associated proteins only. Amyloid fibrils may be formed from proteins, which can also fold into well-defined tertiary structures (for instance myoglobin and lysozyme) suggesting that the ability to form amyloid fibrils may be a general property of polypeptides. However, the main propensity for a particular protein or peptide to form amyloid fibrils is largely dependent on amino acid sequence. This is because for a globular protein to adopt an amyloid state, it has to be partly unfolded first before becomes into an amyloid fibril (Chiti and Dobson, 2006). Under suitable circumstances protein can convert into harmful aggregates. Disordered aggregates can be form from partially folded states. These unstable aggregates can convert ordered amyloid fibrils through more stable β-structured aggregates. Amyloid fibrils are thread-like structures with 7 to 13 nm diameter, containing 2-8 protofilaments in them twisted around each other. β-strands in fibrils are perpendicular to the long axis of the fibrils. If the amorphous deposits and native-like deposits form in an uncontrolled manner, these are also associated with pathological conditions (Fig. 1). Amyloid fibrils are formed by a nucleated polymerization mechanism. Their formation follows sigmoid kinetics with a lag phase, growth phase and saturation phase. In the lag phase disordered monomers convert into nuclei, fibrils and then they grow by addition of monomers fast (Chiti and Dobson, 2017) (Fig. 2). Experimental data suggest that critical protein concentrations are required to initiate fibrillation (Blancas-Mejía *et al*., 2017).

Amyloid aggregates are not soluble in the presence of detergents (Nizhnikov *et al*., 2014). Thioflavin-T (ThT), a benzathiole dye, is more commonly used to identify the presence of amyloid fibrils, as its fluorescence emission increases when it binds to the fibrils (Nilsson, 2004).

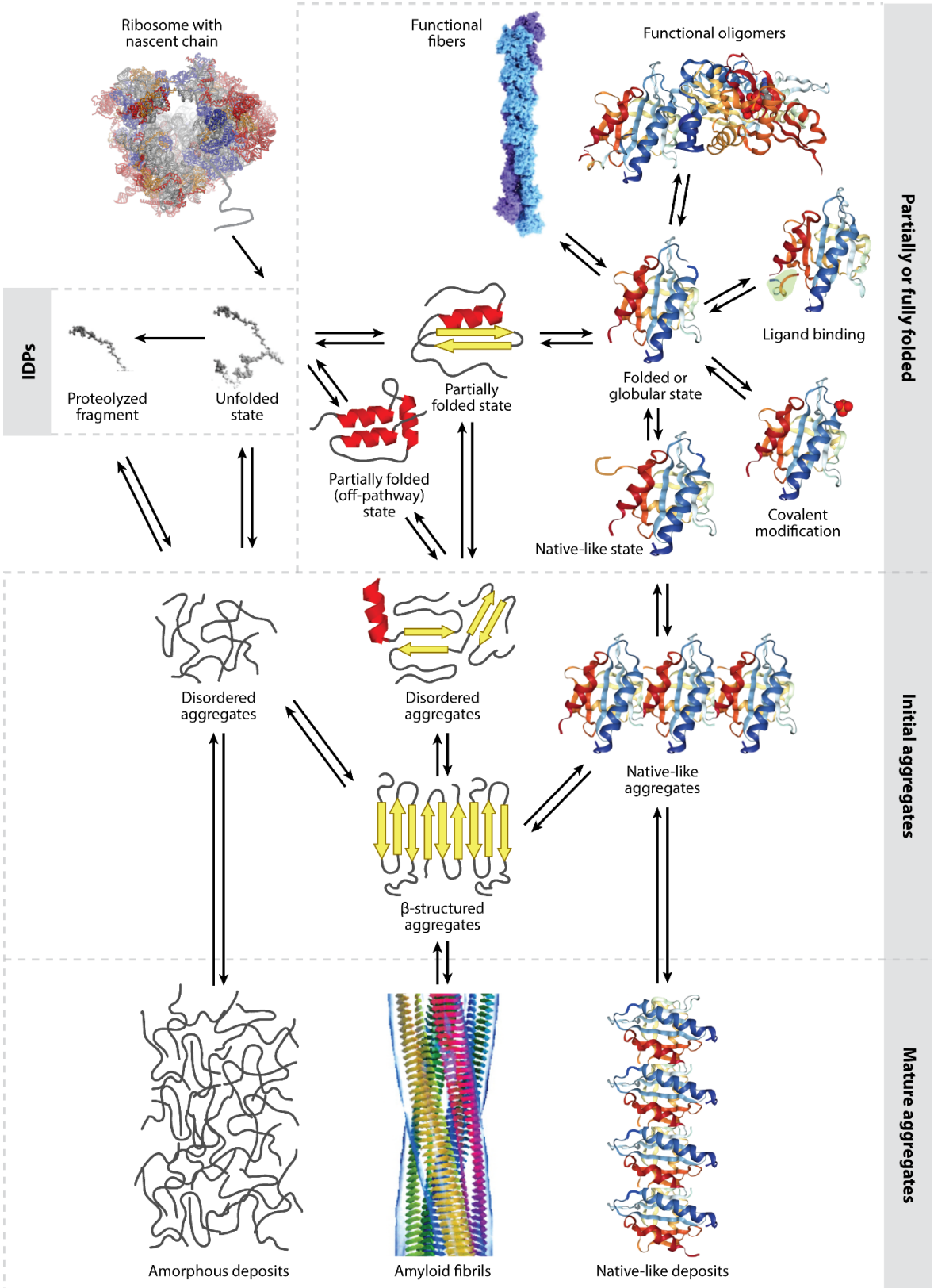


Figure 1. Multiplicity of conformational states (Chiti and Dobson, 2017). IDPs: Intrinsically disordered proteins.

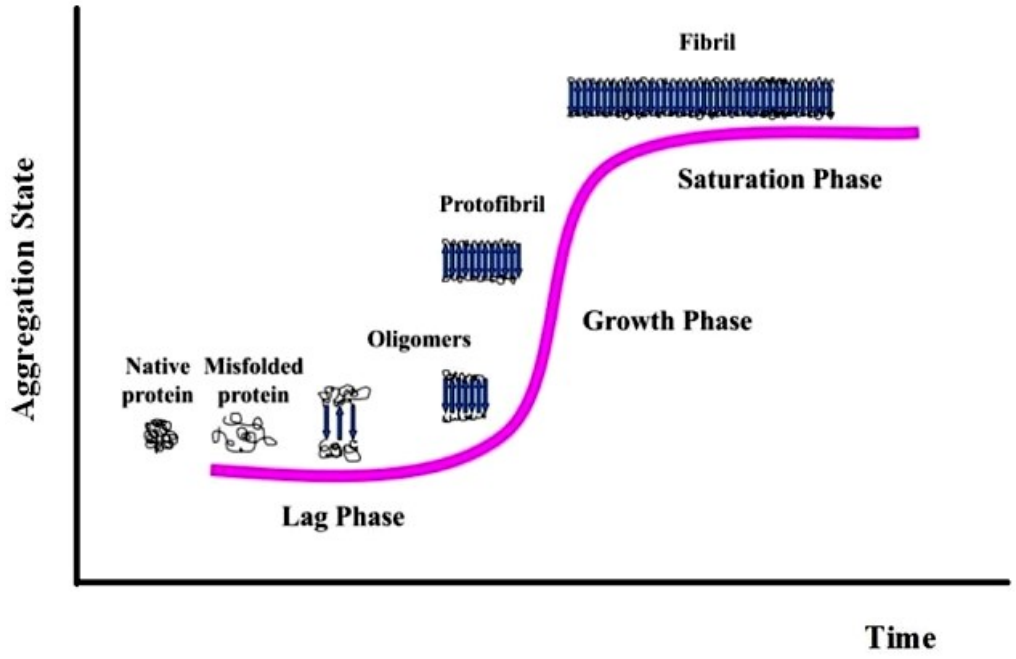


Figure 2. Nucleation dependent amyloid fibril formation (Iannuzzi *et al*., 2014).

1.1.1. Amyloid fibrils

Fibrillar amyloid deposits have more physical properties in common. Most importantly, they exhibit specific optical behaviors when linked to certain dye molecules. Fibrils show green birefringence under polarized light after being staining with Congo red (Frid *et al*., 2007). However, the use of Congo red to detect the presence of amyloid is frequently a labor-intensive process, and only provides a quality measurement of amyloid fibrils present.

In addition to binding to fibrils, ThT has both dramatic and standard enhancement of its emission and a maximum shift of its excitation spectrum, which show the presence of amyloid fibrils as an effective and efficient indicator (Nilsson, 2004).

Amyloid fibrils formed from various polypeptides are believed to share a similar morphology known as the cross-β structure (Chiti and Dobson, 2006). Independent measurements of the fibrillary structure have so far confirmed the cross-β structural core of amyloid fibrils. X-ray fiber diffraction studies have shown that the diffraction pattern of

fibrils is characterized by main orthogonal reflections along the meridional and equatorial directions corresponding to a 4.8 Å inter-peptide separation, and a 10 Å inter-sheet separation respectively (Sipe and Cohen, 2000; Nilsson, 2004; Sunde *et al*., 1997) (Figure 3).

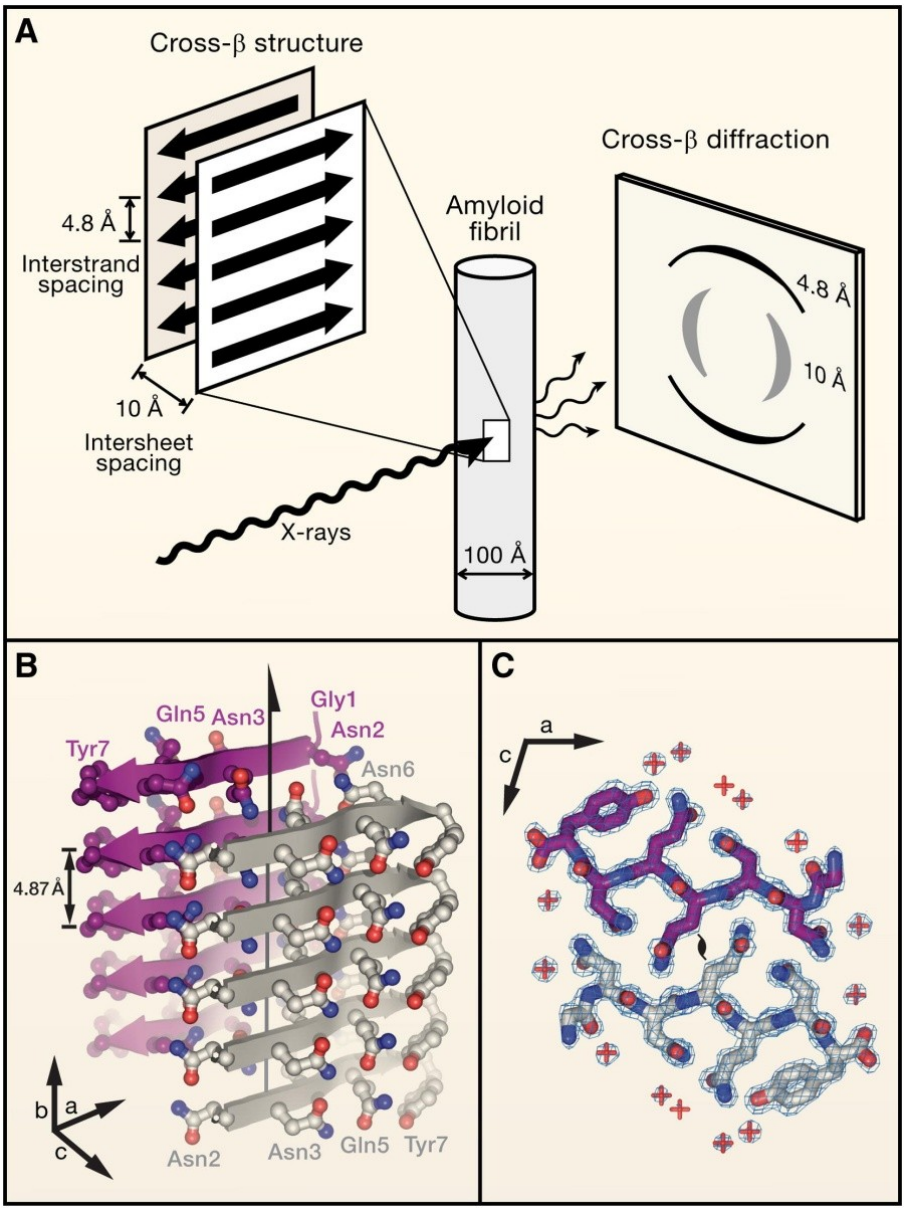


Figure 3: The cross-β structure and the diffraction pattern of fibrils (Eisenberg and Jucker, 2012).

The separations between the inter-peptide and the inter-sheet are parallel and perpendicular to the long-axis of the fibril. This diffraction pattern is now regarded as

indicative of the presence of cross-β structure, and thus the presence of amyloid fibrils. When fibrils are stained, the macromolecular morphology of fibrils could be determined by the transmission electron microscopy (TEM): fibrillary structures are long, non-branched, and ribbon-like structures (Chiti and Dobson, 2006) (Figure 4). The typical diameter of amyloid fibrils is ≈ 10 nm and a length up to a few micrometers (Petkova *et al*., 2002).

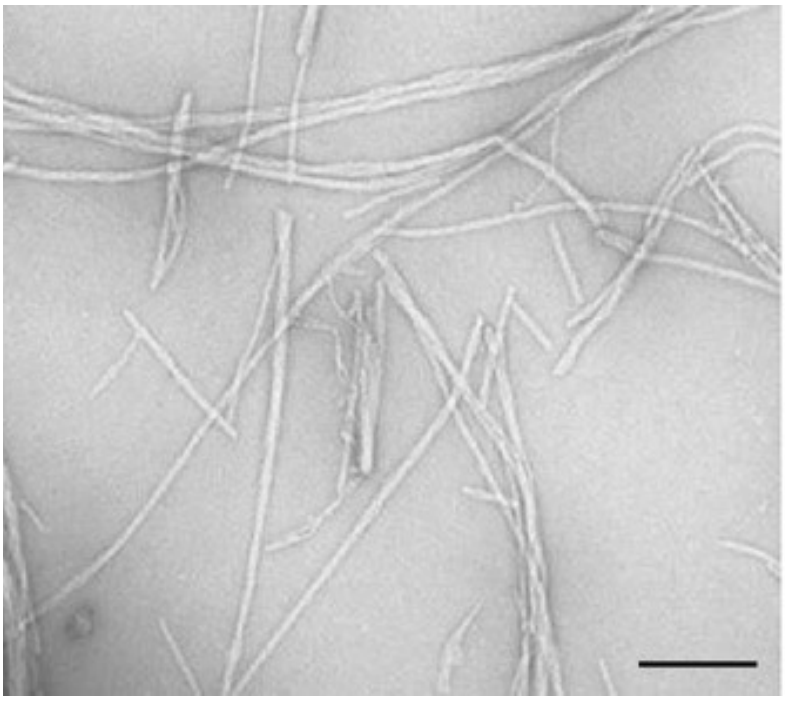


Figure 4: TEM image of negatively-stained Alzheimer's Aβ fibrils. The scale bar is 200 nm (Gras *et al*., 2011).

Over the last decade, the progress of solid-state NMR (SSNMR) and X-ray crystallography explained the molecular atomic details of amyloid fibrils. One of the first SSNMR models of an amyloid fibril used was that of Aβ40, a peptide affected by Alzheimer's disease. Its core fibril unit consists of a parallel in-register β-sheet, wherein each strand is a β-hairpin peptide-peptide backbone with hydrogen bonds which run parallel to the long-axis of the fibril (Petkova *et al*., 2002; Petkova *et al*., 2006).

The individual packing arrangement of polypeptides in amyloid fibrils may change with the changing experimental conditions in which the fibrils are formed. The especial structural polymorphism includes the length of the β-strands, side chain orientations and inter-proto filament packing (Kodali and Wetzel, 2007). Fibril polymorphism may play an important role in amyloid diseases, as the various morphologies show different toxic effect depending on which residues are exposed on the surface. For instance, they have been shown that calmly formed fibrils of Aβ(1-40) to be more toxic than agitated fibrils (Petkova *et al*., 2005).

1.1.2. Non-fibrillar oligomers

Due to their structural disorder and transient nature, it is difficult to obtain high resolution structural details of amyloid oligomers, using conventional normal structural determination techniques. Studies in TEM and atomic force microscopy (AFM) using low-resolution techniques have shown that transient, unstable particles may appear before the formation of fibrils (Chromy *et al*., 2003; Caughey and Lansbury, 2003; Kayed *et al*., 2003). These protein aggregates are mentioned in the literature as amyloid proto fibrils (Chiti and Dobson, 2006). Those that do not progress to form fibrils are regarded off-pathway. Nevertheless, off-pathway oligomers formed in the presence of detergents, lipids, and certain small molecules are generally ignored to be biologically-relevant. Contrary to fibrils, amyloid oligomers do not have a generic structural element and instead, they have a wide spectrum of sizes and morphologies. Size exclusion chromatography (SEC) studies of Aβ40 oligomers (isolated *in vitro* from the brains of persons who died of Alzheimer's disease) have shown the existence of oligomers ranging from dimer size to big oligomers of hundreds of peptides. Annular, spherical, or curvilinear oligomeric assemblies in shape have been published in literature (Kayed *et al*., 2003; Chimon *et al*., 2007).

Although there are big differences in morphologies, oligomers formed from different polypeptide sequences may exhibit similar activities during cell metabolic assays. It is important to note that many oligomers of different sizes always share the ability to interact with a single oligomer-specific antibody. Numerous studies have shown that oligomers can have high β-sheet content (Walsh *et al*., 1999; Chimon *et al*., 2007; Campioni *et al*., 2010). In addition, some non-fibrillar oligomers may contain common structural elements. Based on the high-resolution structural analysis of non-fibrillar oligomers of Aβ42 and of prion-like peptides, it is assumed that they may contain cross-β like fragments (Stroud *et al*., 2012; Walsh *et al*., 2010).

1.2. Amyloid involvement in diseases

All proteins may form unbranched, long, amyloid fibrils with many beta-sheets *in vitro* under suitable conditions i.e., formation of amyloid might be a general property of the polypeptide backbone (Simon *et al*., 2012; Dorta-Estremera *et al*., 2013; Kotormán *et al*., 2017). The aggregation behavior of various peptides and proteins shows remarkable similarities. Unfolded or partially unfolded proteins connect to each other forming small, soluble aggregates that undergo further assembly into proto fibrils or proto filaments. These structures are usually short, thin, in some cases curved, fibrillar structures that can be assumed to compile into mature fibrils. *In vitro* experiments suggest that the formation of such species will generally begin with a lag phase, followed through rapid growth. This phenomenon is increasingly associated with common and highly debilitating diseases.

As numerous diseases share the amyloid plaque pathology and toxicity, fibrils were originally hypothesized to be the toxic species in these diseases (Hardy and Selkoe, 2002). However, recent research has shown that non-fibrillar oligomers are more likely to be the toxic agents causing multiple neurodegenerative diseases (e.g., Parkinson's, Alzheimer's,

Huntington's diseases and spongiform encephalopathies) and type II diabetes (Xue *et al*., 2009; Berthelot *et al*., 2013). At present, the mechanism of toxicity of amyloid oligomers has not been identified, and it is an area under intensive research. Oligomers formed from many peptides, including those not related to amyloid disorders (e.g. lysozyme, β2-microglobulin, transthyretin), all exhibited toxicity, which suggests that the toxicity of amyloid oligomers may be independent of the peptide sequence (Kayed *et al*., 2003; Fändrich, 2012). Experimental evidence broadly supports the hypothesis that amyloid toxicity is based on a generic mechanism involving the interactions of oligomers with cellular membranes and other compounds (Martins *et al*., 2008). Specifically, it is assumed that oligomeric aggregates may at last induce cell death by interacting with and disrupting the integrity of the cellular membrane (Berthelot *et al*., 2013). Moreover, the aggregation of amyloidogenic peptides was found to occur faster in the presence of membrane surfaces, which lead to the claim that membrane-catalyzed fibril formation may cause cellular toxicity.

Diseases related to amyloid fibrils (Table 1) can be classified as degenerative diseases (for instance Alzheimer's disease) and prion diseases (for instance bovine spongiform encephalitis). Prions cause the formation of amyloid fibril *in vivo* in other systems (Scott *et al*., 1999; Hill *et al*., 1997). In some amyloid-related illnesses, patients die because of organ failure resulting from amyloid fibril deposits (Siqueira-Filho *et al*., 1981).

There is no simple way to treat amyloid diseases. Currently, it is limited to treating the symptoms of illnesses. Although some forms of amyloidosis can be treated by chemotherapy and organ transplantation, large amyloid fibril masses can only be removed surgically (Pepys, 2006).

Table 1: A list of some amyloid associated diseases and their precursor polypeptides (Ow and Dunstan, 2014).

Precursor polypeptide	Amyloid classification	Associated syndrome/disease
Aβ peptide	Aβ	Alzheimer's disease
Atrial natriuretic factor	AANF	Cardiac arrhythmias
Serum amyloid A protein	AA	Rheumatoid arthritis
Apolipoprotein A1	AApoA1	Atherosclerosis
Cystatin C	Acys	Cerebral amyloid angiopathy (Icelandic type)
Gelsolin	AGel	Familial Finnish amyloidosis
Immunoglobulin light chain	AL	Systemic Al amyloidosis
Islet amyloid polypeptide	AIAPP	Diabetes mellitus, type II
Kerato-epithelin	Aker	Lattice corneal dystrophy
Lysozyme	Alys	Hereditary non-neuropathic systemic amyloidosis
Median	AMed	Aortic medial amyloid
β2-Microglobulin	Aβ2M	Dialysis related amyloidosis
Prion protein	APrP	Spongiform encephalopathies
Prolactin	APro	Prolactinomas
Transthyretin	ATTR	Familial amyloid polyneuropathy

1.3. Alzheimer's disease

Alzheimer's disease (AD) was first described in 1907 by a German physician named Alois Alzheimer. It is a progressive neurodegenerative disorder (Hippius and Neundörfer, 2003). AD is the most common cause of dementia in elderly people, accounting for 60-70% of cases (Fratiglioni *et al*., 2000). Age is the most significant risk factor for AD, the prevalence for the disease is around 1% at age 65 and around 20% at 85

years of age. Upon investigation, the postmortem brains of AD patients show significant neuronal dystrophy. With the growing old population of the world the number of AD patients will tremendously increase and with it the economic cost to be paid by society. From a pathological point of view, AD is characterized by the presence of extracellular deposits of senile plaques and neurofibrillary tangles (NFTs), each of which appear as lesions on stained neuronal tissue under visible light microscopy (Figure 6). The global prevalence of dementia was estimated at 24 million and is predicted to double every 20 years by at least 2040. As the world's population is constantly aging, the number of vulnerable individuals is growing, especially among the very old. The disease is closely linked to aging and it has been estimated that around 81.1 million people worldwide will suffer from AD by 2040 (Ferri *et al*., 2005; Mayeux and Stern, 2017).

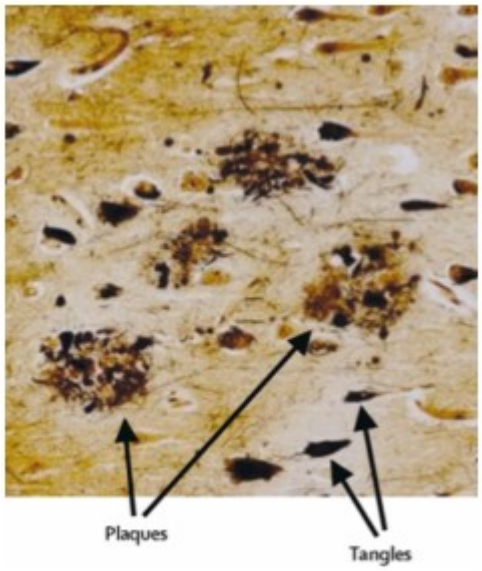


Figure 6: Lesions in the cerebral cortex tissue of an AD brain formed from amyloid plaques and NFTs (Blennow *et al*., 2006).

The first symptoms of AD are often loss of short-term memory, and, with the progression of the disease, disorientation and aphasia followed by a general decrease in cognitive functions.

Despite the fact that there are different tests and criteria available for clinical diagnosis, a definite AD diagnosis can only be made post mortem by a pathological exam of the brain to check the existence of the aforementioned neuropathological changes.

AD has three clinical disease stages: pre-symptomatic, prodromal or amnestic or non-amnestic mild cognitive impairment (MCI) and AD dementia. The pre-symptomatic stage comprises of a cognitively normal person that have begun to develop the pathological features of AD, amyloid plaques and NFTs. The next stage, MCI, the appearance of cognitive symptoms, often deficits in episodic memory with amnestic MCI and attention or language with non-amnestic MCI and may include some progress in cognitive dysfunction. This stage may also be associated with irritability, anxiety and depression. The last stage of dementia, as defined above, consists of impairments in several cognitive domains, such as executive functions, language, and visuospatial functions. At this stage, behavioral changes such as aggression, agitation, emotional distress, restlessness, and sleep disturbance may appear. Patients are mostly diagnosed in the MCI stage as their concerns about changes in cognition increase. The disease course generally lasts from 3 to 8 years depending on the age of the patient at diagnosis (Cappai and Barnham, 2008), out of which ~40% of the time is spent in the most severe stage of the disease (Arrighi *et al*., 2010).

Patients with illness can usually be divided into two subgroups depending on the age of beginning. The initial AD, which accounts for about 2% of all AD cases, occurs before the age of 65 whereas the more frequent form, late onset AD called sporadic, appears after the age of 65. Familial AD belongs to the group of the early AD group of AD and is caused by amyloid-precursor protein (APP), Presenilin 1 or Presenilin 2 mutations.

In 1985, the amyloid-β protein or Aβ was identified as the major constituent of these senile plaques. Monomer Aβ is about 4 kDa peptide which is produced by the intramembrane proteolytic cleavage of a bigger protein, the APP (Hardy and Selkoe, 2002).

APP is sequentially processed with aspartyl proteases β-secretase and γ-secretase, producing a set of Aβ peptides of lengths varying from 38 to 43 residues depending on the position of the cleavage with γ-secretase (Figure 7) (Gandy, 2005).

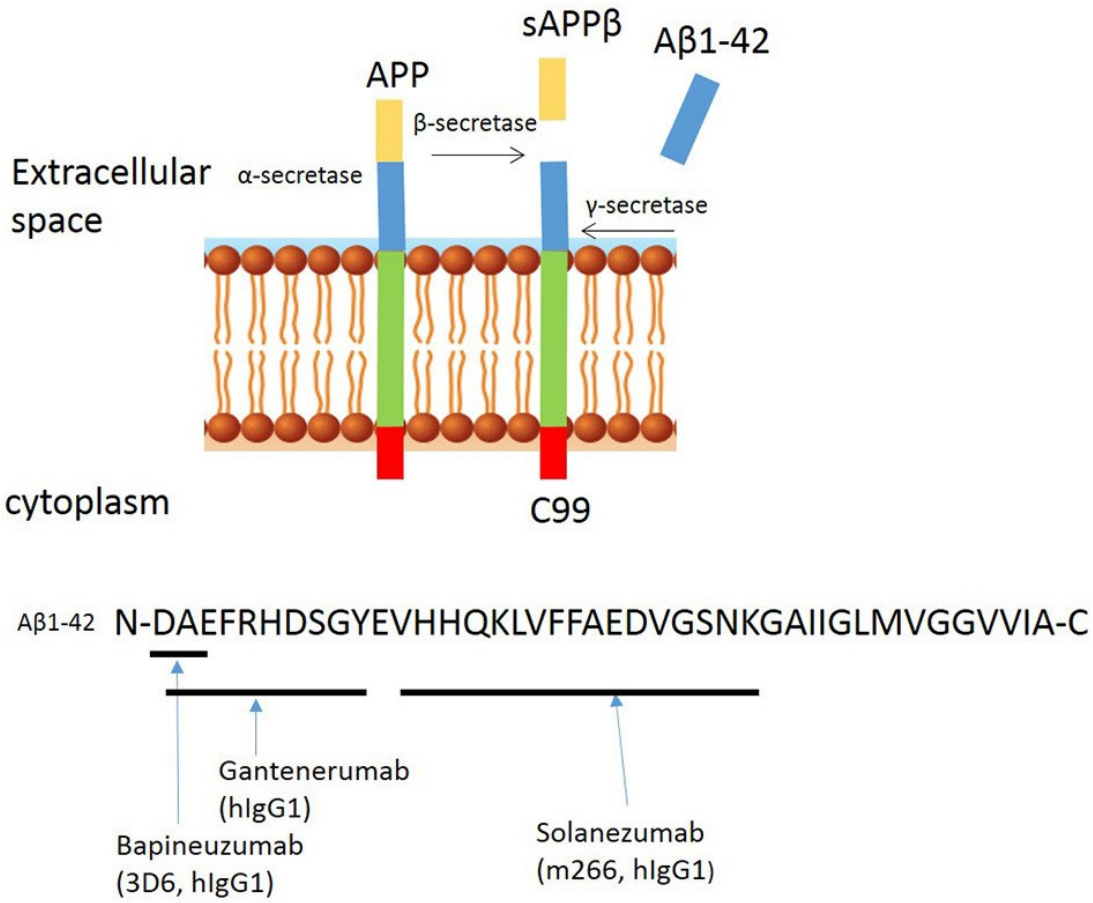


Figure 7: A schematic representation of the production of A*β* through proteolytic processing of APP (Sumner *et al*., 2018).

Peptides containing residues 1-40 (Aβ40) or 1-42 (Aβ42) are predominantly found in AD-associated plaques (Golde *et al*., 2000; Holtzman *et al*., 2011). Although the plaques contain various isoforms of the Aβ peptide, Aβ42 is likely to be the more harmful form of Aβ. *In vitro*, Aβ42 exhibits a significantly higher propensity for aggregation compared to Aβ40, despite the fact that only two amino acids differ from each other (El-Agnaf *et al*., 2000; Barrow *et al*., 1992).

Furthermore, genetic mutations within presenilin 1 and 2 (genes encoding enzymes that cleave APP to produce Aβ peptides) also cause an aggressive early-onset form of AD, leading to an increase in the ratio of Aβ42 to Aβ40 peptides produced (Kumar-Singh *et al*., 2006; Bentahir *et al*., 2006; Hardy, 1997). Genetic evidence strongly supported the amyloid hypothesis: a chromosome responsible for APP encoding in trisomy 21 (Down's syndrome), and the overproduction of Aβ causes an early-onset of dementia with AD-like plaque load (Goate *et al*., 1991; Levy-Lahad *et al*., 1995). Additionally, in individuals with early-onset familial AD, genetic mutations on the APP induce the production of Aβ peptides that exhibit an increased aggregation tendency (Tam and Pasternak, 2012).

In addition to the presence of amyloid plaques, the other marker of AD is the intracellular deposition of NFTs which consists of aggregated hyper phosphorylated forms of the microtubule-associated τ protein (Ballatore *et al*., 2007). The role of the τ protein and interaction of the amyloid in AD pathogenesis is still established (Ittner and Götz, 2011). Investigations with mouse models suggest that the role of NFTs in AD may be lower than Aβ since Aβ plaque pathology was not developed in a τ transgenic mouse model, whereas Aβ formation in APP transgenic mice was found to induce hyper phosphorylation of τ, leading to the formation of NFTs (Götz *et al*., 2004).

The mysterious aspect of AD is that in the brain of dementia patients, plaque load does often not correlate with the progression and severity of the disease (Stroud *et al*., 2012; Näslund *et al*., 2000). Instead, more pieces of evidence show that the synaptic loss and the severity of cognitive impairment are correlated with the concentration of soluble Aβ oligomers in the brain (Lue *et al*., 1999). For instance, oligomers extracted from AD brain may damage synapse structure and function. Furthermore, when injected into the brains of animal models of AD, Aβ oligomers reduced the number of synapses and decreased learning ability (Lesné *et al*., 2006; Cleary *et al*., 2005). In addition, cellular

models of toxicity exhibited characteristic symptoms of neurotoxicity that lead to possible apoptosis by adding of Aβ oligomers prepared either *in vitro* or extracted from cell cultures (Cappai *et al*., 2008). In summary, current experimental evidence suggests that preventing the formation of Aβ oligomeric forms is a promising method of the treatment for AD.

1.4. Amyloid inhibition by small molecules can be a promising method for treating for AD.

With the increasing lifespan of our population, AD approaches epidemic proportions and there is no cure or preventive treatment. In 2010, it was estimated that 36 million people in the world were suffering from AD, and this number is expected to grow to 115 million people by 2050 (Alzheimer's Association, 2012). Compounds have been reported to help the reduction of amyloid deposition in the brain either by inhibiting the enzymes or by inhibiting Aβ peptide aggregates using of antibodies, small peptides, or small organic molecules, natural product extracts, or by increasing amyloid degradation using amyloid vaccines.

Furthermore, there are no drugs that can target the underlying disease: currently licensed treatments such as donepezil (a cholinesterase inhibitor), and memantine (an N-methyl-D-aspartate antagonist), only mitigate cognitive symptoms. At present, five drugs are approved by the Food and Drug Administration (FDA) for use at AD cases and are divided into two categories: acetylcholinesterase inhibitors and glutamate modulators. All five drugs were licensed between 1993 and 2003, none address the underlying etiology of AD, and their effectiveness differs greatly among individuals. During the first year, the performance of a patient can be significantly improved in measures of cognition, activities of daily living behavioral symptoms, and clinical global impression of change for 10−20%

of patients, plateau for 30−50% of patients, or continue to deteriorate for 20−40% of patients (Atri, 2011).

Although AD does not currently have therapeutics, progress has been made. Intensive structural and biochemical analysis of the amyloid structure has led to the development of potential therapeutics for treating underlying diseases. In the last years, small-molecule compounds have been developed that reduce the formation, deposition and accumulation of Aβ amyloid aggregates as a promising method of AD treatment. *In vitro* screenings led to the discovery of many small molecules that may affect the amyloid aggregation pathway (Ryan *et al*., 2012). Some of these drug-like molecules inhibit the formation of amyloid fibrils, while others prevent or reduce non-fibrillar oligomer formation (Levine, 2007; Necula *et al*., 2007).

The most important pharmacological requirement of drugs targeting AD and other neurodegenerative diseases is their ability to penetrate the blood-brain barrier (BBB) at appropriate concentrations in the brain to effectively reach their therapeutic effects (i.e., to inhibit amyloid formation) (Hawkes *et al*., 2009; Hubbard, 2011). Although many small molecules prove to be effective in inhibiting amyloid formation *in vitro* and amyloid toxicity in cell cultures, many of these small molecules have poor BBB penetration and are very toxic, and therefore not readily used as genuine therapeutics.

1.4.1. Dye-based molecules

Among the first compounds that bind amyloid fibrils are the dye molecules used to identify amyloids (Figure 8). Congo red (CR) was initially used for the histological detection of amyloid binding, where fibrils exhibit red-green birefringence when bound to CR if viewed with polarized light (Frid *et al*., 2007). The binding affinity of CR is within the range of 0.1 - 1.5 μM for different fibrils. Like CR, ThT shows Kd's in the low μM

range, with values ranging from 0.033 to 23 μM published in the literature (Groenning, 2010).

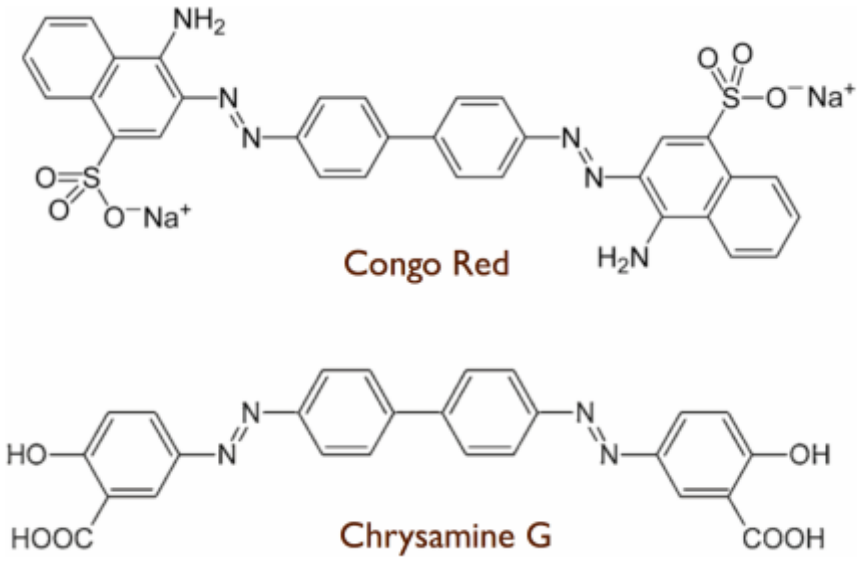


Figure 8: Chemical structure of amyloid-binding dyes Congo red and its derivative, Chrysamine G.

However, although ThT is closely bound to amyloid fibrils, binding has not been found to affect amyloid aggregation, unlike CR. Early amyloid detection using CR showed that CR is not only bound to fibrils but may also affect the amyloid aggregation pathway by interacting with one or more amyloidogenic species (Caspi *et al*., 1998). The fibril formation of amyloidogenic Aβ fragments, prion proteins, and the immunoglobulin light chain variable domain have been found to be promoted by the presence of CR at low molar ratios, and inhibit at high molar ratios (Esler *et al*., 1997).

Although CR has anti-amyloidogenic and anti-prion properties, its carcinogenic properties make CR a poor therapeutic candidate (Hawkes *et al*., 2009). Therefore, efforts have been made to find CR-based analogues that maintain anti-fibrillar aggregation activity but have improved toxicity profile and BBB bioavailability. Chrysamine G (CG) is one such analogue of CR (Figure 8). CG exhibits higher lipophilicity and lower toxicity than

CR and is able of inhibiting aggregation and amyloidogenic toxicity both *in vitro* and *in vivo* (Ishii *et al*., 2002; Reinke and Gestwicki, 2007).

1.4.2. Polyphenols

Polyphenols are a class of molecules naturally occurring in plants, and contain one or more aromatic phenolic rings with more hydroxyl groups (Figure 9 and 10).

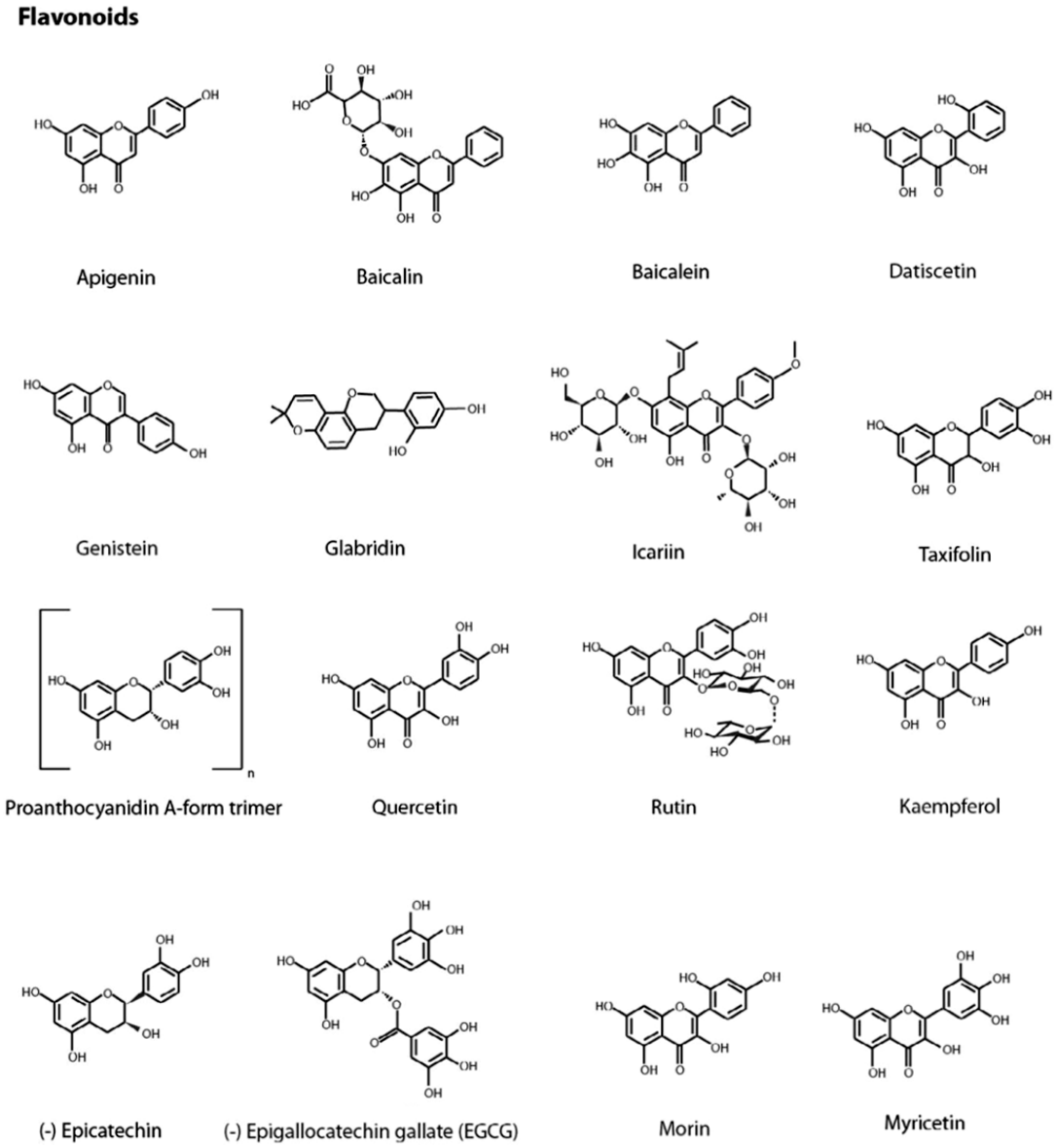


Figure 9. Chemical structures of identified flavonoid natural product-based amyloid inhibitors (Velander *et al*., 2017).

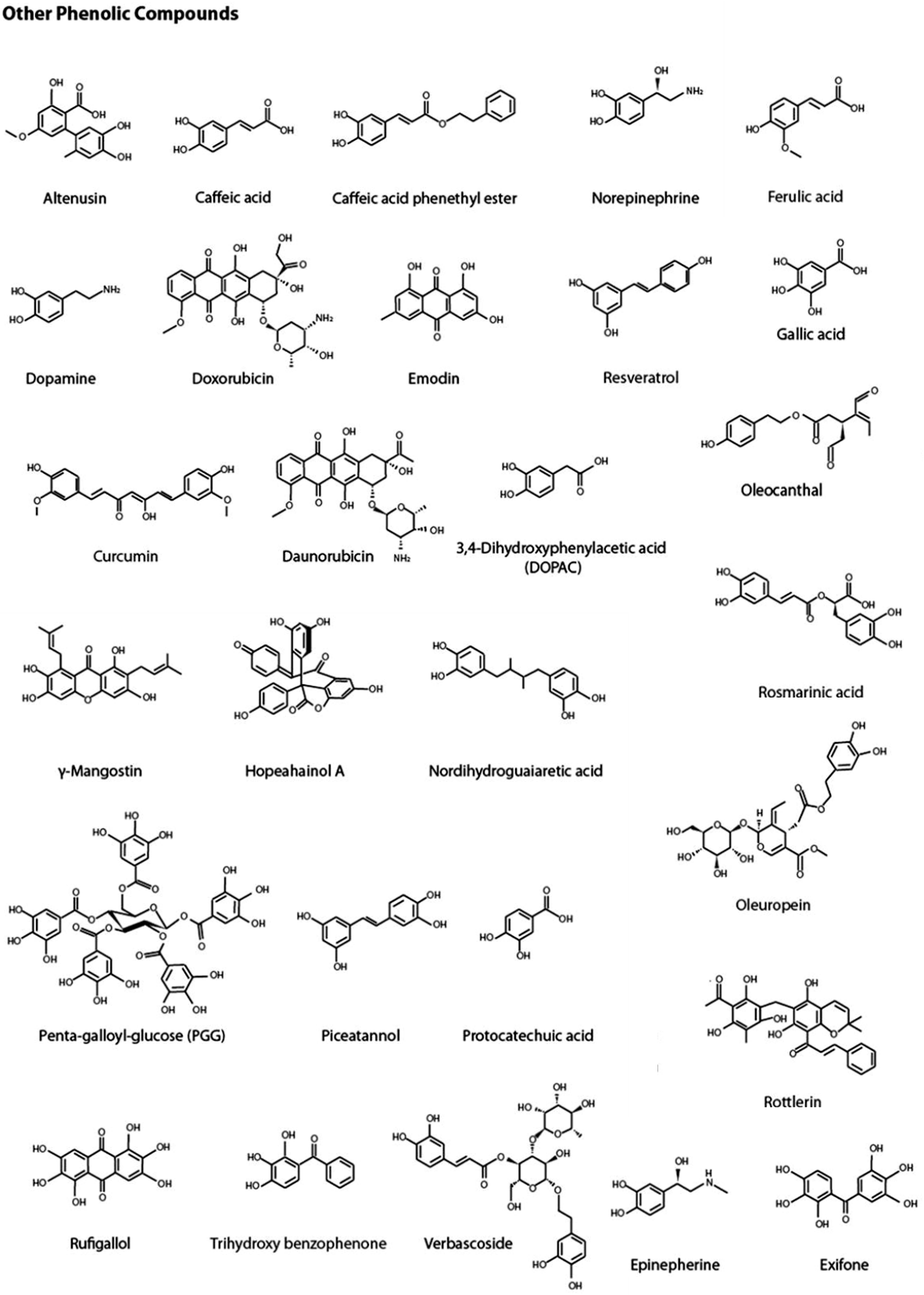


Figure 10. Chemical structures of identified phenolic natural product-based amyloid inhibitors (Velander *et al*., 2017).

Due to their antioxidant properties, the consumption of polyphenols has been reported to have a beneficial effect on health. For instance, epigallocatechin-3-gallate (EGCG), a main phenolic component of green tea and resveratrol, a polyphenol present in red wine, has cancer-preventing properties (Baur and Sinclair, 2006; Singh *et al*., 2011). In the last years, polyphenols were given more attention due to their ability to treat AD (Porat *et al*., 2006).

Furthermore, based on their various functions including anti-oxidant, anti-inflammatory, metal chelating capacities, polyphenols provide a rich source for many different structural backbones to find multifunctional anti-amyloid agents that can be used in rational drug planning efforts (Savelieff *et al*., 2014; Korshavn *et al*., 2016).

Examples include oleuropein and oleocanthal are present in olive oil, resveratrol is in fruit and red wine, curcumin is in turmeric, as well as EGCG and myricetin is in green tea. Other polyphenols found in healthy foods comprise cinnamaldehyde found in cinnamon, caffeic acid and rosmarinic acid found in culinary herbs, and genistein found in legumes. Contrary to the flavonoids or phenolic acid derivatives that included the majority of structures found within polyphenol amyloid inhibitors, many inhibitors with remarkable various structures were identified: cyclodextrin, a cyclic carbohydrate byproduct formed from enzymatic starch degradation; squalamine, an aminosterol isolated from dogfish with previously documented antiviral and anti-bacteria effects (Savelieff *et al*., 2014; Korshavn *et al*., 2016); vitamin A, a fat soluble vitamin (Takasaki *et al*., 2011); hematin, a porphyrin used in treatment of porphyria (D'Avola *et al*., 2016); rifampicin, an antibiotic for the treatment of bacterial infections; and scyll-inositol, a plant sugar alcohol there is plenty in coconut palm. Attention should be paid to the fact that the amyloid-inhibitory functions of many of these compounds have not been validated *in vivo*.

1.5. Recent results in elucidating the molecular mechanism of small-molecule binders and inhibitors of amyloidogenic species

Amyloid fibril formation is a multi-stage process involving various species at each stage. Because of the heterogeneous nature of prefibrillar species, the experimental determination of the molecular structures of these amyloid species remains a challenge. Further compounding experimental challenges, these small molecules can interact with amyloidogenic species at various stages of aggregation that are not known a priori. In addition, the binding mechanism of small molecule amyloid aggregation inhibitors is not fully described by the classical enzyme-inhibition model. The observation of amyloid inhibition often requires an inhibitor concentration in the micro-molar to milli-molar range (Hawkes *et al*., 2009), which suggests that these small-molecule inhibitors are non-specific binders. In contrast, substrates of folded enzymes have higher binding specificity, their binding affinities are often in the nanomolar range (Singh *et al*., 2011). In summary, the above-mentioned challenges significantly inhibited the determination of the molecular base of amyloid inhibition by using experimental methods with small-molecule inhibitors.

In contrast, computer simulations are not limited by these experimental challenges, and are able to give the atomistic level of details required to clarify the effect of small molecules in inhibiting of amyloid formation. Because of these molecular dynamics (MD), simulations have played a key role in understanding the binding mechanism of these small molecule inhibitors. However, clarification of the molecular basis of these small-molecule inhibitors poses many difficulties for simulation studies. The structural disorder of amyloid-forming peptides makes statistically meaningful attributes of MD simulations difficult. Furthermore, since it is unknown whether small-molecule inhibitors can interact with amyloidogenic monomers or aggregates, their binding should be frequently studied

with a number of amyloidogenic species in order to fully understand their mechanism of action.

With the combination of isothermal titration calorimetry, NMR, and MD simulations, the interaction of EGCG molecules with monomeric Aβ42 as modulated by temperature, salt concentration, pH, and ligand: protein molar ratio was examined by Wang et. al (Wang *et al*., 2010). The simulations were performed in the presence of EGCG by increasing molar ratio of the Aβ peptide in α-helical conformation. The results of this study showed that both hydrogen bonding and hydrophobic (aromatic) interactions are important for EGCG binding, and the balance of these interactions is especially sensitive to ligand: protein stoichiometry.

1.6. Drug discovery strategies against amyloid related disorders

Several therapeutic strategies are used to identify disease modifiers against amyloid related disorders. To identify the natural compound, the source of information is derived from epidemiological studies suggesting preventive effects for AD, dementia or diabetes may be associated with diets that contain high intake of polyphenolic compounds and flavonoids (Wang *et al*., 2010). The Mediterranean diet, characterized by a high intake of fruits, vegetables, olive oil, and cereals, is associated with reduced risk for mild cognitive impairment and AD in multiethnic community studies in New York (Scarmeas *et al*., 2006; Scarmeas *et al*., 2009). According to several cohort studies, moderate intake of red wine (which contains resveratrol) is associated with reducing the risk of AD, dementia, or cognitive decline (Arntzen *et al*., 2010; Luchsinger and Mayeux, 2004). Curcumin, present in yellow curry spice, EGCG and myricetin, polyphenolic compounds found in green tea, and turmeric found in traditional Southeast Asian diets are associated with cognitive functional health (Yamada *et al*., 2015). However, the protective effects of the food are not

the same as the specific effects of a single active ingredient. It is not known how diet specific natural compounds may have an effect on health. However, information reported by alternative and complementary medicine and information from these epidemiological sources resulted to testable hypotheses and experimental efforts which have successfully identified a number of natural amyloid inhibitor compounds (Ardah *et al*., 2015; Rigacci *et al*., 2010; Ono *et al*., 2004; Velander *et al*., 2016). One of the current strategies for identifying therapeutic lead compounds for amyloidosis focuses on inhibition of amyloid aggregation, inhibition of toxic amyloid formation and/or stabilization its native form from aggregation and degrading or remodeling toxic amyloid oligomers and/or insoluble fibrils. Different approaches were applied. Several platforms, including *in vitro* (Chen *et al*., 2010) and cell-based approaches (Kim *et al*., 2006), were applied in a semi-to-high throughput capacity to screen for small molecules that modulate or prevent amyloid aggregation.

One selection criterion used to choose the library of compounds for screening emphasizes the overall diversity and quantity of compounds not the specific underlying physicochemical characteristics (Chen *et al*., 2010). For example, Chen et al. developed a high throughput small molecule microarray assay which can be used for identifying amyloid inhibitors by judging binding affinity with amyloid β-peptide with ~11,000 various small molecule leads per array slide. Activities were evaluated from several natural and synthetic compounds and diversity-oriented synthetized compounds. Numerous high-resolution crystal structures of the fragment of amyloidogenic protein sequences are in accordance with atomic structural analysis on small molecules binding these structures (Wiltzius *et al*., 2008; Nelson *et al*., 2005) revealing many molecular scaffolds that either modulate or inhibit the formation of amyloid. These structures, some of which are potentially recommended as potential pharmacophores that can presumably target the generic cross β spine architecture common to all amyloids, are being used for structure-

based drug design efforts at present. For instance, Eisenberg's group, uses amyloid binding dye, Orange G, generated a high throughput screening platform that uses experimental approaches and iterative computational, and examined and fine-tuned structure activity relationships for lead compounds with optimum activity against Aβ amyloid (Landau *et al*., 2011). Furthermore, working with molecular dynamics and molecular docking simulation is a common approach to screen small molecule libraries, getting mechanistic insights into drug – target interactions, and optimizing lead compounds (Lemkul and Bevan, 2012; Lemkul and Bevan, 2012).

1.6.1. Mechanism of inhibition

The particular stages of aggregation and amyloid species that are targeted by natural product-based inhibitors, and as a result biomolecular and biochemical processes, associated with amyloid induced cytotoxicity, have not been fully discovered yet. However, a detailed understanding of the chemical mechanisms underlying these processes has recently been clarified. Anti-aggregation or anti-amyloidogenic agents can form covalent bonds (Sato *et al*., 2013; Zhu *et al*., 2004; Palhano *et al*., 2013; Ishii *et al*., 2008) and/or non-covalent interactions such as hydrogen bonding, π-π interactions or charge-charge interactions between the side chain residues or backbone of the target protein and an inhibitor (Landau *et al*., 2011; Cheng *et al*., 2013; Tu *et al*., 2015; Cao and Raleigh, 2012) which may influence one or all stages of the aggregation processes. The Eisenberg group revealed at least two various binding modes of amyloid pharmacophores which non-covalent interactions can be outlined by comparatively "tight" (e.g., co-crystal structure of Orange G bound to Aβ fibril fragment KLVFFA) or "less tight" binding (e.g., co-crystal structure of curcumin bound to τ fibril fragment VQIVYK) inside the cross β spine of single crystal structures of amyloid fragment sequences from Aβ and τ (Landau *et al*.,

2011). Nevertheless, the formation of a common amyloid pharmacophore mediated by primarily non-covalent bonding interactions does not explain that in some cases these forces, which characterize EGCG mediated anti-amyloid activities in a number of amyloid systems, are ineffective in others (Popovych *et al*., 2012). On this basis, a number of articles have shown that covalent adduct formation may happen between the nucleophilic side chain thiols, amines (as well as the amino terminal amine) and the electrophilic carbonyls inside o-quinone intermediates and/or aldehyde groups (Palhano *et al*., 2013; Ishii *et al*., 2008). Covalent adduct formation influences the anti-aggregation activities of baicalein on α-synuclein materially (Hong *et al*., 2008; Meng *et al*., 2009) and probably also on amylin. Adduct formation also influences binding affinity of EGCG and remodeling of Aβ plus amylin$_{8-24}$ fibrils (Palhano *et al*., 2013). This mechanism seems to be essential that inhibits phosphatase-cleaved amyloid precursor peptide (PAP)$_{248-286}$ amyloid production (Popovych *et al*., 2012). Similar mechanisms have been suggested for noticed inhibitory effects of taxifolin on Aβ aggregation and for oleocanthal and cinnamaldehyde on τ amyloid formation (Li *et al*., 2009; George *et al*., 2013).

1.6.2. Non-covalent and covalent inhibition mechanism

The specificity and type of the non-covalent interactions that mediate amyloid inhibitory activity may depend on the protein or the degree of aggregation that is targeted (Ehrnhoefer *et al*., 2008; Thapa *et al*., 2016). So, numerous of the non-covalent interactions do not imply a comprehensive description of all non-covalent interactions that may mediate the amyloidogenic activities of an inhibitor. Rather it is a summary of the key interactions that are relevant within the given context of a particular inhibitor. Two widely studied compounds, EGCG and curcumin are used, as exemplary non-covalent and covalent interactions.

Curcumin has been reported to modulate amyloid assembly in different amyloid systems. Since curcumin is a potential pan-assay interference compound (Nelson *et al*., 2017), it is especially important to conduct several orthogonal studies to validate the biological activities of curcumin. Nevertheless, extensive literature indicates that curcumin prevents amyloid induced cytotoxicity, amyloid formation, and provides advantageous in vivo effects including reduced plaque burden and clearance via to inhibit amyloid aggregation in the instances of amylin or Aβ to accelerate α-synuclein aggregation, resulting in less harmful intermediates and non-soluble aggregates (Jha *et al*., 2016). Using experimental structural data, computer simulation work has gained insights into key noncovalent interactions between curcumin and the general cross β spine structure present in fibrillar amyloid structures**.**

According to amyloidogenic "steric zipper" hexa-peptide amyloid β fragments and full length $A\beta_{1-40}$ docking simulations, curcumin binds within the inter strand space (maintained at typical 10 Å distance of protein fibrils; Figure 11A) in a planar fashion, its phenyl heads are parallel to the fibril axis. Subsequent analysis has shown that curcumin formed the most important inter-residue side chain interactions that targeted the bolded residues inside segment KLVFFA of the octamer hexa-peptide assembly and residues HQKLVFFA in amyloid β peptide complete lengthwise (Rao *et al*., 2015). In both cases, interatomic distances inside these residues and specific regions of the curcumin molecule indicated a number of important non-covalent relationships that were mediated by hydrogen bonding and hydrophobic interactions (Figure 11A). These include interactions between His14 (π stacking) as well as aromatic phenyl rings of curcumin and between Val18 (π-alkyl stacking) and the phenyl ring of curcumin.

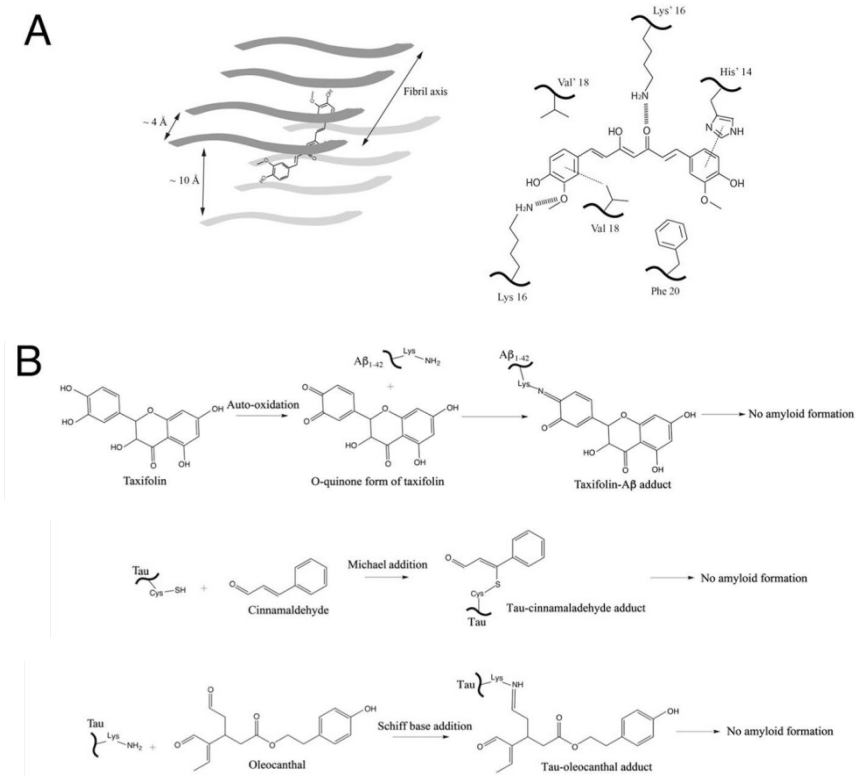


Figure 11. Illustrative representations a number of proposed mechanisms between inhibitors and amyloid proteins. (A) Mechanisms of non-covalent interaction with curcumin. Left panel: The planar molecule of curcumin is depicted by a cartoon schematic within the cross-β spine of an octomeric fibrillar backbone. (B) Mechanisms of covalent interaction. Small molecule naturally occurring compounds that contain electrophilic functional groups as an example o-quinones and aldehydes form covalent adducts with anti-amyloidogen proteins and prevent amyloid fibril formation (Velander *et al*., 2017).

Other interactions include hydrogen bond between two separate Lys16 groups (projected inward from opposite threads of the cross-β spine) (Rao *et al*., 2015). As a result of binding of curcumin, β-sheet perturbations occur within the cross-β spine that may have experimentally important consequences (Jha *et al*., 2016) relevant to inhibition of fibril formation (Ono *et al*., 2004; Yang *et al*., 2005; Singh *et al*., 2013). In fact, a similar mechanism of "binding and destabilization" has lately been reported with τ hexa-peptide VQIVYK that binds of curcumin (Berhanu and Masunov, 2015). Recently, the Eisenberg group has solved the structure of the curcumin-bound τ hexapeptide crystal, which shows that the curcumin is intercalated within the inter-β-sheet pocket of four interacting

oligomeric chains, with curcumin oriented in a longitudinally parallel to the fibril axis (Landau *et al*., 2011). In another recent study, MD simulations were used to investigate molecular relationships mediating amylin-curcumin interactions. MD simulations have supported that multiple curcumin molecules associate primarily themselves, and form a nucleation site, characterized by exposed hydrogen bonding and hydrophobic relationships that bind to and stabilize small order amylin oligomeric "Nano-assemblies" that alleviate the higher order amyloid aggregation (Nedumpully-Govindan *et al*., 2016).

The non-covalent interactions observed in these complexes show not only a mechanistic approach to the effects of curcumin (Thapa *et al*., 2016; Jha *et al*., 2016), with its anti-amyloid effects (Yang *et al*., 2005), the molecular scaffold of the curcumin can of targeting the cross beta spine in all fibrillary amyloid structures, as well as α-helical oligomers that can play an important role during the early occurrences of amyloid aggregation (Knight *et al*., 2006; Abedini and Raleigh, 2009).

EGCG exerts strong anti-amyloid effects against many amyloidogenic proteins. It is capable of preventing the toxic prefibrillar oligomers' formation (while stabilize non-toxic off pathway oligomers), transform previously existing amyloid fibrils into insoluble less toxic aggregates and can inhibit amyloid fibril formation (Bieschke *et al*., 2010; Engel *et al*., 2012; Lopez del Amo *et al*., 2012). Several studies have described some of the key non-covalent binding interactions and events that mediate these effects. Multiple studies show that EGCG exhibits non-specific hydrogen bonding and hydrophobic interactions that can mediate its anti-amyloid activities: NMR data as well as nitro blue tetrazolium dye staining analysis suggest that EGCG can bind to amyloid β peptide and natively unstructured α-synuclein or denatured bovine serum albumin but no any other native globular proteins. Based on these data EGCG may tends to target natively unstructured or unfolded proteins, presumably via non-especial backbone interactions (Ehrnhoefer *et al*.,

2008). EGCG may convert the produced amyloid created using the mutant form of acetylated fragment of yeast prion protein Sup35 (GNNQQNFQQF) but not the native fragment (GNNQQYQQY). Such differential effects may be due to the mutant fragment which contains larger hydrophobic binding regions that may interact with EGCG (Palhano *et al*., 2013). Using a series of amylin mutants, Raleigh's group examined the importance of covalent or residue-specific aromatic/hydrophobic interactions that could affect EGCG-induced amyloid inhibition and/or transformation. It was concluded that non-of them were critical, and that backbone hydrophobic/hydrogen bonding interactions likely convey the effects of EGCG (Cao and Raleigh, 2012).

Other studies have shown that EGCG inhibits amylin amyloid aggregation or transformation of preformed $amylin_{8-24}$ fibrils attenuated in the presence of negatively charged lipid bilayers (Palhano *et al*., 2013; Engel *et al*., 2012). Based on these data, key non-polar and polar regions of amylin that mediate the hydrogen bonding and non-specific hydrophobic interactions, as suggested by Raleigh *et al*. (Cao and Raleigh, 2012), are sequestered by both detergent and lipid bilayers, resulting in less interactions with EGCG.

The anti-amyloid activities of EGCG can be promoted by the binding protein specific regions and/or residue: Data from ion mobility mass spectrometry, biochemical assays, computational simulations and 2D NMR spectroscopy suggest that EGCG-Aβ binding results in compact dimeric and monomeric conformations, as well as higher order SDS-stable Aβ oligomers that are mediated by non-polar and polar interactions between EGCG and the hydrophobic aromatic core of Aβ (Hyung *et al*., 2013).

In summary, the anti-amyloid activity of EGCG is mediated by a wide spectrum of non-covalent interactions whose overall contribution to the observed anti-amyloid activity and specificity is determined by the interacting protein: for amylin, non-specific interactions (i.e., hydrogen bonding/hydrophobic interactions) are sufficient to promote the

activity of anti-amyloid EGCG; this is in contrast sharply with its inhibition of PAP amyloid, wherein specific interactions are indispensable. The above discussed dichotomous behavior is also a deviation from an inhibitory mechanism which is defined by a common phenolic pharmacophore, as described by studies experimenting with curcumin and Orange G (Landau *et al*., 2011). As far as we know, high-resolution structural information is yet to be obtained for EGCG in complex with an amyloid peptide.

From certain flavonoid or catechol-containing phenolic compounds, the presence of o-quinone mediated covalent adduct formation with amyloid proteins has been suggested or verified by several studies (Palhano *et al*., 2013; Popovych *et al*., 2012; Hong *et al*., 2008). Further covalent mechanisms have also been reported to occur between nucleophilic amines, thiols of amyloidogenic proteins and electrophilic reactive groups on inhibitors like aldehydes (Figure 11B, Middle and Lower Panels; (George *et al*., 2013)). Selected cases and major covalent inhibition mechanisms are discussed below.

In order to present instances for oquinone mediated covalent mechanisms, we will use EGCG, taxifolin, baicalein, and catecholamines. Site specific adduct formation to lysine residues in $(PAP)_{248-286}$ is vital for EGCG-mediated anti-amyloid activity as well as SDS-stable insoluble aggregate formation of $(PAP)_{248-286.}$ This was confirmed by a series of NBT binding assays, transmission electron microscopy and NMR analyses (Hong *et al*., 2008). In addition, mass spectrometry implies that gallocatechin (GC), which does not have the gallic ester moiety presented in EGCG but possesses the three contiguous hydroxyl groups on the B-ring, undergoes less conjugation with $(PAP)_{248-286}$, neither stabilizing low molecular weight SDS-stable PAP oligomers nor inhibiting PAP amyloid formation. This indicates that the gallic ester moiety present in EGCG is required for yielding a sufficient amount of adduct formation (i.e., 35% in EGCG-PAP samples versus 10% observed in GC-PAP samples) needed to confer anti-amyloid activities. These effects may be

consequences caused by higher stoichiometric amounts of gallol functional groups capable of oquinone formation in EGCG (versus GC). However, the gallo ester may facilitate initial noncovalent EGCG-PAP interactions as suggested by NMR experiments, that locates EGCG in a more favorable orientation and position (as compared to GC) for covalent conjugation, suggest that the gallo ester may make initial noncovalent EGCG-PAP interactions easier (Popovych *et al*., 2012). This explanation is similar to the thought of how aromatic/hydrophobic residues within the hydrophobic amyloid core may facilitate proper pharmacophore positioning of certain polyphenols along the cross-beta spine of amyloid fibrils (Porat *et al*., 2006).

Taxifolin does not inhibit monomeric or seeded amyloid beta fibril formation in anaerobic conditions, neither if a mild reducing reagent is present. But when it is incubated under aerobic conditions, taxifolin attenuates beta sheet rich secondary changes and prevents Aβ42 fibril formation, a phenomenon quickened in the presence of an oxidizing reagent, sodium periodate. Studies working with mass spectrometry and site directed mutagenesis proved that the chemical mechanism responsible for taxifolin mediated anti-amyloid activity transpires via site specific covalent adduct formation, through Michael addition at residues Lys16 and Lys28 (Sato *et al*., 2013). Additional characterizations with catechol-type (myricetin and quercetin) and noncatechol type (morin, kaempferol and datiscetin) flavonoid compounds indicated that anti-amyloid activities against $A\beta_{1-42}$ were exhibited by all compounds. The results suggest that myricetin and quercetin control their anti-amyloid activities through o quinone-Lys covalent adduct formation similar as taxifolin but that such a mechanism may not be generalized to all flavonoid molecules.

Studying a relevant case, baicalein inhibits α-synuclein amyloid formation. It is believed that the primary chemical mechanism responsible for this activity takes place via formation of baicalein- α- synuclein covalent adducts. When autoxidized into the quinone

form of baicalein versus freshly prepared baicalein, anti-amyloid activities of baicalein against α-synuclein aggregation are considerably enhanced, but rather weakened under conditions of anaerobic quality (Zhu *et al*., 2004; Meng *et al*., 2009).

The disruption of free radical cycling vital to o-quinone auto-oxidation via 5,5-dimethyl-1-pyrroline-N-oxide radical quenching reagent reduced anti-amyloid effects of baicalein in a significant way (Meng *et al*., 2009). At last, the fact, that the o-quinone form of baicalein forms covalent adducts with α−synuclein, was confirmed by results from mass spectrometry (Zhu *et al*., 2004; Meng *et al*., 2009). Also, inhibitory effects of baicalein against amylin oligomerization, amylin amyloid-induced toxicity and fibril formation have been described and validated using varying analytical approaches. Moreover, systematic structure activity studies working with a series of baicalein analogues produced definite evidence for a key role in catechol moiety in mediating the above discussed effects. Importantly, Mass spectrometric evidence showed schiff-base mediated baicalein-amylin adducts (Velander *et al*., 2016).

It is worth to consider that this mechanism is explainable by the ability of baicalein to undergo auto-oxidation to the o-quinone form and in turn to form covalent adducts with amylin. Several quinones and amino chromes derived from oxidized catecholamines undergo o-quinone mediated protein covalent adduct formation leading to the dissolution of both preformed Aβ and α-synuclein amyloid fibrils, as suggested by numerous studies (Conway *et al*., 2001; Li *et al*., 2004; Mazzulli *et al*., 2016). Cinnamomum (tree) verum extract has been shown to prevent amyloid fibril formation from tau and hen egg-white lysozyme (Ramshini *et al*., 2015; Peterson *et al*., 2009). More recently, it has been confirmed that cinnamaldehyde, a significant component of cinnamon bark oil, inhibits Tau187 amyloid formation without having an effect on tau mediated microtubule assembly (George *et al*., 2013).

Titration of lysine into the mixtures having both tau and oleocanthal reduces tau amyloid formation, in a lysine concentration dependent way. A reduction like that is consistent with the potential of lysine to form adducts with the aldehyde reactive-functional groups in oleocanthal (possibly even more readily than tau), resulting in fewer oleocanthal molecules available for inhibiting tau amyloid (Li *et al*., 2009).

1.7. Natural product and dietary supplement based amyloid inhibitors

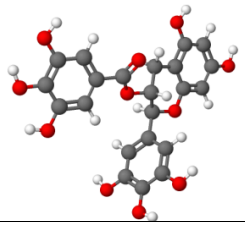
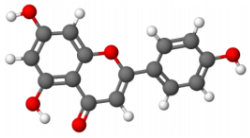
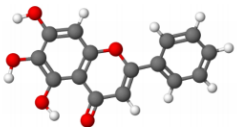
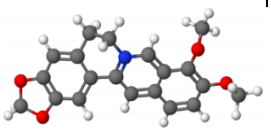
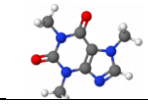
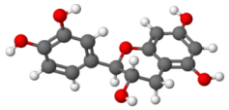
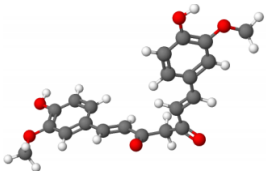
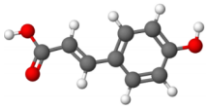
Natural compounds are complex chemical multiple-target molecules found most commonly in plants and microorganisms. These agents have been extensively studied because of their antioxidant and anti-inflammatory qualities. However, additionally to their potential to prevent damage caused by oxidative stress, they have been proven to modulate multiple signal transduction pathways through direct effects on enzymes like kinases, regulatory proteins and receptors (Bagli *et al*., 2016). Dietary natural products are a rich in phytochemicals and active compounds that inspire future drug development for AD (Table 2.). In the last decade, it was reported that certain fruits, vegetables, spices, drinks and beverages have the potential to counteract AD pathogenesis. To illustrate this, some dietary habits, like a Mediterranean diet (MeDi) and marine products, have a promising effect for attenuating the clinical features of AD patients (Modi *et al*., 2015; Essa *et al*., 2012; Mosconi *et al*., 2014; Teixeira *et al*., 2013). They effectively inhibit oxidative stress, Aβ accumulation and toxicity, tau phosphorylation, and neuroinflammation and exhibit antioxidant and anti-inflammatory qualities. Also, associations with reductions in other pathophysiological conditions of AD while learning ability, improving memory, cognitive function and protecting against the death of neuronal cells, were associated with these dietary components (Essa *et al*., 2012; Eskelinen *et al*., 2009).

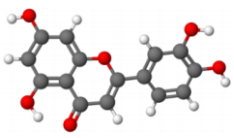
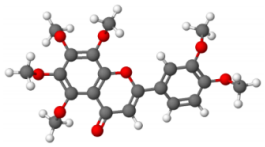
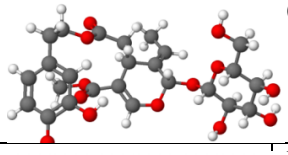
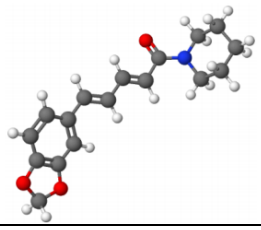
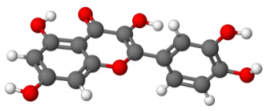
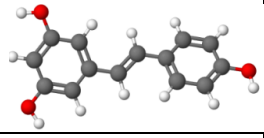
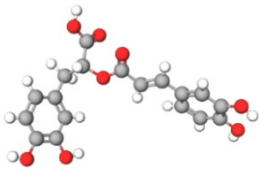
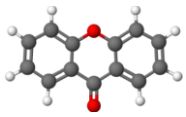
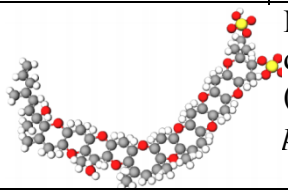
Nature gifted humanity with a plethora of flora-bearing fruits, vegetables and nuts. The wide range of bioactive nutrients present in these natural products is crucial in preventing and curing various neurodegenerative diseases, for example AD, multiple sclerosis, Parkinson's disease and other neuronal dysfunctions. Piles of evidence suggest that common phyto-compounds, flavonoids like polyphenolic antioxidants found in fruits, vegetables, herbs and nuts, may potentially hinder neurodegeneration, and improve memory and cognitive function. Nuts such as walnut have also demonstrated neuroprotective effect against AD (Essa *et al*., 2012).

The molecular mechanisms behind the curative effects mainly rely on the action of phytonutrients on distinct signaling pathways, which are associated with neuroinflammation and protein folding. The emphasis is put on traditional herbal medicines and small molecules (usually plant secondary metabolites) as potential anti-inflammatory drugs, particularly in respect to cytokine suppression by many studies. To illustrate this, ω-3 polyunsaturated fatty acids and a number of polyphenolic phytochemicals have been shown to work against inflammation in cell and animal models. Some of these plant secondary metabolites have also been shown to have effects of antioxidant, anti-inflammatory, anti-amyloidogenic, neuroprotective, and cognition-enhancing qualities (Apetz *et al*., 2014).

Most of the active ingredients of medicines are gained from natural products (Vickers and Zollman, 1999). Five FDA-approved drugs agents received by screening natural sources like plants, marines and microbes has led to the discovery of numerous clinically useful drugs, which are much needed in the treatment of human diseases (Brahmachari, 2010). Natural flora and fauna have always been and is an essential medical reservoir, with a vast number of modern FDA approved products having been derived from them (Harvey, 2008; Newman and Cragg, 2012**)**.

Table 2. Potential natural bioactive compounds with protective effect against Aβ Alzheimer's disease.

No.	Active compounds	Structures*	Sources	Activities
1	(-)-Epigallocatechin gallate		Green and black tea	Reduces Aβ mediated cognitive impairment and modulates tau pathology (Rezai-Zadeh *et al*., 2008; Sgarbossa and Antonella, 2012).
2	Apigenin		Parsley, celery, cloves, spinach and red wine	Relieves Aβ burden, suppresses the amyloidogenic process, inhibits oxidative stress and restores ERK/CREB/BDNF pathway (Zhao *et al*., 2013).
3	Baicalein		*Scutellaria baicalensis* (Root)	Reduces Aβ production and improves cognitive performance (Zhang *et al*., 2013).
4	Berberine		European barberry, goldthread and Oregon grape	Ameliorates Aβ pathology, gliosis and cognitive impairment (Durairajan et al., 2012).
5	Caffeine		Coffee	Restores memory and decreases brain Aβ levels (Arendash *et al*., 2009).
6	Catechin		Cocoa	Inhibits Aβ-induced apoptosis (Heo and Lee, 2005).
7	Curcumin		Turmeric	Reduces amyloid aggregation *via* metal chelation, which may reduce amyloid aggregation or oxidative neurotoxicity in AD (Baum and Ng, 2004).
8	Hydroxycinnamic acids		Virgin olive oil	Exerts neuroprotective effects against Aβ-induced cytotoxicity and oxidative stress (Villareal *et al*., 2016).

9	Luteolin		Parsley, celery, peppers, olive oil, lemons, peppermint and sage	Downregulates the expression of APP and reduces the secretion of Aβ (Liu *et al*., 2011).
10	Nobiletin		Peels of citrus fruits like tangerine and orange	Decreases Aβ burden and improves memory impairment (Onozuka *et al*., 2008).
11	Oleuropein		Olive	Inhibits Tau aggregation (Daccache *et al*., 2011).
12	Piperine		Black pepper and long pepper	Decrease lipid peroxidation and acetylcholineesterase enzyme (Chonpathompikunlert *et al*., 2010).
13	Quercetin		Apples, berries, black and green tea and red wine	Inhibits the formation and aggregation of Aβ (Ansari *et al*., 2009).
14	Resveratrol		Majorly found in grapes and red wine	Helps in clearance of Aβ peptide (Marambaud *et al*., 2005).
15	Rosmarinic acid		Basil, lemon balm, rosemary, sage, thyme and peppermint	Inhibits β sheet in tau protein and β-secretase activity, decrease mitochondrial dysfunction (Cornejo *et al*., 2017).
16	Xanthone		Purple mangosteen	Protects against Aβ-induced cytotoxicity and oxidative stress (Moongkarndi *et al*., 2010).
17	Yessotoxin		Marine dinoflagellate (*Lingulodinium polyedrum*)	Inhibits tau hyperphosphorylation and Aβ accumulation (Alonso *et al*., 2013).

*The structures were re-generated from http://molview.org/. Here, the different colors represent various atoms in the molecules, where grey=carbon, white=hydrogen, red=oxygen, blue=nitrogen and yellow=sulfur.

In the field of Alzheimer's, the experimental outcomes have already confirmed a number of conclusions on the possibility of natural product leads; the majority of the compounds examined to date in direct connection with AD primarily come from one source; plants which have comparatively few molecules. These molecules are derived from marine and microbial sources. The greatest successes, which have developed two of the five currently approved drugs for the treatment of AD, have been the results of plant-based AChE discovery programs (Houghton and Howes, 2005).

Multiple factors may be the driving force of the increased interest in natural supplementation and for its use in Alzheimer's and other neurodegenerative diseases; it is obvious that the lack of a safe, effective, proven therapy is a contributing factor regarding the search for alternatives. There is a wide range of raw plant extracts/ herbal formulations, which have a great use as natural remedies in the treatment of AD and other neurodegenerative diseases (Houghton and Howes, 2005; Howes and Houghton, 2003; Howes *et al*., 2003).

Traditional natural medical systems such as Indian, Chinese, Native American, and medieval European have offered various "brain tonics" and memory enhancers. Including "Ashwagandha" (*Withania somnifera*; Solanaceae) and Brahmi (*Bacopa monnieri* L. Pennell; Scophulariaceae) described in Indian Ayurveda as memory enhancers, the common 'Sage' plants (*Salvia species*; Labiatae) is said to be "good for the memory" in Roman texts, and *Gingko biloba* (Ginkgoaceae) is studied in Chinese literature as a possible herb for memory loss as early as 2800 BC. Indian turmeric (*Curcuma longa*; Zingiberaceae) contains an antiinflammatory compound and antioxidant called curcumin, which is found to be most effective in the treatment of AD. Vegetables like pumpkins, carrots, spices like zinger, sesame and sunflower seeds containing various chemical agents are quite nurturing for the function of the brain. More recently, consumption of

blueberries/grapes and pomegranate juice has been confirmed to beneficially effect the state of AD patients. In addition, food supplements of vitamin B_6, B_{12}, E, and C, folic acid and co-enzyme Q10 have been found to have positive effect in AD patients (Tildesley *et al*., 2003; Scholey *et al*., 2008; Wang *et al*., 2008).

Natural phenolic compounds, being a long family of plant substances, are one of the most actively researched categories of potential amyloid inhibitors (Stefani and Rigacci, 2013; Shariatizi *et al*., 2015; Jayamani and Shanmugam, 2014). Currently, more than 8,000 plant polyphenols are discovered including more than 4,000 flavonoids identified (Pandey and Rizvi, 2009). Resveratrol inhibits Aβ42 fibril formation and islet amyloid polypeptide aggregation dose-dependently (Feng *et al*., 2009; Evers *et al*., 2009). This inhibitory effect of flavone derivatives dependents on the position and number of hydroxyl groups around the flavone backbone (Šneideris *et al*., 2015). The position of phenolic hydroxyl moieties on the aromatic rings is a strong determinant of the potent anti-aggregation effect. In contrast, the number of hydroxyl groups is less important (Porat *et al*., 2006).

Natural phenolic and polyphenolic substances are to stabilize native states, or to remodel and to inactivate toxic amyloid oligomers (Stefani and Rigacci, 2013). A number of observations implies that polyphenols prevent amyloid fibril formation via specific aromatic interactions with the amyloidogenic core (Porat *et al*., 2006). It may be that aromatic rings of polyphenolic compounds and the aromatic residues found in proteins associate via π-π stacking interactions (Bhat *et al*., 2015). The hydrophobic and/or aromatic character of these compounds is a contributing factor to the antiamyloidogenic effect, whereas the antioxidative potency is mostly related to the destabilization of fibrils (Shoval *et al*., 2007). According to studies, the presence of vicinal dihydroxyphenyl moieties, irrespective of their position in the aromatic rings, is vital regarding the inhibitory property of polyphenols (Bhattacharya *et al*., 2014; Ghosh *et al*., 2013). Non-flavonoids exhibited

higher anti-aggregation activity compared to flavonoids. Polyphenols contain more of aromatic rings, hydroxyl and keto groups, and have a high degree of planarity, exhibit the largest inhibitory activity (Lakey-Beitia *et al*., 2015). According to a study on structure–function relationship, the presence of minimum two phenolic rings with two to six atom long linkers, along with at least three hydroxyl groups on the aromatic rings, are crucial for efficient inhibition exerted by polyphenols (Shariatizi *et al*., 2015). The mechanism of inhibition of amyloid formation is not the same each natural polyphenol. Some of them inhibit the formation of oligomers, but promote fibril formation, others are known to inhibit the formation of fibrils, but not oligomers, and there are even those inhibiting both. A different group of polyphenols redirect amyloid fibrils from fibrillogenic forms to nonfibrillogenic oligomers. Conformational changes are induced in the oligomer aggregate by polyphenols. H-bonds are disrupted by these changes, and the amyloid aggregate is perturbed. The β-sheets are separated by polyphenol molecules, resulting in a loosely packed structure. H-bonding capacity of polyphenols is the cause of this behavior (Ngoungoure *et al*., 2015). Flavonoids, especially phenols, are essential components of a normal human diet. Correlation between an elevated intake of dietary polyphenols and the prevention of amyloidosis is to be considered. It is possible, that nutraceutical strategies become a way to increase the chances of preventing certain amyloid diseases. For many of the drugs being used today are natural products or derivatives of natural products (Berhanu *et al*., 2015; Yamada *et al*., 2015).

1.7.1. Medicinal herbs & spice extracts

In spite of the fact that we, humans, have been using a quite wide range of spices for more than 2000 years, the research of their biological activities has just recently begun (Mirmosayyeb *et al*., 2017). Nuts, fruits, spices and herbs also possess important bioactive

compounds – without unwanted side effects – needed for the prevention and cure of many diseases (Iriti *et al*., 2010; Essa *et al*., 2012). Extracts from kitchen spices are known to contain therapeutic natural compounds considered to be highly promising. The main components of spice extracts contain biologically active flavonoids, di- and triterpenoids, phytosterols, tocopherols, phytosterols, alkaloids, phenolic acids, carotenoids, vitamins and other compounds (Misharina, 2016). Molecules known to inhibit protein misfolding have a great promise regarding therapeutics for the treatment of diseases, which are caused by the formation of amyloid fibrils (Barbero *et al*., 2014).

Cayenne pepper (*Capsicum annuum* L) contains the five major capsaicinoids: nordihydrocapsaicin, capsaicin, homocapsaicin, dihydrocapsaicin, and homodihydrocapsaicin. Two major capsaicinoids are capsaicin and dihydrocapsaicin, representing between 79% and 90% of total capsaicinoids, respectively, also depending on fruit ripening (Barbero *et al*., 2014). Cayenne pepper contains more capsaicin than dihydrocapsaicin (Bajer *et al*., 2015). Carotenoids are the second group of main components of pod. In pepper, thirty-four carotenoids were discovered and identified. Four main carotenoids are responsible for the red color of pepper. (capsorubin, capsanthin, cryptoxanthin, zeaxanthin). Among these four, capsanthin is found to be the main and the most stable compound, and accounts for up to 60% of all the carotenoids. In fresh rods, carotenoids are stable, but exposed to autoxidation in milled or dried ones. Although pod pepper has vitamins, flavonoids and about 50 volatile compounds causing the flavor, their total content is insignificant, they only sum up to less than 0.01% (Misharina, 2016). The more peppers grew and the concentration of carotenoids and capsaicinoids got higher, the less they contained phenols and flavonoids (Tundis *et al*., 2013; Menichini *et al*., 2009).

Saffron (*Crocus sativus*) is contained of polar carotenoids. Its main secondary metabolites are safranal responsible for odour, crocin for colour and picocrocin for taste.

Experimental results show that *Crocus sativus* stigma extract shows inhibitory action on fibrillogenesis and Aβ aggregation. The main carotenoid trans-crocin 4, the digentibiosyl ester of crocetin is held responsible for the anti-amyloidogenic activity. It is possible that the molecular mechanism inhibiting fibril formation is due to the capability of crocin to bind to the hydrophobic regions of Aβ (Essa *et al*., 2012; Papandreou *et al*., 2006). The anti-amyloidogenic effect of crocin is exerted by the inhibition of Aβ amyloid formation, but the disruption of amyloid aggregates also exerts it. So, crocin may prove to be important, while searching for therapies inhibiting or disrupting aggregation (Ghahghaei *et al*., 2013).

Oligomerization of Aβ, human islet amyloidogenic polypeptide promoting fibril deposition, and hen egg white lysozyme is inhibited by curcumin derived from turmeric, while it also inhibits both the fibrillation and the oligomerization of PrP (Stefani and Rigacci, 2014; Pithadia *et al*., 2016). Curcumin alters the aggregation of proteins, reduces the toxicity of the aggregates (Ahmad *et al*., 2017) and inhibits alpha-synuclein oligomerization into higher molecular weight aggregates (Pandey *et al*., 2008). Basil is rich in phenolic acids contributing to a potent antioxidant capacity. Substantial concentrations of rosmarinic acid have been associated with the medicinal qualities of the herb. In the literature rosmarinic acid is described to be the most prevalent basil phenolic, however, high concentrations of other caffeic acid derivatives, for example chicoric acid, are also found (Kwee and Niemeyer, 2011). Hen egg white lysozyme amyloid aggregate formation was effectively inhibited by rosmarinic acid (Ramazzotti *et al*., 2016). The aqueous extract of clove has a significant amount of polyphenols (Suantawee *et al*., 2014). Cinnamon is a great source of proanthocyanidins, for instance, a group of plant polyphenols (Williams *et al*., 2015). Fifty-two phenolic compounds were discovered in culinary herbs (rosemary,

thyme, bay and oregano) and spices (cumin and cinnamon) (Vallverdú-Queralt *et al*., 2014).

Spices are proven to be great polyphenol sources. Their high polyphenolic content was found to be related to numerous health benefits (Hossain *et al*., 2011). It is possible that the aromatic rings of polyphenol interact with aromatic residues in amyloidogenic proteins competitively, block the self-assembly process and inhibit the π-π interaction. Amyloid fibril formation may be inhibited by the phenolic hydroxyls of polyphenols, through binding the hydrophobic residues in amyloidogenic proteins (Cheng *et al*., 2013). The key determinant for the anti-amyloidogenic properties was found not to be the number, but to be the position of hydroxyl groups on the aromatic ring (Porzoor *et al*., 2015). According to reports, many herbs contain flavonoids and other phenolic antioxidant. Good examples are basil, chives, coriander, dill, lovage, mint, oregano, parsley, rosemary, and safflower, sage, tarragon and thyme (Justesen and Knuthsen, 2001; Yu *et al*., 2013). Pinocembrin, identified in a number of plants, for example ginger roots and wild marjoram, is a natural flavonoid compound (Lan *et al*., 2016). Pinocembrin is potentially a promising substance for the prevention and therapy of Alzheimer's disease (Liu *et al*., 2012). According to the results, a constituent of ginger named 6-shogaol inspires much hope as a therapeutic agent for the treatment of various neurodegenerative diseases, including Alzheimer’s disease (Na *et al*., 2016).

The anti-amyloid activities of 27 vegetables, spices and herbs were screened by Ana Lucia Fuentes and her co-workers. It was found that mint, peppermint, red bell pepper and thyme had the greatest anti-amyloid activity (Fuentes *et al*., 2016).

Peppermint can prove to be an important source of biologically active compounds (Alexa *et al*., 2018). It is possible that flavones (e.g. luteolin derivatives), flavanones (e.g. eriocitrin derivatives and phenolic acids (e.g. caffeic and rosmarinic acids) are the main

infusion antioxidants. Catechin, (-)-epigallocatechin gallate, syringic, vanillic, gallic and p-coumaric acids were also identified in it. Peppermint is effective at inhibiting the aggregation of islet amyloid polypeptide (Uribe *et al*., 2016). The molecular mechanism, by which EGCG inhibits human islet amyloid polypeptide aggregation, was researched. According to this research, EGCG binding inhibits the aromatic-stacking and inter-peptide hydrophobic interactions responsible for intra-peptide interaction and inter-peptide β-sheet formation. For β-hairpin formation, the last two phenomena are essential.

EGCG binding thus does away with the three-stranded β-sheet structures and leads to the formation of coil-rich three-dimensional structures. The effect of a simple polyphenol called gallic acid (GA) was observed. GA is one of the most significant components in plant tissues, above all in tea leaves. GA inhibits the conformational transition of α-helix → β-sheet usually induced during insulin fibril formation. GA is known to interact with native insulin, inhibiting nuclei formation, which is required for fibril growth, thereby preventing amyloid fibril formation (Jayamani *et al*., 2014). Not only did GA prevent alpha-synuclein fibrillation and toxicity but also disaggregated preformed alpha-synuclein amyloid fibrils. Surprisingly, GA bound to soluble, non-toxic oligomers with no β-sheet content, and stabilized their structure (Ardah *et al*., 2014). An extract of *Salvia officinalis* (garden sage) was supplied with, polyphenolic compounds containing rosmarinic acid (Bakota *et al*., 2015).

In a research studying cultivated and wild nettle leave samples caffeic acid derivative, 2-O-caffeoylmalic acid, chlorogenic acid, kaempferol 3-O-rutinoside, rutin, quercetin 3-O-glucoside and isorhamnetin 3-O-rutinoside were detected by phenolic profile and HPLC analysis. Caffeic acid derivative, p-coumaric acid, chlorogenic acid, rutin, quercetin 3-O-glucoside, 2-O-caffeoylmalic acid, kaempferol 3-O-rutinoside, isorhamnetin 3-O-rutinoside were discovered in wild leaf samples (Otles *et al*., 2012). *Melilotus*

officinalis contains coumarin and related compounds like *o*-coumaric and melilotic acids, flavones, volatile oils, tannins and resins (Martino *et al*., 2006). Analogs of common coumarin were identified as a new kind of inhibitors of Aβ aggregation (Soto-Ortega *et al*., 2011).

1.7.2. Chinese dietary supplements

The traditionally used herbal medicine *Panax ginseng* Meyer shows hypolipidemic and hypoglycemic effects (Jeon *et al*., 2013; Trinh *et al*., 2007; Park *et al*., 2011). It also exhibits beneficial effects on cancer patients (Park et al., 2011) and inflammations (Yuan and Chung, 2010; Seo *et al*., 2005). In addition, it has also been confirmed to have therapeutic effects on allergic rhinitis in clinical patients (Jung etal., 2011). Moreover, neuro- (Chen *et al*., 2012) and cardioprotective (Peng *et al*., 2012) effects of *P. ginseng* were also discussed. Ginsenosides, being the main molecular components found in ginseng, have also been effectively implemented in the treatment of cognitive impairments (Zhao *et al*., 2009), for example the amelioration of Aβ plaques (Tohda *et al*., 2004). The fibril formation of reduced and carboxymethylated κ-casein was dose-dependently inhibited by the ginsenoside Rg1 and Rb1 (Chen *et al*., 2016). However, it appears that other different components of *P. ginseng* have also been described as biologically active, for example polysaccharides, peptides, fatty acids and polyacetylenic alcohols (Attele *et al*., 1999; Kim *et al*., 2013).

The compounds, that inhibit the formation of amyloid fibril, exhibit neuroprotective, anti-aging and antitumor activity, so it is possible that the molecular components of ginseng have similar effects. It is also possible for ginseng to act as a tool to investigate the amyloid aggregation pathway, and its active components may be used as

molecular scaffolds for the development of biologically available and more active drugs (Stefani and Rigacci, 2013).

Terpene trilactones and flavonoid glycosides are the two main groups of constituents in the standard extract of *Ginkgo biloba* leaves. Seven flavonoid glycosides were found to exert moderate to weak inhibitory activity on Aβ42 fibril formation whereas four terpene trilactones had insignificant effect. Considering this, there must be other additional compounds in the extract of *Ginkgo biloba* leaves, responsible for its inhibitory effect on Aβ42 fibril formation (Xie et al., 2014). Catechins and procyanidins, for example gallocatechin, (-)- epigallocatechin, (+)-catechin, (-)-epicatechin, (-)- procyanidins B-1 and B-3 are present in the extracts of its leaves. These polyphenols inhibit Aβ42 aggregation, destabilize preformed fibrils and have been evaluated (Xie *et al*., 2014).

1.7.3. Commercially available coffees and teas

Coffee is a beverage rich in antioxidant and widely consumed worldwide (Pourshahidi *et al*., 2016; Essa *et al*., 2012). Coffee consumption has preventive benefits with many positive health effects. Its inverse correlation with the occurrence of diabetes mellitus, various cancer types, Parkinson's, and Alzheimer's disease were confirmed by excessive research, epidemiological studies, and meta-analyses concerning coffee consumption. These health benefits are due to coffee polyphenols (Ochiai *et al*., 2014; Butt and Sultan, 2011). The chemical structure of polyphenols contains two or more phenol rings with hydroxyl groups in ortho or para positions (Lakey-Beitia *et al*., 2015). It has been reported that numerous natural polyphenols have potent inhibitory effects on amyloid fibril formation (Lakey-Beitia *et al*., 2015; Bhattacharya *et al*., 2014).

Coffee extracts possess three main active components, i.e. caffeic acid, caffeine and chlorogenic acid (CGA). All components show different inhibitory effects on the formation of toxic human islet amyloid polypeptide (hIAPP) amyloids, as suggested by earlier

findings. CA exhibits the most potency in delaying the conformational transition of the hIAPP molecule with the most prolonged lag time, whereas caffeine shows the lowest potency (Cheng *et al*., 2011). It is a proven fact that α-synuclein fibrillization is 90% inhibited by CA at 1:1 ratio (Gazova *et al*., 2013). CA was reported to inhibit Aβ1-42 self-induced aggregation (Wei *et al*., 2016). CA can pass through the blood-brain barrier; therefore, it has been suggested as an anti-amyloidogenic agent (Fazili and Naeem, 2015).

Alkaloids are a group of natural organic nitrogen-containing compounds, which are primarily present in plants, especially in particular families of flowering plants. Caffeine is to be found in many dietary sources such as coffee (71–220 mg caffeine/150 ml), tea (32–42 mg/150 ml), cola (32–70 mg/330 ml) and cocoa (4 mg/150 ml). Caffeine reduce levels of Aβ and Aβ-induced neurotoxicity both *in vitro* and *in vivo* (Ng *et al*., 2015), therefore caffeine may prevent Alzheimer's disease. Caffeine is capable of inhibiting the formation of β-sheets by interacting with the peptide's aromatic side chains of the constituent amino acids. Furthermore, caffeine molecules form hydrogen bonds with peptide backbone, thereby weaken interstrand hydrogen bonds of β-sheets (Sharma *et al*., 2016). The disruption of the blood-brain barrier occurs at an early pathological stage in Alzheimer's disease. It may make it possible for Aβ to accumulate in the brain. If that is the case, it does that by allowing the transport of Aβ produced in the periphery.

The brain may be protected against Alzheimer's disease-associated blood-brain barrier disruptions by caffeine administration (Madeira *et al*., 2017). Moreover, trigonelline is a coffee ingredient too, and it is one of the most often consumed alkaloids. Molecular modelling demonstrated that trigonelline has high affinity to the A-β (1-42) peptide, and acts similar to the anti-Alzheimer's disease drug candidate – cotinine (Makowska *et al*., 2014). The neuroprotective activities of CGA against Aβ-caused toxicity were studied, too (Lee *et al*., 2011). Moderate (three to five cups a day) coffee intake in people is associated

with a considerable decrease in the risk of developing different chronic diseases (Shukitt-Hale *et al*., 2013). According to a recent epidemiological study, higher caffeine consumption over decades decreases the risk of having Alzheimer's disease (Arendash and Cao, 2010).

2. Main objectives and organization of the thesis

One out of eight people aged 65 or older has AD (Citron, 2010). With the increasing longevity of our population, AD is approaching epidemic proportions without cure or preventative therapy in sight. A pathological hallmark of AD is the extracellular deposition of amyloid in the brain. These fibrillar deposits (plaques) are formed from the self-aggregation of the β-amyloid (Aβ) peptide, a 38 to 42 residue protein that is normally produced as part of the cellular metabolism. Similarly, amyloid composed of other peptides or proteins are also found in other neurodegenerative diseases such as Parkinson's, Huntington's, and prion-related diseases.

One therapeutic approach is the screening or development of natural bioactive molecule inhibitors of Aβ aggregation. Dietary natural products can serve as a rich source of phytochemicals and active compounds, having the potential for future drug development for AD. In the last decade, researchers reported that certain fruits, spices, vegetables, drinks and beverages could have the potential to counteract AD pathogenesis. These natural products effectively prevent oxidative stress, Aβ accumulation and toxicity, tau phosphorylation, neuro-inflammation and shows antioxidant and anti-inflammatory properties. Associations with reductions in other pathophysiological conditions of AD while improving memory, learning ability, cognitive function and protecting against neuronal cell death were also mentioned regarding these dietary components (Essa *et al*., 2012). At present, researchers are searching for new, safer and more effective therapies that are capable of more precisely targeting the pathophysiology of AD and inhibiting the Aβ accumulation and toxicity.

Our research group has been working on enzymatic modification of α-chymotrypsin and trypsin on the formation of amyloid-like fibrils in different aqueous organic solvents. We have been also investigating the inhibitory activity of various

bioactive compounds, the effect of dietary supplements, sorts of red wine, fruits and vegetables, coffee, spices, herbs, grapefruit seed extract and peppermint extracts on the formation of amyloid-like fibrils of α-chymotrypsin and trypsin in different aqueous organic solvents.

Our primary interest here was the discovery of novel molecules possessing anti-neurodegenerative and anti-amyloidogenic properties through screening of a diverse library of natural compounds. By considering these main objectives, our aims were:

- Collection, extractions of bioactive compounds and total phenolic analysis from 5 categories or groups of natural products.
- Modification and examination of trypsin with phenylmethylsulfonyl fluoride (PMSF) for the formation of amyloid-like fibril in aqueous organic solvents and analysis of inhibitory effect of extracted compounds.
- *in vitro* screening by turbidity, size exclusion chromatography, aggregation kinetics, CR binding, FTIR, CD and TEM on amyloid-like fibril formation of trypsin by coffees, spices, medicinal herbs, Chinese dietary supplements, grapefruit seed extract and peppermint extracts in aqueous organic ethanol.
- Investigation of inhibitors or suitable therapeutic agents for amyloid related neurodegenerative diseases.

In each of my studies, I have comparatively investigated 5 categories or groups of natural product extracts for screening of anti-amyloidogenic properties on the formation of amyloid-like fibrils of trypsin in different aqueous organic solvents.

From these studies, we aim to provide the anti-amyloidogenic and anti-neurodegenerative natural bioactive extracts from diverse library.

During the last three decades, tremendous progress was made in the understanding of the mechanism of AD. However, discovery or screening of new pharmacological

approaches, especially of disease-modifying drugs, has not been successful to date. Aggregation of β-amyloid peptides in brain tissue is the likely cause of AD, which means that the alpha helical structural changes to the beta sheet are to be considered of physiological importance, and they lead to the formation of amyloid structures (Ardito *et al*., 2017). Recent studies on Aβ amyloid formation indicate that several natural small aromatic compounds and flavonoids interfere with aggregation pathways possibly by remodelling the amyloid intermediates through the different mechanisms or interactions (Petkova *et al*., 2006).

3. Materials and methods

3.1. Materials

Bovine pancreas trypsin (EC 3.4.21.4; from bovine pancreas), gallic acid, limonin (from citrus seeds) and *N*-benzoyl-L-arginine ethyl ester (BAEE) were purchased from Sigma-Aldrich (Budapest, Hungary). Folin-Ciocalteu's phenol reagent was the product of Merck (Darmstadt, Germany). Ginseng Royal Jelly ampoule *P. ginseng* extract and Royal Jelly content vial with honey food supplement was distributed by Oriental Herbs Kft., Budapest, Hungary. A total of 31 dried culinary spices were purchased from the local supermarket. Chili, rosemary, dill, clove, lovage, tarragon, parsley, thyme, sage, bear's onion and saffron distributed by Kotányi Hungária Kft., Budapest, Hungary, basil, allspice, turmeric, caraway seed, safflower, bay leaf and cinnamon imported by R COOP3 Kft., Szeged-Szőreg, Hungary, golden cress, coriander seed, oregano, marjoram, cumin, pepper, mace, anise, ginger and juniper berry packed by Szilasfood Kft., Kistarcsa, Hungary, hibiscus, celery and peppermint were used disassembled by Toldi Fűszer és Delicates Bt., Budapest, Hungary. Folin-Ciocalteu's phenol reagent was the product of Merck (Darmstadt, Germany). The different ground, roasted coffees were obtained from commercial sources. Eduscho Wiener Extra was purchased from Tchibo Budapest Kft., Budaörs, Hungary, Douwe Egberts Omnia Classic from Douwe Egberts Hungary, Budapest, Hungary, Bravos Classic from Mocca Negra Zrt., Szentendre, Hungary, Brasil Santos, Panama arabica and Kenya Kiandi from Latin Negyed Kft., Szeged, Hungary. Grapefruit seed drops with ginseng was purchased from Oriental Herbs Kft., Budapest, Hungary. Acai oral liquid was distributed by Big Star Street Kft., Budapest Hungary. Grapefruit seed extract was distributed by Bioextra Zrt., Hatvan, Hungary. Virgin Tea Ampoule was distributed by Oriental Herbs Kft., Budapest, Hungary. The different herbs (peppermint leaves (*Menthae piperitae folium*), medical sage leaves (*Salviae folium*),

marigold (*Calendulae flos*), walnut leaves (*Juglandis folium*), thornapple buds (*Crataegi folium cum flore*), yarrow (*Millefolii herba*), field horse tail (*Equiseti herba*)) were purchased from Mecsek-Drog Kft., Pécsvárad, Hungary. All other reagents and buffer components used were of analytical grade.

3.2. Preparation of water-soluble extracts

15 ml of distilled boiling water was added to 200 mg of tea. 3 g coffee was added to 22.5 ml hot (100 ^{o}C) distilled water, then it was boiled three times. 600 mg of spice was added to 6 ml distilled water, then it was brought to boil. After the samples cooled down to room temperature, they were being centrifuged for 1 min at 13,000 rpm. The supernatants were stored in the freezer until use. The extracts were diluted in distilled water prior to use as required.

3.3. General methods

Trypsin concentration was determined based on the UV absorbance of the constituent aromatic and cystine residues at 280 nm, using a calibration curve measured for trypsin in the 0-1 mg/ml concentration range. Spectroscopic samples have been corrected for appropriate solvent backgrounds in all experiments where applicable.

3.4. Assay of enzyme activity

The activity of trypsin, with BAEE as a substrate, was determined following the method of Schwert and Takenaka (Schwert and Takenaka, 1955). The increase in absorption at 253 nm was followed in a 3 ml reaction mixture, which contained 46.7 mM Tris/HCl buffer and 0.9 mM BAEE at pH 8.0. The reaction was initiated by the addition of 20 μl enzyme solution at a concentration of 0.15 mg/ml.

3.5. Modification of trypsin with phenylmethylsulfonyl fluoride (PMSF)

Chemical modification of the serine residue at the active site of trypsin with PMSF irreversibly inactivates the enzyme. This is required to suppress the autolysis at pH = 7.0, which could lead to artefacts in the observation of amyloid formation. 8.4 μl 100 mM PMSF in 2-propanol and 20 μl 50 mg/ml trypsin in 0.001 N HCl were added to 2 ml 0.05 M potassium phosphate buffer at pH = 7.0 at 24 ^{o}C while stirring. Such aliquots were added to the solution on four occasions. The reaction mixture was left to be stirred for 30 minutes, then it was fractionated by filtration on a Sephadex G-25 column, with 0.05 M potassium phosphate buffer at pH = 7.0 as eluent, to remove excess reagents. The degree of modification was calculated from enzymatic activity assays. It was determined that 95% of the trypsin had been modified.

3.6. Turbidity measurements

Turbidity assay was performed as an indicator to suggest the formation of protein aggregates and fibrils (Bhat and Bano, 2014; Fazili and Naeem, 2015). Turbidity was monitored using a Cecil CE 5501 spectrophotometer. The absorbance of the samples was measured at 350 nm wavelength at 24 ^{o}C in a 10 mm path length cell in the presence or absence of different inhibitory agents in 60% ethanol/10 mM phosphate buffer at pH 7.0 after 24 h incubation, to estimate the rate of aggregation. The concentration of PMS-trypsin was 0.13 mg/ml in the turbidity measurements. Respective blank corrections were done in the case of each experiment. Inhibition percentages were calculated from the turbidity measurements. We subtracted the relative absorption for the given sample from 1, and then we multiplied it by 100. Data represent the turbidity measurements of at least three independent experiments. All data were presented as mean ± standard error of the mean (SEM).

3.7. Determination of total phenolic content

Spectrophotometric analysis of the total phenolic content of samples by means of Folin Ciocalteu colorimetric method was performed following the protocol of Waterhouse (Waterhouse, 2002). The reaction mixtures were kept for 2 h at room temperature, and absorptions were measured at 765 nm using a UV-Vis Cecil CE 5501 spectrophotometer. The same procedure was repeated for different concentrations of gallic acid solutions (0–50 mg/l) as standard, and the calibration curve was plotted. The total phenolic contents were expressed as mg gallic acid equivalents per litre (GAE/l) of samples. The data presented are the average of three independent measurements.

3.8. Determination of limonin concentration using HPLC-MS

Liquid chromatographic separation was performed on a DionexUltimate 3000 UHPLC system equipped with a membrane degasser, a binary pump, a standard autosampler, a thermostated column compartment and a variable wavelength detector. The components of the sample were separated on a Gemini-NX C18 (3 µm, 150 x 2 mm) column (Phenomenex) equipped with a Gemini-NX C18 guard column (5 µm, 4 x 2 mm) thermostated at 25 °C. Mobile phase A consisted of water containing 0.1% formic acid, while methanol containing 0.1 % formic acid served as mobile phase B. The gradient elution was performed as follows: 0 min, 20% B; 1 min, 20% B; 12 min, 95% B; 17 min, 95% B; 18 min, 20% B; 25.0 min, 20% B. The flow rate was set to 0.2 ml/min. The injection volume was 5 µl.

Mass analyses were performed on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer. Ionization of analytes was performed using heated electrospray interface (HESI) in negative electrospray ionization. The temperature of ion transfer capillary, spray voltage, sheath gas flow rate, auxiliary gas flow rate were set to 350 °C, 3.5 kV, 35 and 10,

respectively. Detection of limonin was achieved in PRM mode monitoring the characteristic fragment ion (m/z 469.2>249.1) of the deprotonated molecule. The acquired data were processed using Xcalibur™ version 2.2.1 and Trace Finder version 3.3 (Thermo Fisher Scientific).

3.9. Aggregation kinetics

The time dependence of the aggregation of PMS-trypsin in 60% ethanol was being monitored on a Hitachi U-2000 spectrophotometer for 30 minutes by monitoring the increase of absorption at 350 nm at pH 7.0 and 24 ^{o}C, in the absence or presence of different inhibitory agent concentrations. The increase of UV absorption at 350 nm is indicative of the presence of large aggregated particles. The enzyme concentration in the aggregation kinetics assays was 0.13 mg/ml.

3.10. Congo red binding

Absorption spectra of CR (disodium-3,3'[[1,1-biphenyl]-4,4'-diylbis(azo)]bis(4-aminonaphthalin-1-sulphonate)) were recorded between 400 and 600 nm in a 10 mm-path-length quartz cell. The concentration of CR solution was determined using ε_M: 45,000 M^{-1} cm^{-1} at 498 nm (Klunk *et al*., 1989; Chaturvedi *et al*., 2015). Aliquots were prepared with protein concentration of 0.13 mg/ml in 60% ethanol/10 mM phosphate buffer at pH 7.0 in the presence or absence of various inhibitory agents. 200 µl of each 1-day-aged PMS-trypsin sample was added to 800 µl of a solution containing CR in 5 mM phosphate buffer/150 mM NaCl at pH 7.0. Absorption spectra were recorded after incubation for 15 minutes at room temperature. Difference spectra were constructed by subtraction of spectra of CR alone and PMS-trypsin alone from the spectra of PMS-trypsin + CR. 0.3 mg/ml protein concentration was used for absorption spectra by the successive

addition of CR and ginseng extract, and the spectrum of PMS-trypsin was subtracted from spectrum of PMS-trypsin + CR.

3.11. Electronic circular dichroism (ECD) measurements

Electronic circular dichroism spectra were recorded in the far UV range of 185–260 nm, in a 0.1 cm pathlength quartz cell on a Jasco J-815 CD spectropolarimeter at 24 ^{o}C. The ECD spectra of PMS-trypsin and PMS-trypsin with diluted inhibitory agents were recorded in 60% ethanol/10 mM phosphate buffer (pH 7.0) at 0.15 mg/ml protein concentrations. Spectra presented here are the averages of 10 independent scans. Ellipticity was expressed in mdeg units.

3.12. Size exclusion chromatography

Protein samples previously incubated for 1 day were centrifuged at 13,000 rpm for 1 min, and then 0.5 ml supernatants were applied. Sephadex G-75 column was used for size-exclusion chromatography (10 x 300 mm^2) followed by elution with 0.025 M phosphate buffer at pH=7.0. Fractions were collected, and their absorption was measured at 280 nm. Our column was calibrated with bovine serum albumin (67 kDa), trypsin (23.3 kDa) and lysozyme (14 kDa).

3.13. Fourier-transformed infrared (FTIR) spectroscopy analysis

The FTIR spectra were obtained with a Bruker Vertex 70 FTIR spectrometer (Bruker, Karlsruhe, Germany). The spectrometer is equipped with a DTGS detector. All FTIR measurements were performed under ambient conditions. Typical acquisition parameters are listed below: spectral resolution 2 cm^{-1}, scan number 32, zero-filling factor 4, apodization function; Blackman-Harris 3-Term, phase resolution 16, phase correction

mode Mertz, aperture 6 mm, scan speed 10 kHz, acquisition mode; double-sided, forward-backward. The spectrometer is accomplished with a platinum-diamond cell. The spectra range was recorded between 4000 cm^{-1} to 900 cm^{-1} with 2 cm^{-1} resolution and 128 scales were used to measure each of the samples.

3.14. Transmission electron microscopy

The inhibitory agent extracts were filtered through 0.02 μm Whatman inorganic membrane filter before use. 10 μl aliquots of the protein samples were placed on carbon-coated 300-mesh nickel grids (Nisshin EM Co. Ltd. Tokyo) and stained with 2% (w/v) uranyl acetate. Electronmicrographs were taken on a JEOL JEM-1011 transmission electron microscope (operating at 60 kV), using Olympus Morada 11 megapixel camera and iTEM softwer (Olympus).

3.15. Statistical analysis

Quantitative data was presented as mean +/− standard error of the mean. The statistical analysis was made by one-way analysis of variance (ANOVA). Significance of results was determined as ***$P < 0.001$,**$P < 0.01$ and *$P < 0.05$.

Pearson's correlation coefficient value (r) was defined as:

$$r = \frac{\sum_{i=1}^{n} (x_i - \bar{x})\,(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n} (x_i - \bar{x})^2}\,\sqrt{\sum_{i=1}^{n} (y_i - \bar{y})^2}}$$

4. Results and discussion

In these experiments, trypsin was used as a model protein modified with PMSF. The chemically modified enzyme is catalytically inactive, thus there is no autolysis at pH 7.0. Amyloid-like fibrils were prepared using PMS-trypsin as previously reported by the incubation of the soluble protein in the presence of 60% ethanol at 24 ^{o}C at pH 7.0 (Kotormán *et al*., 2015). The turbidity measurement is a procedure for detecting aggregates (Zhao *et al*., 2016). An increase in the UV absorption at 350 nm indicates more protein aggregation due to an increase in the scattering of light by aggregated particles (Ghosh *et al*., 2013). The effects of different inhibitory agents on PMS-trypsin aggregation can also be monitored *via* turbidity measurements.

Congo red is a well-known amyloid-specific dye, when amyloid fibrils are present in the solution, the visible absorption intensity increases, and a characteristic red shift of the CR absorption band occurs (Chaturvedi *et al*., 2016). CR exhibits maximal absorption at 490 nm. The shoulder peak on the CR absorption spectrum at around 540 nm indicates the presence of amyloid fibrils (Mahdavimehr *et al*., 2017). CR mainly binds to β-pleated sheet conformation, especially to amyloid fibrils (Klunk *et al*., 1989). CR binding assay was used to determine whether the aggregated species formed in the PMS-trypsin samples were fibril-like or not. CR binding properties of amyloid aggregates have been widely used to study anti-fibrillation activity of various inhibitors (Awasthi and Saraswathi, 2016). Difference spectra demonstrated the spectral changes of CR upon binding to PMS-trypsin amyloid fibrils.

Amyloid formation and morphology of aggregates were visualized by using transmission electron microscopy in the absence and presence of different inhibitory agents after 24 h incubation.

4.1. Inhibition of protein aggregation using herbal extracts

The sample without inhibitory agent shows maximum absorption value at 350 nm whereas the presence of different herbal extracts shows a marked decrease in it. The results from this comparative study indicated that peppermint extract displayed the greatest amyloid inhibiting function of the 7 samples tested. The maximum decrease of 99.4% in the absorption value was found to be in the case of peppermint extract diluted 5 times among the herbal extracts. Peppermint was also a good inhibitor of α-chymotrypsin aggregation (Kotormán *et al.*, 2018). The percentage of inhibition was calculated based on turbidity assays. The total phenolic content of the tested herb extracts varied from 163.7 ± 12.6 to 722.4 ± 25.5 mg GAE/l. The highest amount of total phenolic content was detected in the peppermint extract, while it also had the strongest aggregation inhibitory effect (Figure 12).

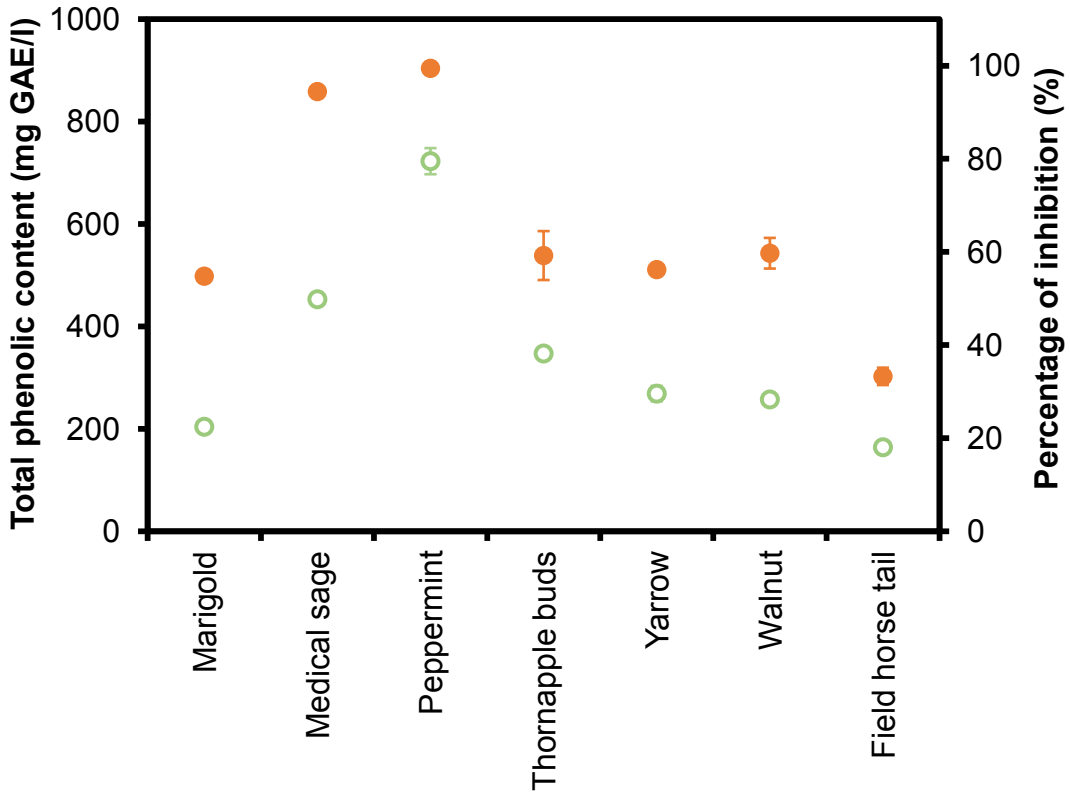


Figure 12. The percentage of inhibition (●) correlates with the total phenolic content (○) in the presence of herb extracts diluted x 5. All data were represented as mean ± standard error of the mean (SEM) from the average of three independent measurements.

The aggregation kinetic assays were used to monitor the time course of aggregation by PMS-trypsin through monitoring the increase of absorption at 350 nm in the absence and presence of peppermint extract at different concentrations. Faster increase in absorption was observed when peppermint extract was not present in the solution. Turbidity values were found to decrease continuously with the increasing concentration of the peppermint extract and its inhibitory effect was dose dependent (Figure 13).

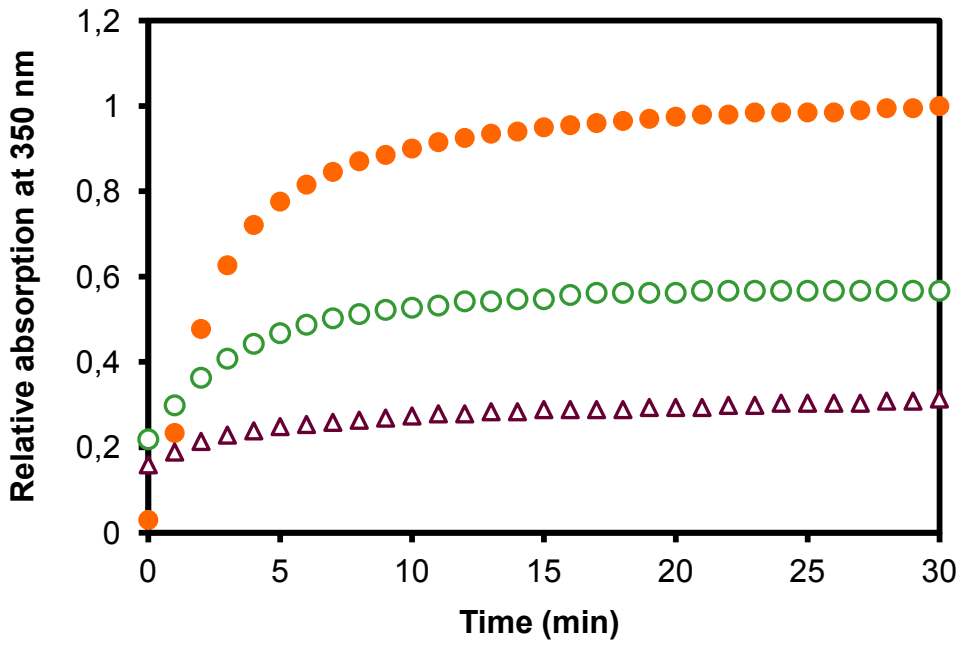


Figure 13. Kinetics of PMS-trypsin fibrillation. Change in OD (A_{350nm}) during PMS-trypsin fibrillation in the absence (●) and presence of peppermint extract diluted 25 times (Δ) and 50 times (o).

The inhibitory effect of the peppermint extract on the PMS-trypsin fibrillation was also observed using CR binding assay. These experiments suggested that the aggregates in the absence of an inhibitory agent have amyloid-like properties. In the presence of the peppermint extract the value of the maximum of CR differential spectrum was lower than in its absence (Figure 14). CR differential spectra indicated that the presence of the

peppermint extract decreased the amounts of amyloid fibrils in 60% ethanol at pH 7.0, and its inhibitory effect was dose-dependent.

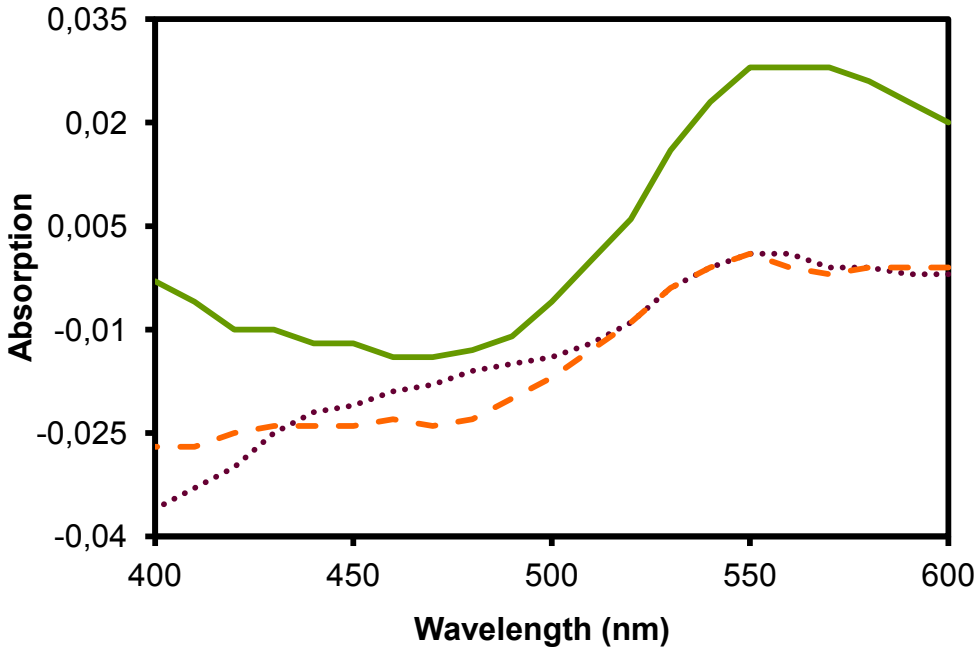


Figure 14. CR absorption difference spectra of PMS-trypsin in the absence (solid line) and presence of peppermint extract x 25 (dotted line) and x 50 (dashed line). CR and protein final concentrations were 3.2 μM and 26 μg/ml, respectively.

TEM image of PMS-trypsin in the absence of the peppermint extact shows fibrillar structure in 60% ethanol at pH 7.0 (Figure 15A). However, in the sample in which inhibitory agent was added to the PMS-trypsin, there was a significant lack of fibrils with only occasional scattered amorphous aggregates were observed (Figure 15B). TEM images demonstrated, that peppermint extract had inhibitory effect on the formation of amyloid-like fibrils of PMS-trypsin.

Based on our results, it was found that the anti-amyloidogenic effects of the herbal extracts could be related to their total phenolic content. Our results showed that the peppermint extract had a preventive effect on aggregation of the PMS-trypsin, and it could effectively inhibit the *in vitro* fibrillation. The process depended on the amount of the

peppermint extract. Peppermint is, therefore, a valuable source of phenolic compounds to prevent fibril formation.

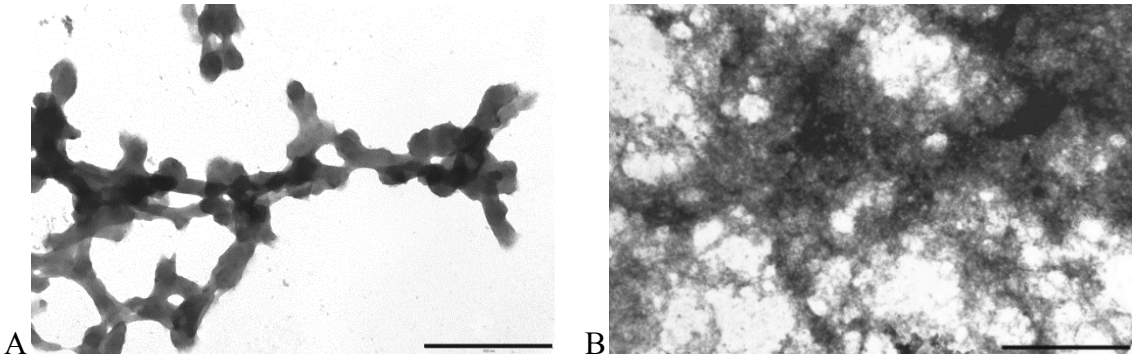


Figure 15. Transmission electron micrographs of PMS-trypsin in the absence (A) and presence (B) of peppermint extract diluted 25 times. The scale bar indicates 500 nm. Enzyme concentration: 0.13 mg/ml.

4.2. Inhibitory effect of the chili extract on the amyloid-like fibril formation

Turbidity measurement was used as an indication of the degree of aggregation in the presence of 27 commercially available spices. All examined agents showed varied effects. These experiments revealed that among the spices investigated chili was the best inhibitor. In the presence of chili extract diluted 500 times, the absorption had decreased to 36.8% relative to the sample not containing inhibitory agent (Figure 16).

Aggregation kinetic assays were performed in the absence and presence of various concentrations of the chili extract. Turbidity values were found to decrease continuously with the increasing concentrations of the chili extract. The results are shown in Figure 17. Our experiments proved, that the inhibitory effect of the chilli extract was dose dependent.

The CR absorption difference at 550 nm was found to decrease with incubation in the presence of chili extract. CR binding assay indicated that the presence of the chili extract decreased the amounts of amyloid-like fibrils, and inhibited the fibril formation dose-dependently (Figure 18).

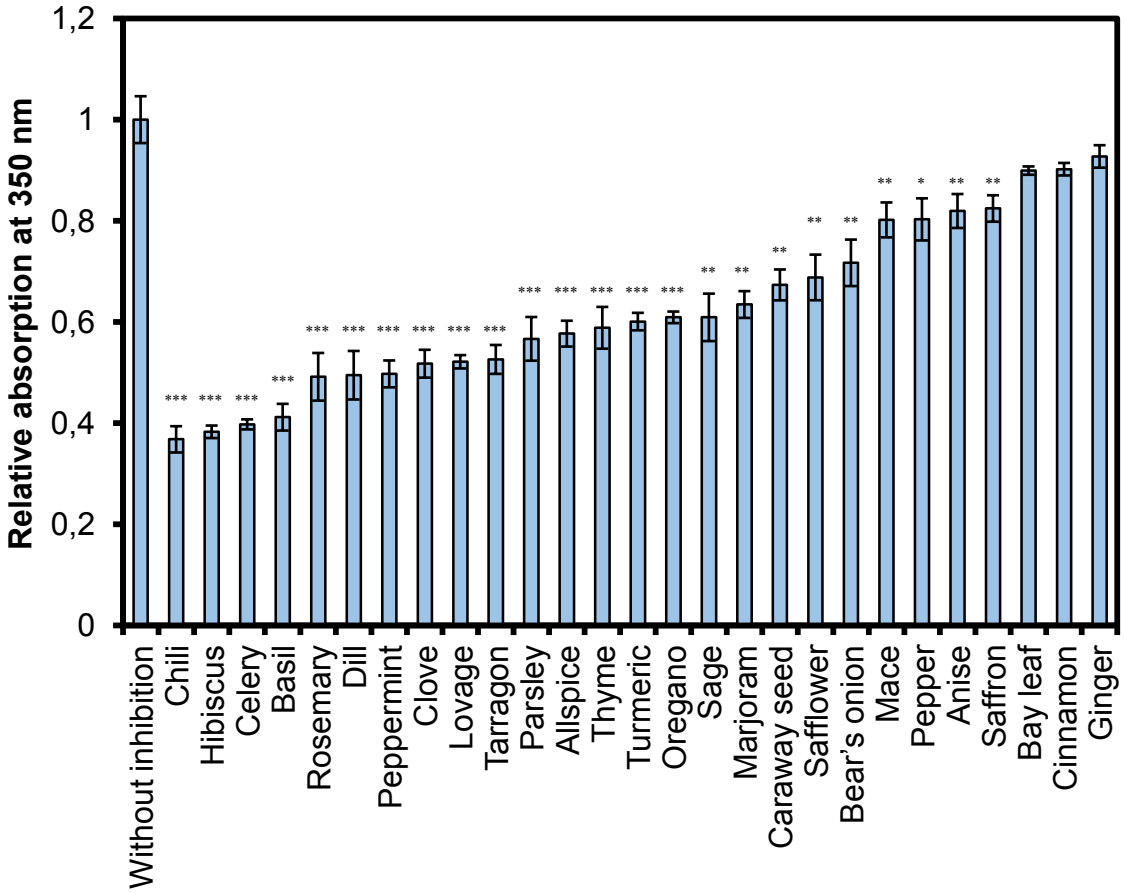


Figure 16. Turbidity changes of PMS-trypsin in the presence of various spice extracts or without them. In the incubation samples the spice extracts were diluted 500 times.

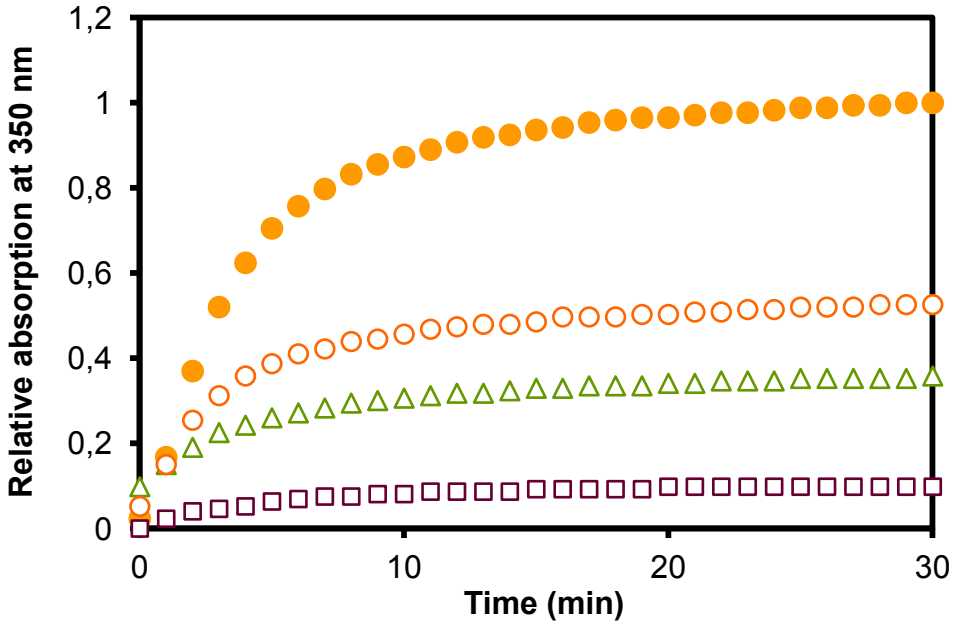


Figure 17. Time course of PMS-trypsin aggregation in the absence (●) and presence of chili extract diluted 50 times (□), 500 times (△) and 1,000 times (o).

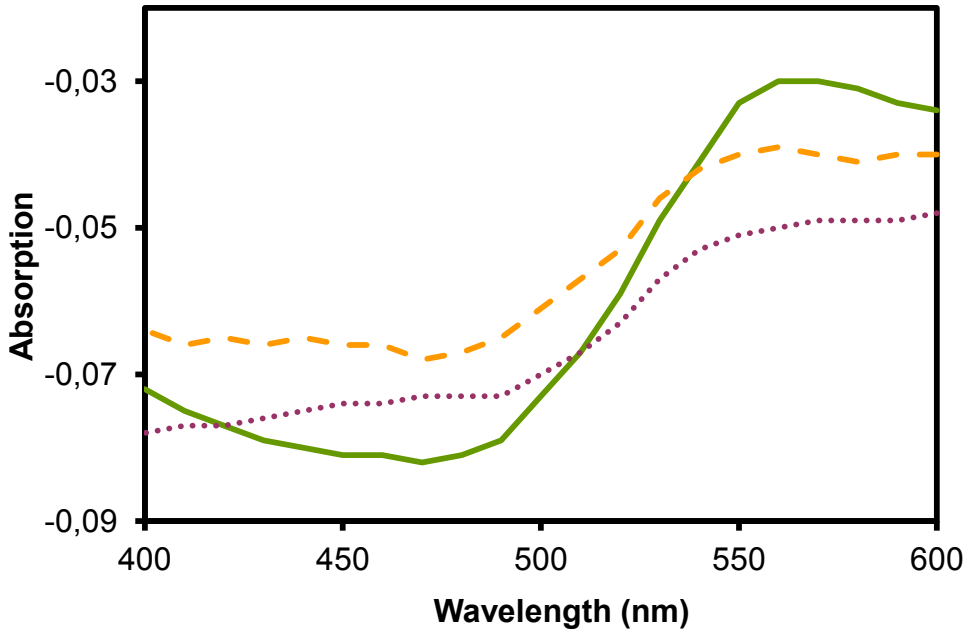


Figure 18. CR differential spectra in the absence (solid line) and presence of chili extract diluted 250 times (dotted line) and 1000 times (dashed line). CR and protein final concentrations were 3 μM and 26 μg/ml, respectively.

FTIR spectroscopy has been used since early 1970 to examine the amyloid fibril structure (Sarroukh *et al*., 2013). The fibrils display a typical anti-parallel β-sheet structure characterized by two bands in the amide I: a low frequency, high intensity band around 1620–1630 cm^{-1} and a high frequency, low intensity band located around 1685–1695 cm^{-1}. In order to demonstrate the inhibitory effect of chili extract on PMS-trypsin fibrillation by structural change, we analysed the FTIR spectrum of protein in 10 mM phosphate-buffered 60% ethanol without chili extract and in the presence of chili extract diluted 500 times. A characteristic peak of 1629 cm^{-1} was obtained when it was measured with the PMS-trypsin, which shows the oligomers have anti-parallel β-sheet organization. 1648, 1641 cm^{-1} strong peaks (Figure 19) were obtained in the presence of chili extract with PMS-trypsin, which clearly shows a change in structural orientation and the transition state of oligomers to a monomer one. It also indicates that the chilli extract inhibited amyloid

aggregation by stabilizing its native form from aggregating and remodelling or degrading toxic amyloid oligomers.

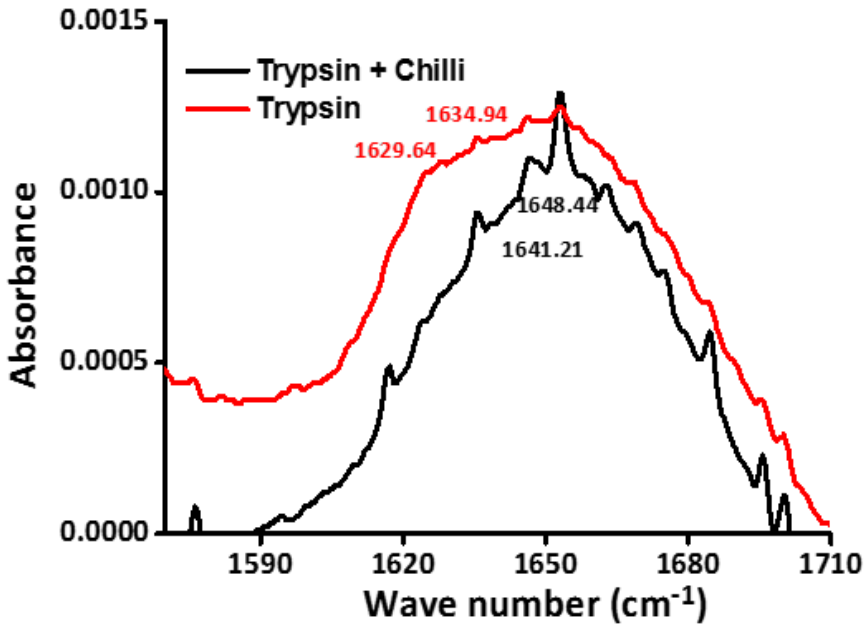


Figure 19. FTIR spectra of PMS-trypsin in 10 mM phosphate-buffered 60% ethanol illustrating the Amide I regions respectively in the presence of chili extract diluted 500 times and without it.

Electronic circular dichroism analysis in the far UV wavelength range (185–260 nm) was conducted to determine the changes in the secondary structure of PMS-trypsin in aqueous ethanol at pH 7.0. The presence of a mild solvent causes an increase in the β-sheet conformation in proteins (Furkan *et al*., 2016). Conversion of the secondary structure of PMS-trypsin in the presence of 60% ethanol takes place immediately. It is known from our research group's previous experiments that in the presence of 10 mM phosphate buffer at pH 7.0 in the structure of the PMS-trypsin, α-helix is present with the dominance of the β-sheet. The ECD spectrum obtained in the presence of 60% ethanol was characteristic of amyloid fibrils (Kotormán *et al*., 2015). Differences between the measured spectra arise from the differences in size distribution of the aggregates evolving gradually. The spectra were detected placing the cell in two ways, in the middle of the sample holder or next to

the detector. The two measurements give the same results with clear, real solution. A remarkable change can be observed in the sample without any inhibitory agent concerning the intensity and blue shift of spectral maxima for the sample closer to the detector. In the absence of chili extract, the large sized colloid aggregates were abundant. The light-dispersion of such colloidal samples is substantial, consequently fewer photons get to the detector, which results in the acquisition of less intense spectra. Furthermore, the concentration of proteins in real solution state is lower in suspensions that also contribute to the decrease of spectral intensities. We observed a remarkable change in the intensity of the spectrum and a blue shift approaching the sample toward the detector without any inhibitory agent. This sample contained the largest-sized aggregates. The two measurements gave almost the same results in the presence of chili extracts diluted 500 times indicating that the chili extract stabilized the protein in a more or less dissolved state (Figure 20).

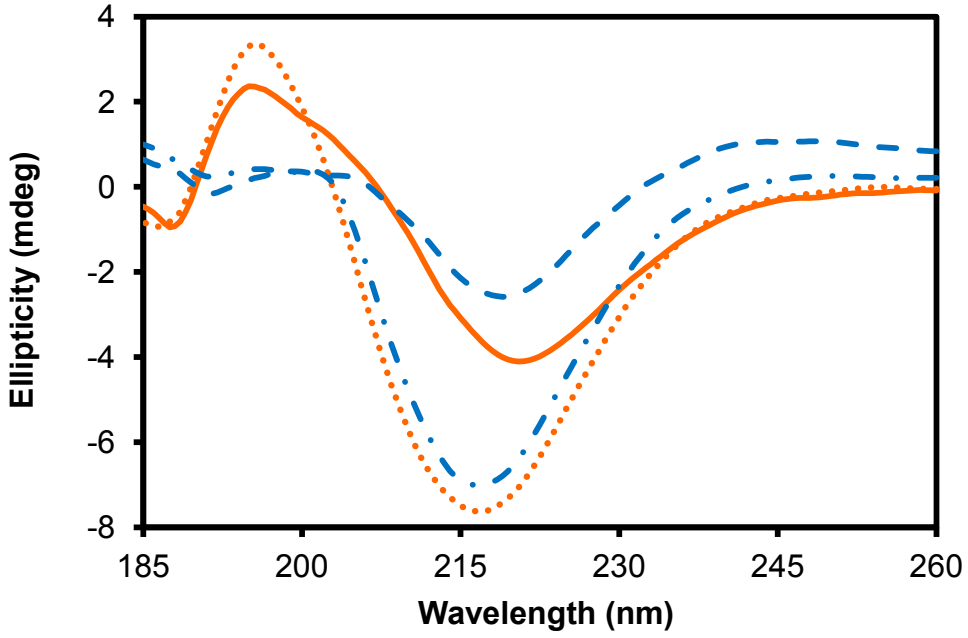


Figure 20. ECD spectra of PMS-trypsin in 60% ethanol without chili extract (solid line, dashed line) and in the presence of chili extract diluted 500 times (dotted-dashed line, dotted line). The sample was set in the middle of the sample holder (solid line, dotted line), or next to the detector (dashed line, dashed-dotted line).

Presumably, the structure of the protein slightly changes in the presence of a chili extract, too, but aggregates do not. If they do, they form rather slowly, indeed.

The ability of chili extract to prevent aggregation was visualised by TEM. TEM image of PMS-trypsin in absence of chili extract shows fibrillar structure in 60% ethanol. However, in the sample in which chili extract was added to the PMS-trypsin, there was a significant lack of fibrils with only occasional scattered amorphous aggregates (Figure 21). The finding suggests that chili bioactive compounds may be potential therapeutic drug candidates.

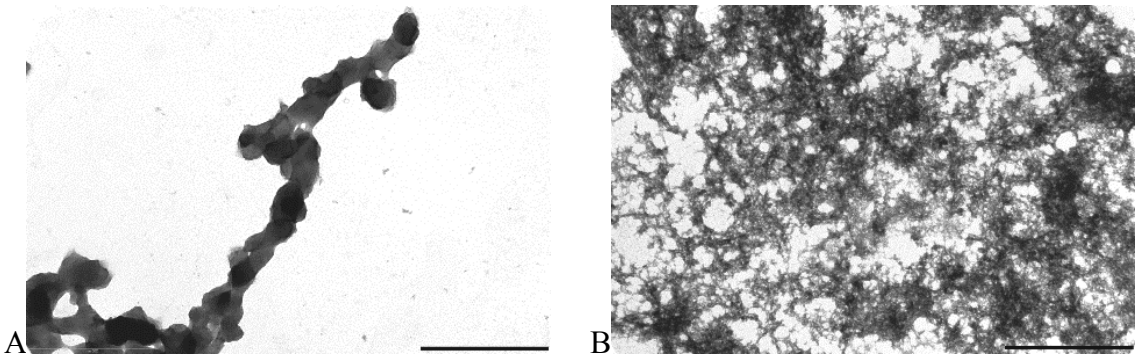


Figure 21. Transmission electron micrographs of PMS-trypsin (A) in the absence and (B) in the presence of chili extract x 500. The scale bar indicates 500 nm. Enzyme concentration: 0.13 mg/ml.

4.3. Examination of the anti-amyloidogenic effect of the *P. ginseng* extract

When I joined this research group's work, experiments on the anti-amyloidogenic effect of the *P. ginseng* extract had already been underway. To find out whether its bioactive components and CR compete to bind to the amyloid-like fibrils, or not, we successively added CR and the ginseng extract to the fibrils. During the first experiment, CR was firstly added to the fibrils, followed by the *P. ginseng* extract. In the second one, the sequence of administration of these agents was reversed. With these experiments we

could show that there is no competition between the CR and the biomolecules of the *P. ginseng* extract to bind to the fibrils or to a different site (Figure 22).

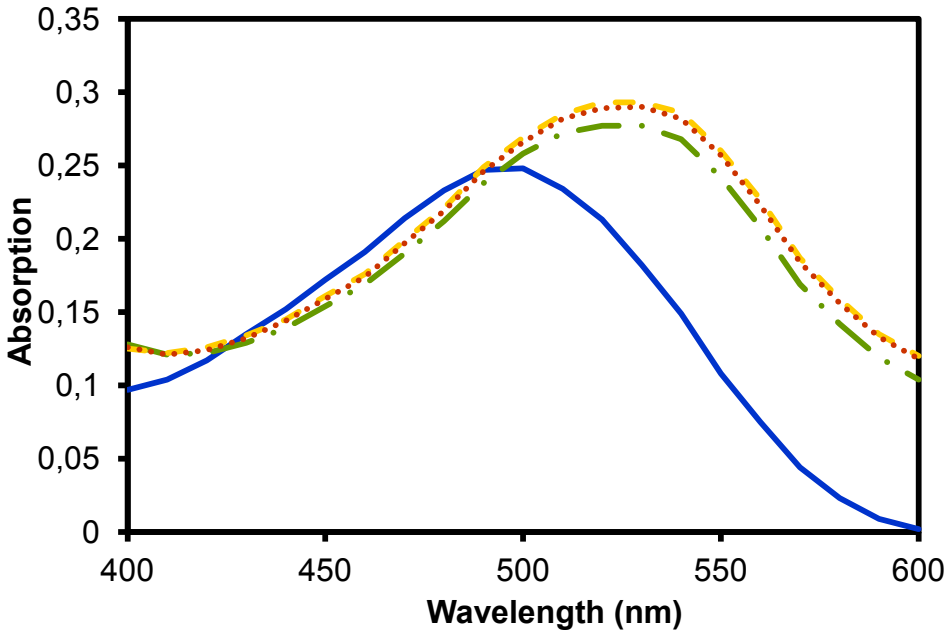


Figure 22. CR absorption spectra by successive addition of CR and ginseng extract. CR alone (solid line), in the absence of *P. ginseng* extract (dashed-dotted line), first adding CR and then the ginseng extract to the protein (dashed line), and the reverse order (dotted line).

The different-sized particles in our samples were selected using size exclusion chromatography. Our results proved that the sample without *P. ginseng* extract contains higher amounts of protein oligomers than in the presence of it (Figure 23). In the presence of *P. ginseng* extract there are more monomer trypsin molecules in the sample. Our results show that the presence of ginseng extract helps at maintaining the native structure of the protein. It was demonstrated that ginsenoside Rg_1 and Rb_1 stabilize the derivative of κ-casein in its native-like state (Liu *et al.*, 2015).

ECD spectroscopy was used to examine the conformational changes of PMS-trypsin in 60% ethanol/10 mM phosphate buffer, in the presence and in the absence of the 500-fold diluted *P. ginseng* extract. ECD experiments were made in two different sample

positions. Significant change can be observed in the sample without the *P. ginseng* extract concerning the intensity and blue shift of spectral maxima for the sample closer to the detector. Adding *P. ginseng* extract to our sample decreased the light dispersion of the enzyme solution (Figure 24). This is due to the decrease of the rate of the protein aggregation or to the smaller size of the aggregates evolved. Although, the intensity of spectral minima varies in the presence of *P. ginseng* extract, a greater shift of the minima cannot be observed.

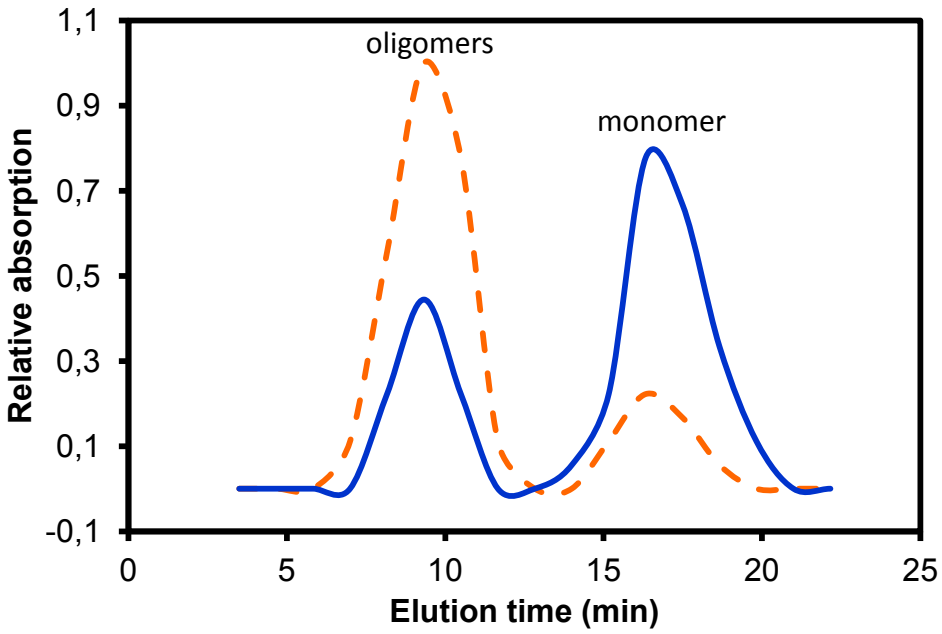


Figure 23. Molecular weight determination using Sephadex G-75 column in the presence of *P. ginseng* extract x 100 (solid line) and without it (dashed line).

The effects of the *P. ginseng* extract on morphology of PMS-trypsin fibrils were further studied by TEM (Figure 25). TEM micrographs confirmed the formation of amyloid-like fibrils without the inhibitory agent in aqueous ethanol at pH 7.0 after incubation for 24 hours. However in the presence of the 500-fold diluted *P. ginseng* extract, TEM image demonstrated that fibril formation was effectively inhibited.

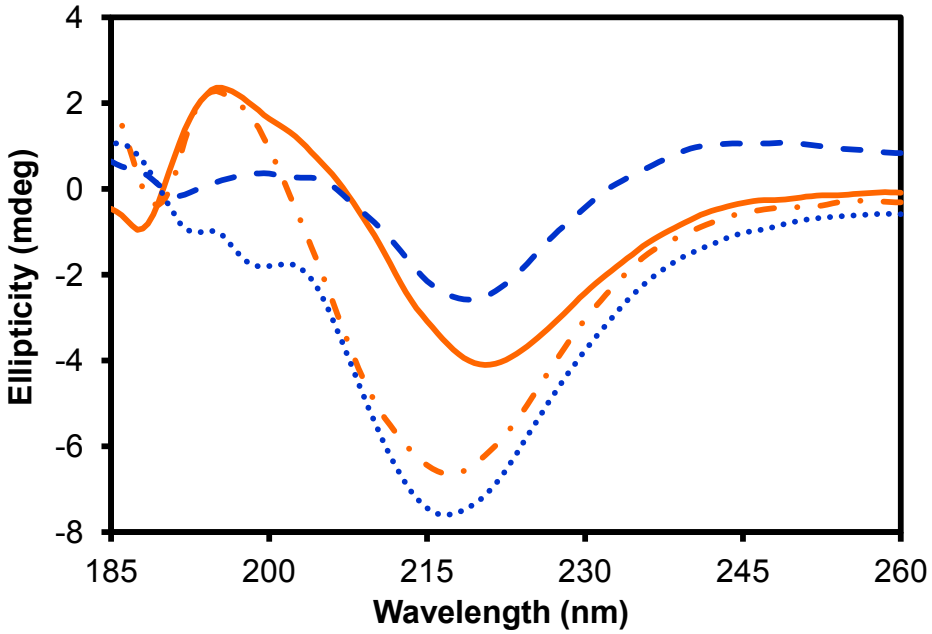


Figure 24. ECD spectra of PMS-trypsin in 10 mM phosphate-buffered 60% ethanol with *P. ginseng* extract x 500 (dotted-dashed line, dotted line) or without it (solid line, dashed line). The sample was set in the middle of the sample holder (solid line, dashed-dotted line), or next to the detector (dashed line, dotted line).

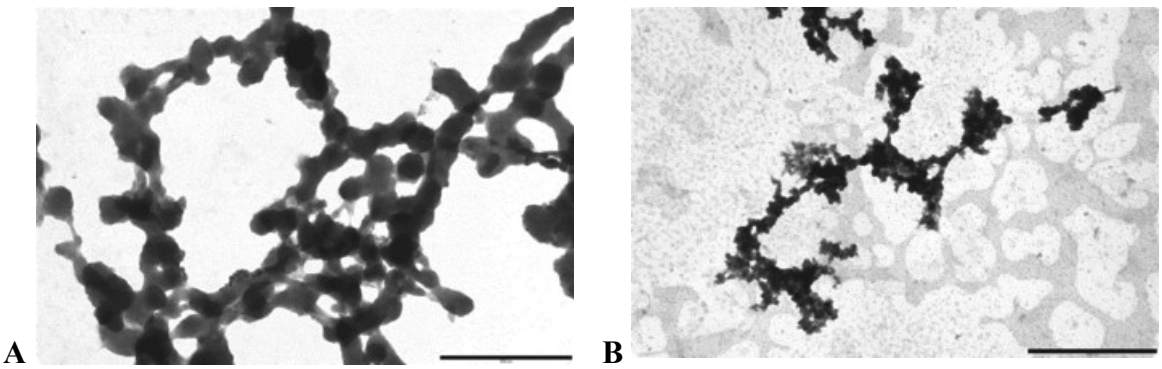


Figure 25. TEM micrographs of PMS-trypsin samples after incubation at 24°C for 24 h. PMS-trypsin in 60% ethanol (A), PMS-trypsin in 60% ethanol in the presence of *P. ginseng* extract x 500 (B). The scale bar indicates 500 nm. Enzyme concentration: 0.13 mg/ml.

4.4. Inhibition of amyloid-like fibril formation using the Eduscho coffee extract

The turbidity measurements were performed to detect the effect of different coffee extracts on PMS-trypsin aggregation. The greatest effect was exerted by the Eduscho coffee extract. The absorption had decreased in the presence of Eduscho coffee extract

diluted 250 times to 18.1% relative to the sample not containing inhibitory agent. The total concentrations of phenolic compounds of different coffee extracts ranged from 4942 ± 361.7 to 7702 ± 528.5 mg GAE/l (Figure 26). The calculated Pearson's correlation coefficient values (r), fell in between 0.896 and 0.997. According to our experiments, the degree of inhibition changed pro rata with the total concentrations of phenolic compounds.

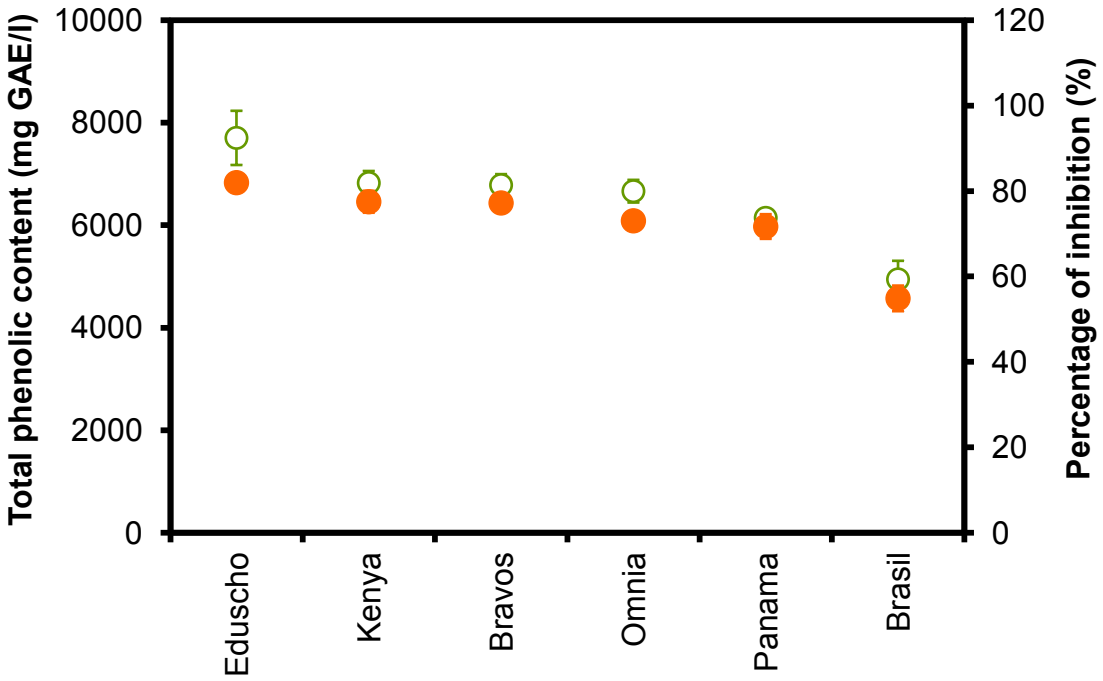


Figure 26. The change of percentage of inhibition (●) with the total phenolic content (o). Coffee extracts were diluted x 250. All data were presented as mean ± standard error of the mean (SEM) from three independent measurements.

CR binding assay can also be utilised to study the anti-fibrillation efficiency of small molecular inhibitors (Awasthi and Saraswathi, 2016). CR binding assay indicated that the presence of the Eduscho coffee extracts decreased the amounts of amyloid fibrils in 60% ethanol at pH 7.0, and their inhibitory effects were dose-dependent (Figure 27). Similar to our experimental results, Cheng *et al*. came to the conclusion that coffee components had inhibited the amyloid fibril formation using human islet amyloid polypeptide (Cheng *et al*., 2011).

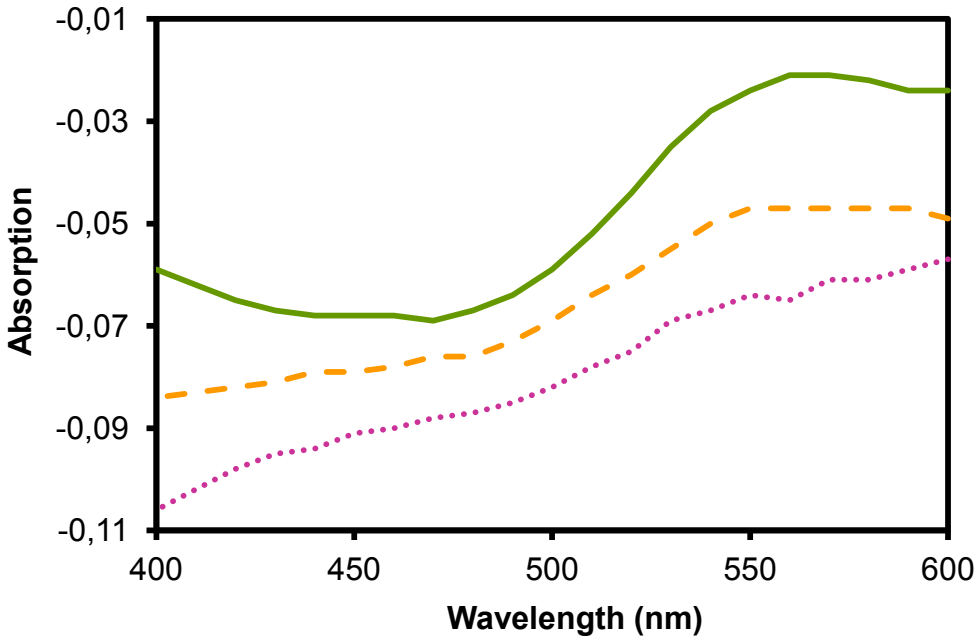


Figure 27. CR difference spectra without coffee extract (solid line) and in the presence of Eduscho coffee extract diluted 250 times (dotted line) and 500 times (dashed line). Congo red and protein concentrations were 5 μM and 26 μg/ml, respectively.

To determine whether there is a competition between polyphenolic compounds of the Eduscho coffee extract and CR in the binding to the PMS-trypsin fibrils, we used the successive addition of the coffee extract and CR (Figure 28). During the first experiment, first CR, then the coffee extract was added to the amyloid-like fibril. In the second experiment the administration of these agents happened in reverse order. With these experiments we could show that the polyphenols in the coffee extract do not bind to the fibrils or the polyphenolic compounds bind to another site.

Gel filtration chromatography was used to separate the particles of different size. The presence of Eduscho coffee extract diluted 100 times increased the amount of the protein oligomers (Figure 29). We demonstrated that the inhibitory effect of the coffee extract on the fibril formation is due to its capacity to stabilize the oligomeric form of the PMS-trypsin. Similarly to our results, phenyl ethyl alcohol had had an inhibitory effect on

hen egg white lysozyme fibril formation by producing mainly oligomers (Seraj *et al.*, 2018).

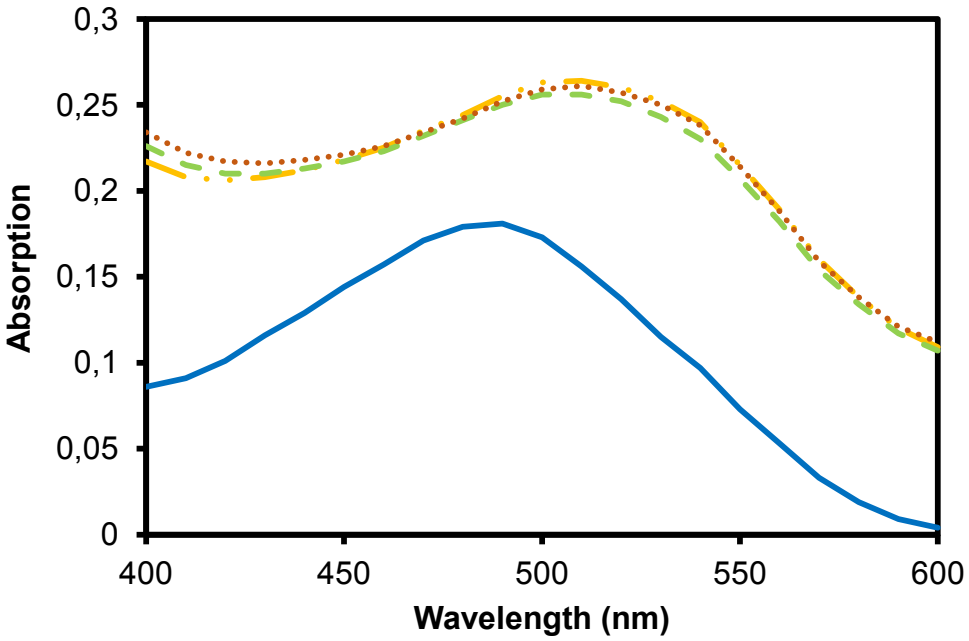


Figure 28. Absorption spectra by successive addition of CR and Eduscho coffee extract. CR alone (solid line), in the absence of Eduscho coffee extract (dashed-dotted line), first adding CR and then the Eduscho coffee extract to the protein (dotted line), and the reverse order (dashed line).

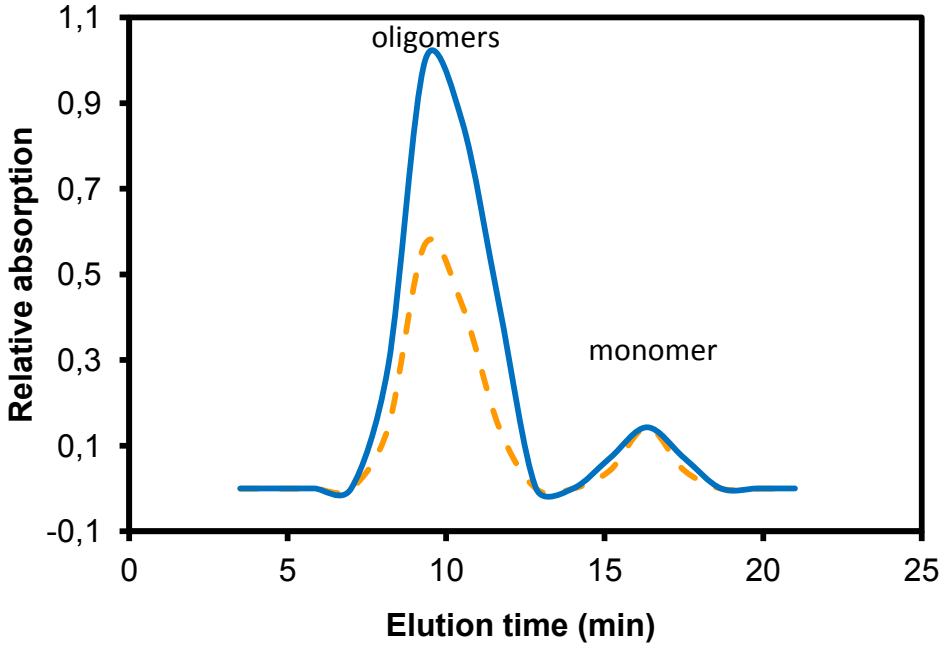


Figure 29. Size-exclusion chromatography using Sephadex G-75 column in the presence of Eduscho coffee extract x 100 (solid line) and without it (dashed line).

ECD spectra of PMS-trypsin were recorded at lower ethanol concentrations, in an attempt to follow the gradual conversion of the secondary structure preceding intermolecular association. Spectra were measured after 0, 20, 30, 60, and 120 min incubation in 10% ethanol/0.01 M phosphate buffer at pH 7.0. In general, spectra measured at 10% ethanol concentration display minor α-helical contributions as opposed to those measured at 60% ethanol concentration, where spectral characteristics of β-sheet structure are dominant (Kotormán *et al*., 2015). In 10% ethanol/0.01 M phosphate buffer the intensity of the shoulder appearing at 200 nm was found to increase to some extent over time, while the shoulder around 218 nm have diminished (Figure 30). ECD spectral changes were complete after 60 min incubation. These experimental results indicated slow helix to coil transition, which could be elucidated as the first structural transitional step of the trypsin aggregation process.

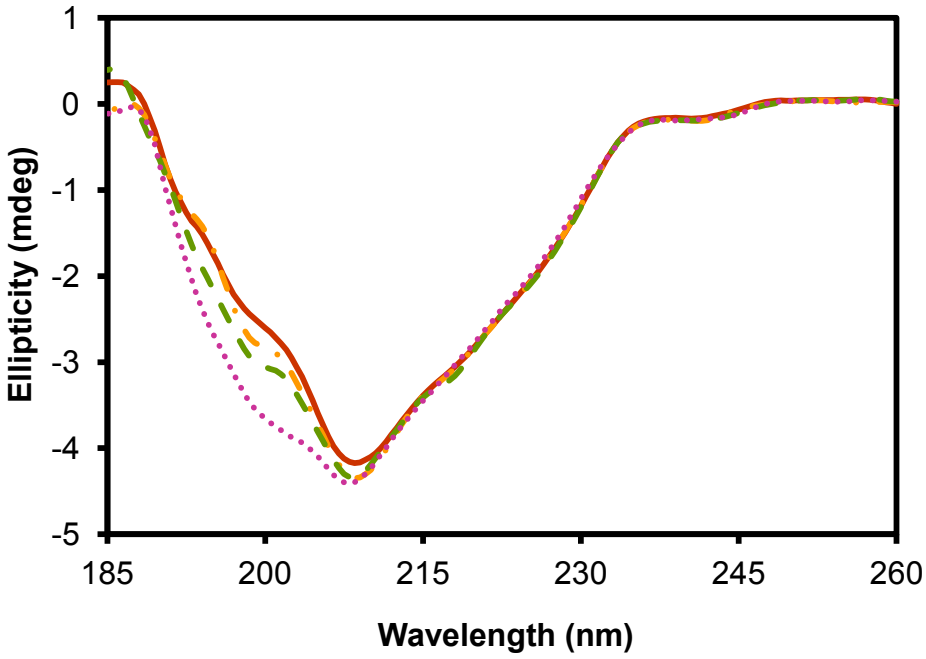


Figure 30. ECD spectra of PMS-trypsin in 10 mM phosphate-buffered 10% ethanol without coffee extract at 0 min (solid line), 20 min (dotted-dashed line), 30 min (dashed line), 60 min (dotted line).

Such structural changes were found to be slower and less pronounced in the presence of the Eduscho coffee extract diluted 250 times (Figure 31). Taking these results into account, we concluded that the addition of the Eduscho coffee extract effectively prevented PMS-trypsin from undergoing helix-to-coil transition.

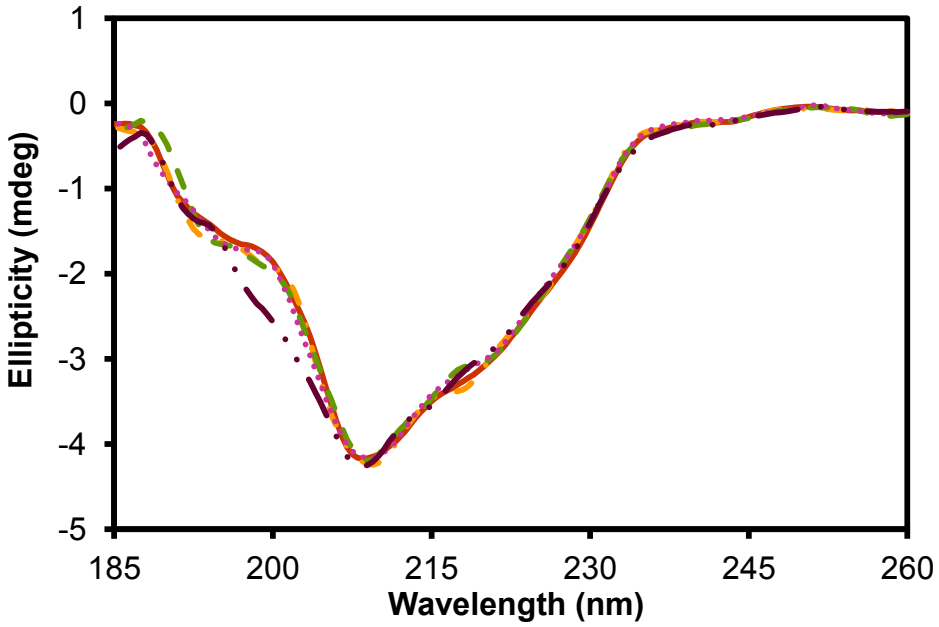


Figure 31. ECD spectra of PMS-trypsin in 10 mM phosphate-buffered 10% ethanol in the presence of Eduscho coffee extract x 250 at 0 min (solid line), 20 min (dotted-dashed line), 30 min (dashed line), 60 min (dotted line), 120 min (double-dotted-dashed line).

ECD spectroscopy in the far UV wavelength range (185–260 nm) was used to detect the secondary structural changes in PMS-trypsin in 60% ethanol in the absence and presence of the Eduscho coffee extract. The sample was either set in the middle of the sample compartment, or at the right side, next to the detector (Figure 37). A change in the intensity and blue shift was observed, when the sample was located next to the detector. This indicated that large aggregates were present in the protein sample without the Eduscho coffee extract. Such a phenomenon was observed to a lesser extent when the Eduscho coffee extract was present in the samples.

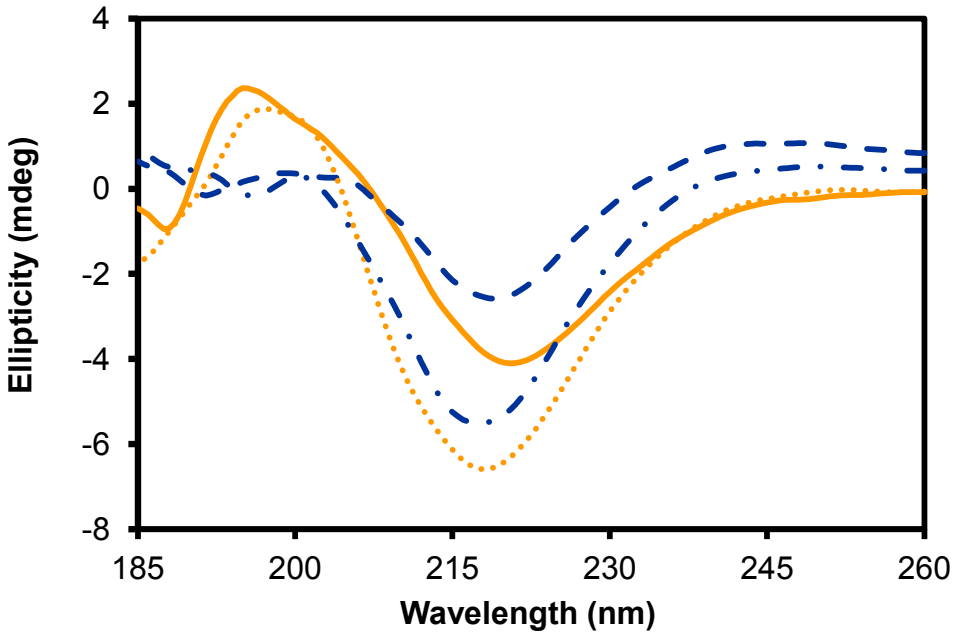


Figure 37. ECD spectra of PMS-trypsin in 60% ethanol without Eduscho coffee extract (solid line, dashed line) and in the presence of Eduscho extract diluted 250 times (dashed-dotted line, dotted line). The sample was set in the middle of the sample holder (solid line, dotted line), or next to the detector (dashed line, dashed-dotted line).

Amyloid formation and morphology of aggregates were visualized by using TEM. TEM images of PMS-trypsin in absence of inhibitory agent show fibrillar structure in 60% ethanol at pH 7.0 (Figure 38A and 39A). The extent of fibril formation was reduced at a significant level in the presence of the Eduscho coffee extract diluted 250 times (Figure 39B).

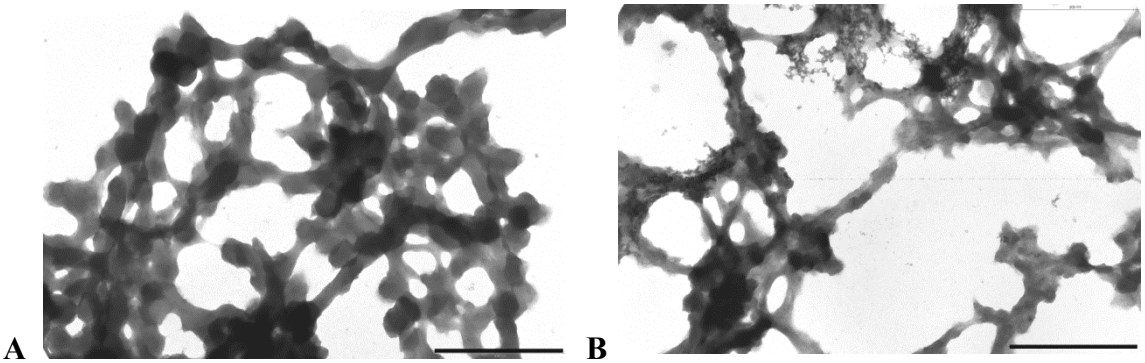


Figure 38. Transmission electron micrograph of PMS-trypsin in the absence (A) and the presence of the Eduscho coffee extract dikuted 250 times (B). The scale bar indicates 500 nm. Enzyme concentration: 0.13 mg/ml.

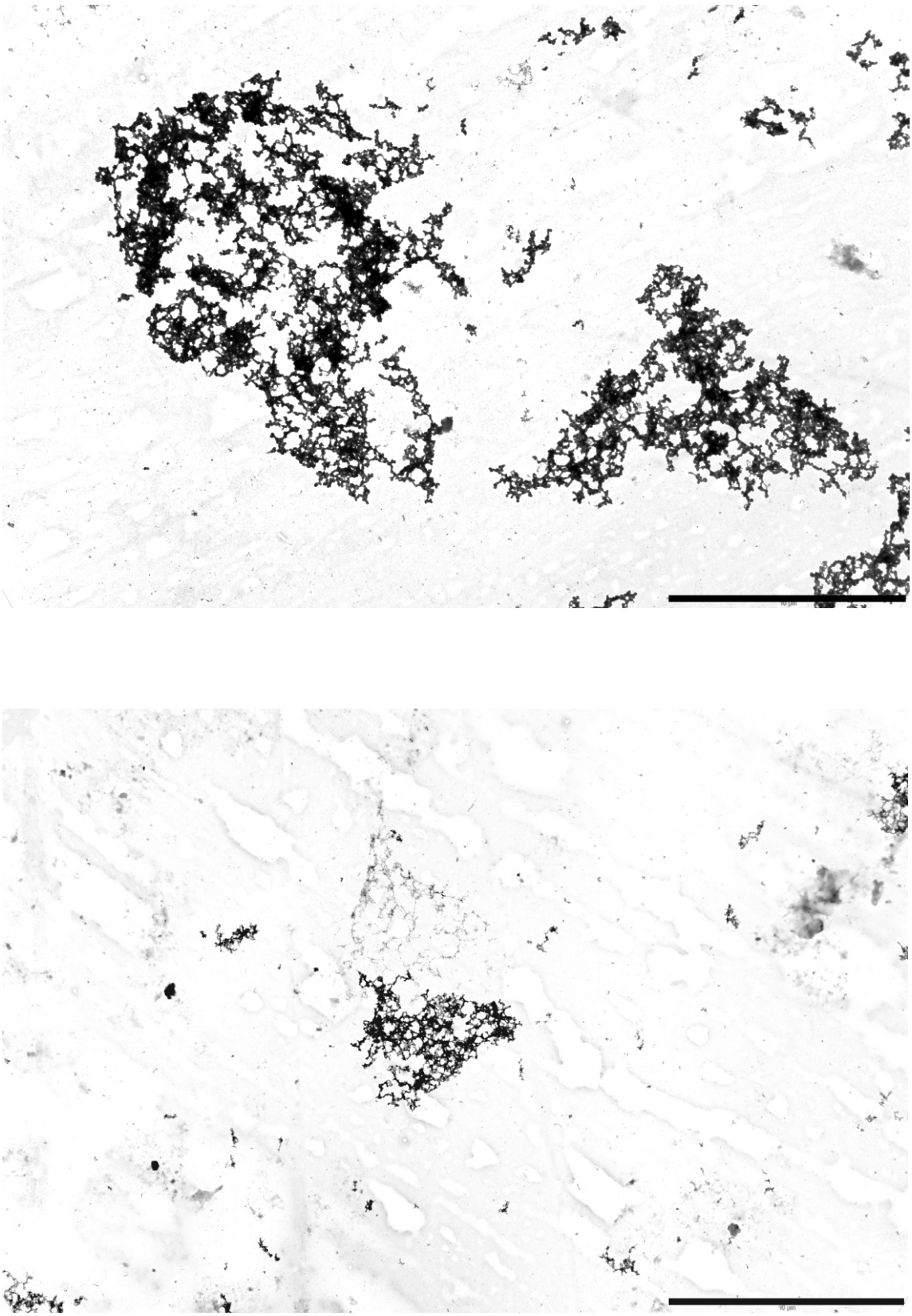


Figure 39. Transmission electron micrograph of PMS-trypsin in the absence (A) and the presence of the Eduscho coffee extract dikuted 250 times (B). The scale bar indicates 10 μm. Enzyme concentration: 0.13 mg/ml.

4.5. Inhibitory effect of the grapefruit seed extract on the amyloid-like fibril formation

The grapefruit seed extract diluted 800 times reduced the absorption to 52.3% compared relative to the PMS-trypsin sample, which contained no inhibitory agent, so, it was the best inhibitor among the food supplements investigated. The total phenolic content of the different inhibitory agents was determined, and it varied between 431.4 ± 62.2 and 2659 ± 59.9 mg GAE/l (Figure 40). The percentage of inhibition of amyloid formation did not change proportionally with the total phenolic content. Based on the results obtained, it is probable that not only polyphenols are responsible for the fibril formation inhibitory effect but also other compounds. Grapefruit seed extract also contains limonoids (Cvetnić and Vladimir-Knezević, 2004), which have neuroprotective effects (Venkatesan *et al*., 2015; Yoon *et al*., 2010).

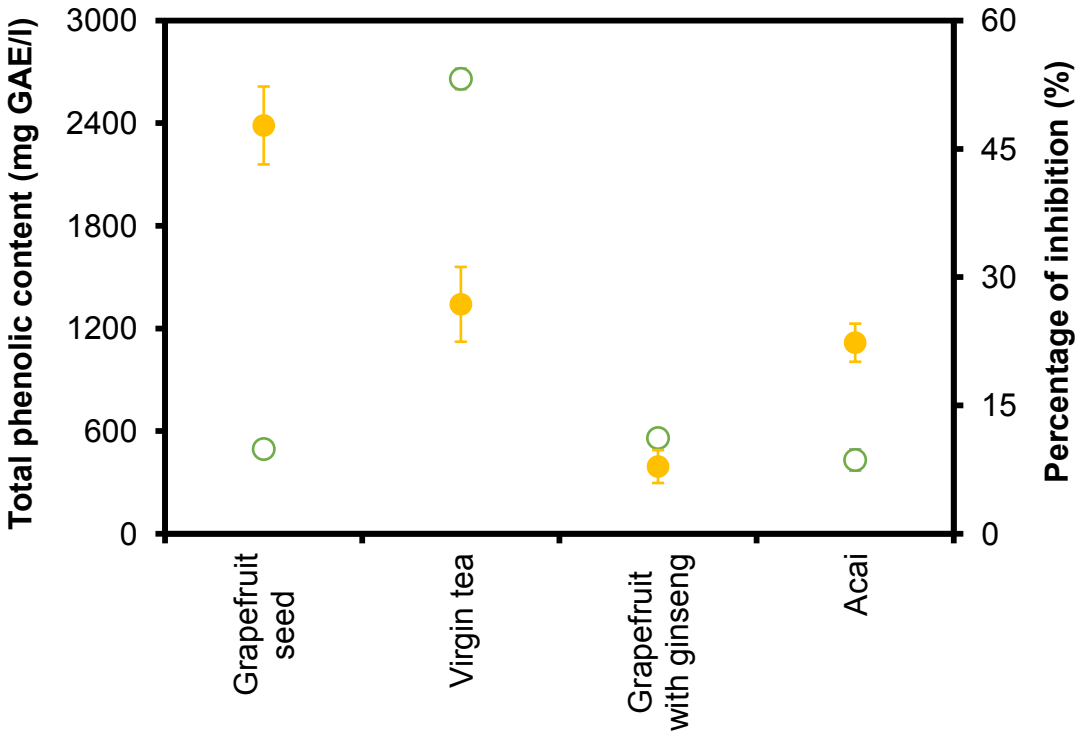


Figure 40. Change of the total phenolic content (○) with the percentage of the inhibition of amyloid-like fibril formation (●) in 60% ethanol in the presence of different inhibitory agents x 800. All data were presented as mean ± standard error of the mean (SEM) from three independent measurements.

The limonin concentration in the grapefruit seed extract was determined: it was 16.8 ng/ml. In acai commercial pulps there are various bioactive compounds, in addition to phenolic compounds and there are also carotenoids in them (Santos *et al*., 2008). Some carotenoids have anti-amyloidogenic effect (Lakey-Beitia *et al*., 2017).

The inhibitory effect of the grapefruit seed extract on the aggregation process was determined at various concentrations. At 100-fold dilution grapefruit seed extract almost eliminated the formation of protein aggregates. The anti-aggregation effect of the grapefruit seed extract was dose dependent (Figure 41).

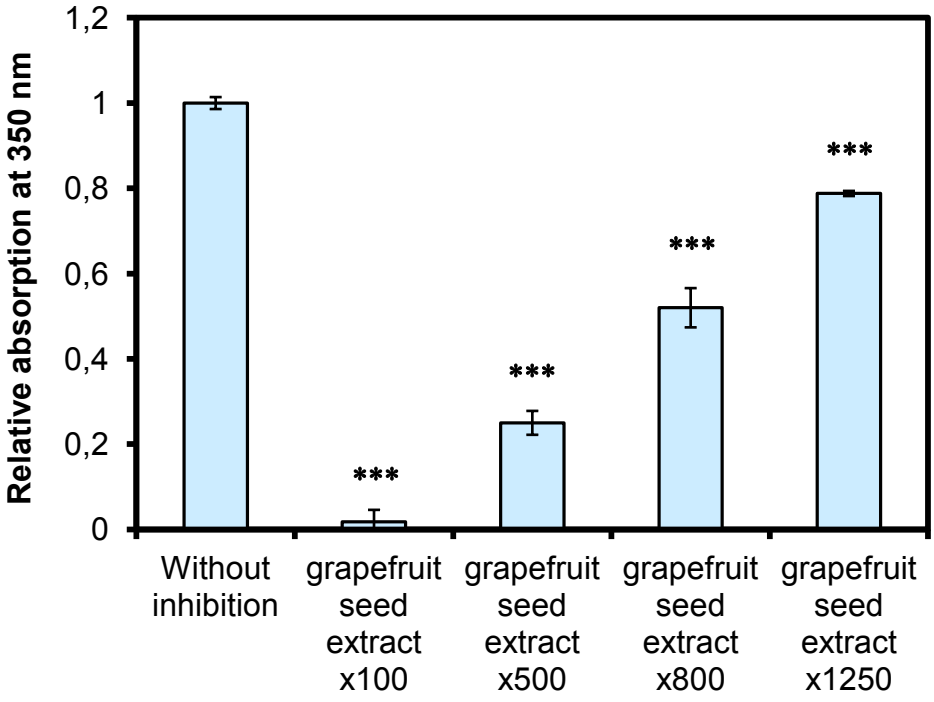


Figure 41. Turbidities at 350 nm of PMS-trypsin in 60% ethanol at pH 7.0 in the absence or presence of different concentrations of grapefruit seed extract.

To determine the efficiency of the grapefruit seed extract and of limonin against the PMS-trypsin fibrillation CR binding assay was used. Both inhibitory agents were able to exert dose-dependent inhibitory effect on the formation of amyloid-like fibrils (Figure 42

and 43). For the first time, our results indicated that limonin was an effective anti-amyloidogenic agent.

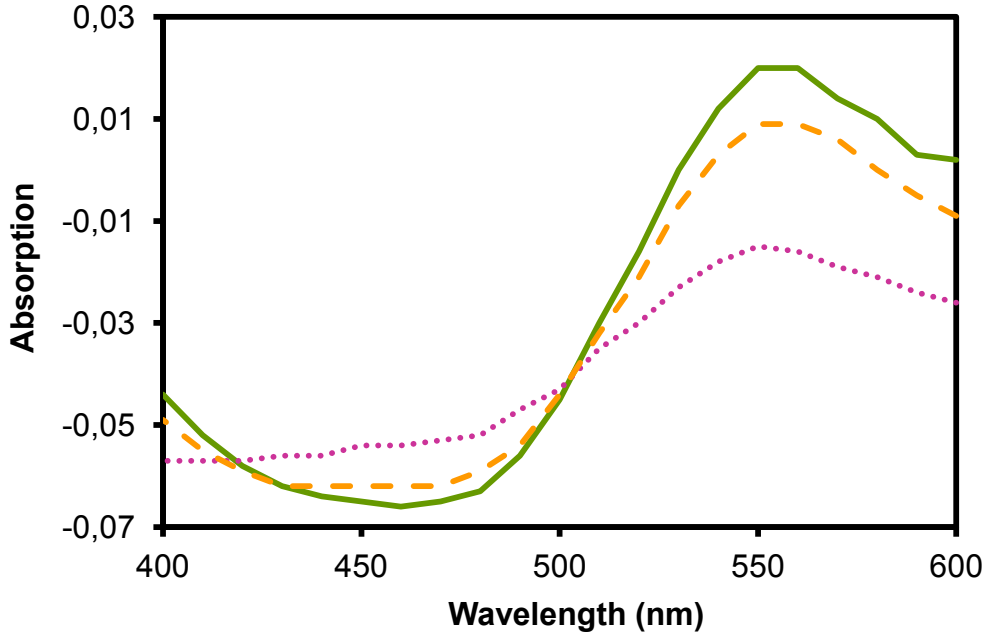


Figure 42. CR absorption difference spectra of PMS-trypsin in the absence (solid line) and presence of grapefruit seed extract diluted 800 times (dashed line) and 100 times (dotted line). Congo red and protein concentrations were 6 μM and 26 μg/ml, respectively.

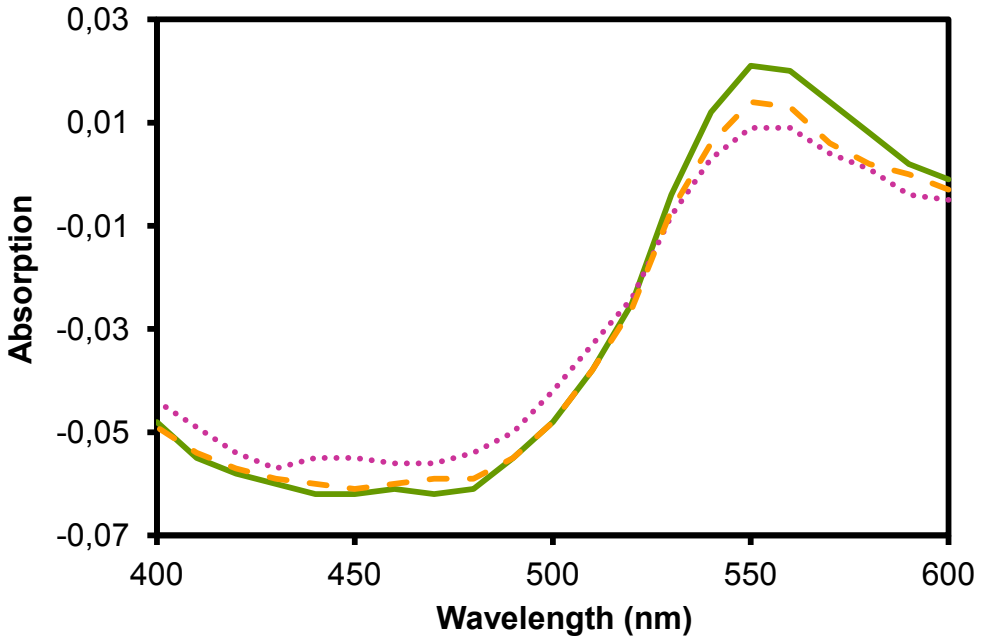


Figure 43. CR absorption difference spectra of PMS-trypsin in the absence (solid line) and presence of 34 nM limonin (dashed line) and 340 nM limonin (dotted line).

Changes in the PMS-trypsin secondary structure can be revealed using far-UV ECD measurements in 60% ethanol in the absence or presence of grapefruit seed extract diluted 500 times. Far-UV ECD measurements were made in two different sample positions. In the presence of grapefruit seed extract light dispersion decreased (Figure 44). This can be explained by the fact that the aggregation speed has decreased, or the size of the aggregates formed has decreased. According to our experiments, the grapefruit seed extract effectively inhibited the amyloid fibril formation.

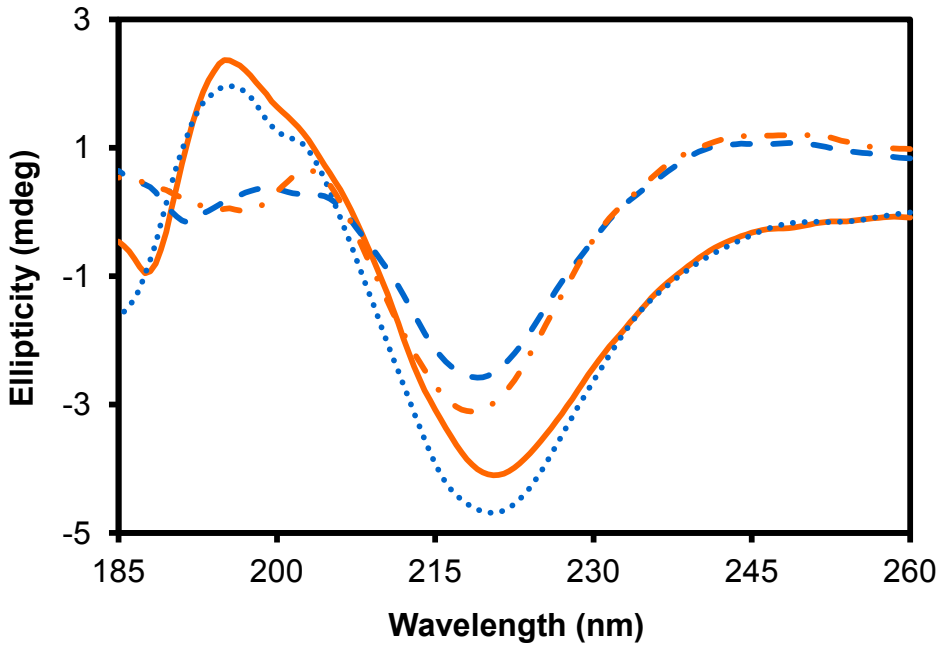


Figure 44. Far-UV CD spectra of PMS-trypsin in the absence (dashed line, solid line) and presence of grapefruit seed extract x 500 (dotted-dashed line, dotted line). The sample was set in the middle of the sample holder (solid line, dotted line), or next to the detector (dashed line, dotted-dashed line).

TEM studies showed that there was no fibril formation in the presence of grapefruit seed extract diluted 500 times, indicating the inhibitory effect of grapefruit seed extract on the formation of amyloid-like fibrils by trypsin (Figure 45).

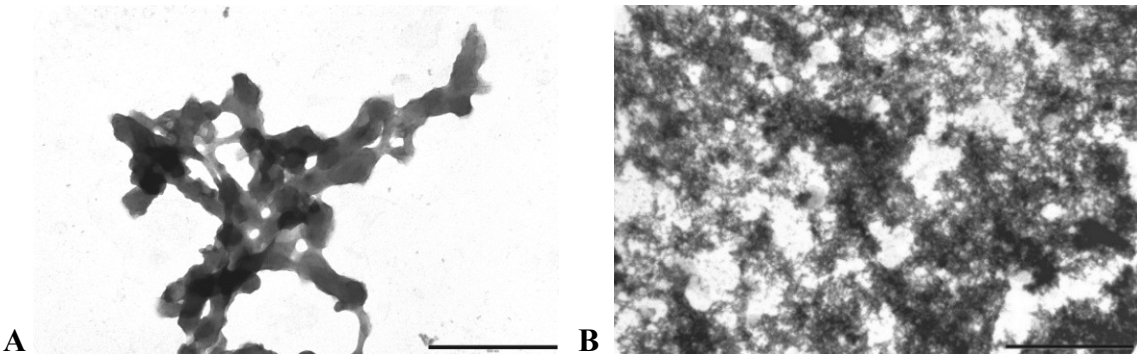


Figure 45. Transmission electron micrographs of PMS-trypsin samples in the absence (A) and presence (B) of grapefruit seed extract diluted 500 times in 60% ethanol at pH 7.0. Protein concentration: 0.13 mg/ml. The scale bar represents 500 nm.

5. Conclusions

The present study can be concluded by the statement that natural product extracts may be efficient anti amyloidogenic agents, as they arrest PMS-trypsin fibrillation in a concentration dependent manner. We summarize the most important findings of our study as follows:

- It has been demonstrated that the peppermint extract could effectively inhibit PMS-trypsin amyloid fibril formation *in vitro*, and the process was concentration dependent of the peppermint extract. Our findings revealed that the anti-amyloidogenic activities of herb extracts might be related to their total phenolic contents. According to our experiments peppermint extract might serve as a valuable source of beneficial phenolic compounds for the prevention of protein aggregation.
- Our study demonstrated that the chili cayenne extract got the highest inhibitory effect on the formation of amyloid fibrils among all the spices investigated. The findings also suggest that the bioactive compounds in the chili extract may be a potential therapeutic drug candidate for amyloid related and neurodegenerative diseases including Alzheimer's disease. This study concludes that the possible nutritional importance of kitchen spices in the food system and the inhibitory effects of the chili extract clearly indicate that they could be used as a pharmacological agent to inhibit or retard amyloid fibril formation in Alzheimer's disease.
- The present study revealed that the *P. ginseng* extract is an efficient anti-amyloidogenic agent against PMS-trypsin fibrillation in aqueous ethanol at pH 7.0. Our results showed that the presence of the *P. ginseng* extract helped to maintain the native structure of the protein.

- According to our experiments, the degree of inhibition was found to change pro rata with the total concentrations of phenolic compounds of the coffee extracts. It can be concluded that the Eduscho coffee extract may be an effective anti-amyloidogenic agent, as it arrests PMS-trypsin fibrillation in a concentration dependent manner.
- We have successfully shown that the grapefruit seed extract inhibits the aggregation process of PMS-trypsin in 60% ethanol at pH 7.0. The inhibition of PMS-trypsin aggregation positively correlated with increased grapefruit seed extract concentration. Our results indicated, for the first time, that limonin effectively inhibited fibril formation.

6. References

Abedini A, Raleigh DP. A critical assessment of the role of helical intermediates in amyloid formation by natively unfolded proteins and polypeptides. Protein Eng Des Sel 2009;22:453–9.

Ahmad B, Borana MS, Chaudhary AP. Understanding curcumin-induced modulation of protein aggregation. Int J Biol Macromol 2017;100:89–96.

Alexa E, Danciu C, Radulov I, Obistioiu D, Sumalan RM, Morar A, Dehelean CA. Phytochemical screening and biologica activity of *Mentha×piperita* L. and *Lavandula angustifolia* Mill. extracts. Anal Cell Pathol (Amst) 2018;2018:2678924.

Alonso E, Vale C, Vieytes MR, Botana LM. Translocation of PKC by Yessotoxin in an in Vitro Model of Alzheimer's Disease with Improvement of Tau and β-Amyloid Pathology. ACS Chem Neurosci 2013;4:1062–70.

Alzheimer's Association. 2012 Alzheimer's disease facts and figures. Alzheimer's Dement 2012;8:131–68.

Ansari MA, Abdul HM, Joshi G, Opii WO, Butterfield DA. Protective effect of quercetin in primary neurons against Abeta(1-42): relevance to Alzheimer's disease. J Nutr Biochem 2009;20:269–75.

Apetz N, Munch G, Govindaraghavan S, Gyengesi E. Natural compounds and plant extracts as therapeutics against chronic inflammation in Alzheimer's disease--a translational perspective. CNS Neurol Disord Drug Targets 2014;13:1175–91.

Ardah MT, Paleologou KE, Lv G, Abul Khair SB, Kazim AS, Minhas ST, et al. Structure activity relationship of phenolic acid inhibitors of α-synuclein fibril formation and toxicity. Front Aging Neurosci 2014;6:197.

Ardah MT, Paleologou KE, Lv G, Menon SA, Abul Khair SB, Lu J-H, et al. Ginsenoside Rb1 inhibits fibrillation and toxicity of alpha-synuclein and disaggregates preformed fibrils. Neurobiol Dis 2015;74:89–101.

Ardito F, Giuliani M, Perrone D, Troiano G, Lo Muzio L. The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review). Int J Mol Med 2017;40:271–80.

Arendash GW, Mori T, Cao C, Mamcarz M, Runfeldt M, Dickson A, et al. Caffeine Reverses Cognitive Impairment and Decreases Brain Amyloid-β Levels in Aged Alzheimer's Disease Mice. J Alzheimer's Dis 2009;17:661–80.

Arendash GW, Cao C. Caffeine and coffee as therapeutics against Alzheimer's disease. J Alzheimers Dis 2010;20 Suppl 1:S117-26.

Arntzen KA, Schirmer H, Wilsgaard T, Mathiesen EB. Moderate wine consumption is associated with better cognitive test results: a 7 year follow up of 5033 subjects in the Tromsø Study. Acta Neurol Scand 2010;122:23–9.

Arrighi HM, Neumann PJ, Lieberburg IM, Townsend RJ. Lethality of Alzheimer Disease and Its Impact on Nursing Home Placement. Alzheimer Dis Assoc Disord 2010;24:90–5.

Atri A. Effective pharmacological management of Alzheimer's disease. Am J Manag Care 2011;17 Suppl 13:S346-55.

Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. Biochem Pharmacol 1999;58:1685–93.

Awasthi S, Saraswathi NT. Vanillin restrains non-enzymatic glycation and aggregation of albumin by chemical chaperone like function. Int J Biol Macromol 2016;87:1–6.

Bagli E, Goussia A, Moschos MM, Agnantis N, Kitsos G. Natural Compounds and Neuroprotection: Mechanisms of Action and Novel Delivery Systems. In Vivo 2016;30:535–47.

Bajer T, Bajerová P, Kremr D, Eisner A, Ventura K. Central composite design of pressurised hot water extraction process for extracting capsaicinoids from chili peppers. J Food Compos Anal 2015;40:32–8.

Bakota EL, Winkler-Moser JK, Berhow MA, Eller FJ, Vaughn SF. Antioxidant activity and sensory evaluation of a rosmarinic acid-enriched extract of *Salvia officinalis*. J Food Sci 2015;80:C711–7.

Ballatore C, Lee VM-Y, Trojanowski JQ. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. Nat Rev Neurosci 2007;8:663–72.

Barbero GF, Ruiz AG, Liazid A, Palma M, Vera JC, Barroso CG. Evolution of total and individual capsaicinoids in peppers during ripening of the Cayenne pepper plant (Capsicum annuum L.). Food Chem 2014;153:200–6.

Barrow CJ, Yasuda A, Kenny PT, Zagorski MG. Solution conformations and aggregational properties of synthetic amyloid beta-peptides of Alzheimer's disease. Analysis of circular dichroism spectra. J Mol Biol 1992;225:1075–93.

Baum L, Ng A. Curcumin interaction with copper and iron suggests one possible mechanism of action in Alzheimer's disease animal models. J Alzheimers Dis 2004;6:367-77-9.

Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 2006;5:493–506.

Bentahir M, Nyabi O, Verhamme J, Tolia A, Horre K, Wiltfang J, et al. Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. J Neurochem 2006;96:732–42.

Berhanu WM, Masunov AE. Atomistic mechanism of polyphenol amyloid aggregation inhibitors: molecular dynamics study of Curcumin, Exifone, and Myricetin interaction with the segment of tau peptide oligomer. J Biomol Struct Dyn 2015;33:1399–411.

Berthelot K, Cullin C, Lecomte S. What does make an amyloid toxic: Morphology, structure or interaction with membrane? Biochimie 2013;95:12–9.

Bhat WF, Bhat SA, Bano B. Evaluation of polyphenols as possible therapeutics for amyloidoses: Comparative analysis of Kaempferol and Catechin. Int J Biol Macromol 2015;81:60–8.

Bhat SA, Bano B. Conformational behaviour and aggregation of chickpea cystatin in trifluoroethanol: Effects of epicatechin and tannic acid. Arch Biochem Biophys 2014;562:51–61.

Bhattacharya S, Pandey NK, Roy A, Dasgupta S. Effect of (−)-epigallocatechin gallate on the fibrillation of human serum albumin. Int J Biol Macromol 2014;70:312–9.

Bieschke J. Natural Compounds May Open New Routes to Treatment of Amyloid Diseases. Neurotherapeutics 2013;10:429–39.

Bieschke J, Russ J, Friedrich RP, Ehrnhoefer DE, Wobst H, Neugebauer K, et al. EGCG remodels mature alpha-synuclein and amyloid-beta fibrils and reduces cellular toxicity. Proc Natl Acad Sci U S A 2010;107:7710–5.

Blancas-Mejía LM, Misra P, Ramirez-Alvarado M. Differences in Protein Concentration Dependence for Nucleation and Elongation in Light Chain Amyloid Formation. Biochemistry 2017;56(5):757–66.

Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. Lancet. 2006;368(9533):387–403.

Brahmachari G. Handbook of pharmaceutical natural products. Wiley-VCH; 2010.

Butt MS, Sultan MT. Coffee and its consumption: benefits and risks. Crit Rev Food Sci Nutr 2011;51:363–73.

Campioni S, Mannini B, Zampagni M, Pensalfini A, Parrini C, Evangelisti E, et al. A causative link between the structure of aberrant protein oligomers and their toxicity. Nat Chem Biol 2010;6:140–7.

Cao P, Raleigh DP. Analysis of the inhibition and remodeling of islet amyloid polypeptide amyloid fibers by flavanols. Biochemistry 2012;51:2670–83.

Cappai R, Barnham KJ. Delineating the Mechanism of Alzheimer's Disease Aβ Peptide Neurotoxicity. Neurochem Res 2008;33:526–32.

Caspi S, Halimi M, Yanai A, Sasson SB, Taraboulos A, Gabizon R. The anti-prion activity of Congo red. Putative mechanism. J Biol Chem 1998;273:3484–9.

Caughey B, Lansbury PT. Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. Annu Rev Neurosci 2003;26:267–98.

Chaturvedi SK, Alam P, Khan JM, Siddiqui MK, Kalaiarasan P, Subbarao N, Ahmad Z, Khan RH. Biophysical insight into the anti-amyloidogenic behavior of taurine. Int J Biol Macromol 2015;80:375–84.

Chaturvedi SK, Khan JM, Siddiqi MK, Alam P, Khan RH. Comparative insight into surfactants mediated amyloidogenesis of lysozyme. Int J Biol Macromol 2016;83:315–25.

Chen L, Lin Z, Zhu Y, Lin N, Zhang J, Pan X, et al. Ginsenoside Rg1 attenuates β-amyloid generation via suppressing PPARγ-regulated BACE1 activity in N2a-APP695 cells. Eur J Pharmacol 2012;675:15–21.

Chen F, Wang Y, Yang M, Yin J, Meng Q, Bu F, et al. Interaction of the ginsenosides with κ-casein and their effects on amyloid fibril formation by the protein: Multi-spectroscopic approaches. J Photochem Photobiol B Biol 2016;160:306–17.

Chen J, Armstrong AH, Koehler AN, Hecht MH. Small molecule microarrays enable the discovery of compounds that bind the Alzheimer's Aβ peptide and reduce its cytotoxicity. J Am Chem Soc 2010;132:17015–22.

Cheng B, Gong H, Xiao H, Petersen RB, Zheng L, Huang K. Inhibiting toxic aggregation of amyloidogenic proteins: A therapeutic strategy for protein misfolding diseases. Biochim Biophys Acta - Gen Subj 2013;1830:4860–71.

Cheng B, Liu X, Gong H, Huang L, Chen H, Zhang X, et al. Coffee components inhibit amyloid formation of human islet amyloid polypeptide in vitro: possible link between coffee consumption and diabetes mellitus. J Agric Food Chem 2011;59:13147–55.

Chimon S, Shaibat MA, Jones CR, Calero DC, Aizezi B, Ishii Y. Evidence of fibril-like β-sheet structures in a neurotoxic amyloid intermediate of Alzheimer's β-amyloid. Nat Struct Mol Biol 2007;14:1157–64.

Chiti F, Dobson CM. Protein Misfolding, Functional Amyloid, and Human Disease. Annu Rev Biochem 2006;75:333–66.

Chiti F, Dobson CM. Protein Misfolding, Functional Amyloid, and Human Disease: A Summary of Progress Over the Last Decade. Annu Rev Biochem 2017;86:27–68.

Chiti F, Webster P, Taddei N, Clark A, Stefani M, Ramponi G, et al. Designing conditions for in vitro formation of amyloid protofilaments and fibrils. Proc Natl Acad Sci U S A 1999;96:3590–4.

Chonpathompikunlert P, Wattanathorn J, Muchimapura S. Piperine, the main alkaloid of Thai black pepper, protects against neurodegeneration and cognitive impairment in animal model of cognitive deficit like condition of Alzheimer's disease. Food Chem Toxicol 2010;48:798–802.

Chromy BA, Nowak RJ, Lambert MP, Viola KL, Chang L, Velasco PT, et al. Self-assembly of Abeta(1-42) into globular neurotoxins. Biochemistry 2003;42:12749–60.

Citron M. Alzheimer's disease: strategies for disease modification. Nat Rev Drug Discov 2010;9:387–98.

Clamp M, Fry B, Kamal M, Xie X, Cuff J, Lin MF, Kellis M, Lindblad-Toh K, Lander ES. Distinguishing protein-coding and noncoding genes in the human genome. Proc Natl Acad Sci U S A 2007;104:19428–33.

Cleary JP, Walsh DM, Hofmeister JJ, Shankar GM, Kuskowski MA, Selkoe DJ, et al. Natural oligomers of the amyloid-β protein specifically disrupt cognitive function. Nat Neurosci 2005;8:79–84.

Conway KA, Rochet JC, Bieganski RM, Lansbury PT. Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. Science 2001;294:1346–9.

Cornejo A, Aguilar Sandoval F, Caballero L, Machuca L, Muñoz P, Caballero J, et al. Rosmarinic acid prevents fibrillization and diminishes vibrational modes associated to β sheet in tau protein linked to Alzheimer's disease. J Enzyme Inhib Med Chem 2017;32:945–53.

Cvetnić Z, Vladimir-Knezević S. Antimicrobial activity of grapefruit seed and pulp ethanolic extract. Acta Pharm 2004;54:243–250.

Daccache A, Lion C, Sibille N, Gerard M, Slomianny C, Lippens G, et al. Oleuropein and derivatives from olives as Tau aggregation inhibitors. Neurochem Int 2011;58:700–7.

D'Avola D, López-Franco E, Sangro B, Pañeda A, Grossios N, Gil-Farina I, et al. Phase I open label liver-directed gene therapy clinical trial for acute intermittent porphyria. J Hepatol 2016;65:776–83.

Dill KA, Ozkan SB, Shell MS, Weikl TR. The Protein Folding Problem. Annu Rev Biophys 2008;37:289–316.

Dobson CM. Protein folding and misfolding. Nature 2003;426:884–90.

Dobson CM. The Amyloid Phenomenon and Its Links with Human Disease. Cold Spring Harb Perspect Biol 2017;9:a023648.

Dorta-Estremera SM, Li J, Cao W. Rapid generation of amyloid from native proteins in vitro. J Vis Exp 2013:50869.

Durairajan SSK, Liu L-F, Lu J-H, Chen L-L, Yuan Q, Chung SK, et al. Berberine ameliorates β-amyloid pathology, gliosis, and cognitive impairment in an Alzheimer's disease transgenic mouse model. Neurobiol Aging 2012;33:2903–19.

Eisenberg D, Jucker M. The amyloid state of proteins in human diseases. Cell 2012;148(6):1188–203.

Ehrnhoefer DE, Bieschke J, Boeddrich A, Herbst M, Masino L, Lurz R, et al. EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. Nat Struct Mol Biol 2008;15:558–66.

El-Agnaf OMA, Mahil DS, Patel BP, Austen BM. Oligomerization and Toxicity of β-Amyloid-42 Implicated in Alzheimer's Disease. Biochem Biophys Res Commun 2000;273:1003–7.

Engel MFM, vandenAkker CC, Schleeger M, Velikov KP, Koenderink GH, Bonn M. The polyphenol EGCG inhibits amyloid formation less efficiently at phospholipid interfaces than in bulk solution. J Am Chem Soc 2012;134:14781–8.

Eskelinen MH, Ngandu T, Tuomilehto J, Soininen H, Kivipelto M. Midlife coffee and tea drinking and the risk of late-life dementia: a population-based CAIDE study. J Alzheimer's Dis 2009;16:85–91.

Esler WP, Stimson ER, Ghilardi JR, Felix AM, Lu Y-A, Vinters H V., et al. Aβ deposition inhibitor screen using synthetic amyloid. Nat Biotechnol 1997;15:258–63.

Essa MM, Vijayan RK, Castellano-Gonzalez G, Memon MA, Braidy N, Guillemin GJ. Neuroprotective effect of natural products against Alzheimer's disease. Neurochem Res 2012;37:1829–42.

Evers F, Jeworrek C, Tiemeyer S, Weise K, Sellin D, Paulus M, et al. Elucidating the mechanism of lipid membrane-induced IAPP fibrillogenesis and its inhibition by the red wine compound resveratrol: a synchrotron X-ray reflectivity study. J Am Chem Soc 2009;131:9516–21.

Fändrich M. Oligomeric intermediates in amyloid formation: structure determination and mechanisms of toxicity. J Mol Biol 2012;421:427–40.

Fazili NA, Naeem A. Anti-fibrillation potency of caffeic acid against an antidepressant induced fibrillogenesis of human α-synuclein: Implications for Parkinson's disease. Biochimie 2015;108:178–85.

Feng Y, Wang X, Yang S, Wang Y, Zhang X, Du X, et al. Resveratrol inhibits beta-amyloid oligomeric cytotoxicity but does not prevent oligomer formation. Neurotoxicology 2009;30:986–95.

Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, et al. Global prevalence of dementia: a Delphi consensus study. Lancet (London, England) 2005;366:2112–7.

Fratiglioni L, Wang H-X, Ericsson K, Maytan M, Winblad B. Influence of social network on occurrence of dementia: a community-based longitudinal study. Lancet 2000;355:1315–9.

Frid P, Anisimov S V., Popovic N. Congo red and protein aggregation in neurodegenerative diseases. Brain Res Rev 2007;53:135–60.

Fuentes AL, Hennessy K, Pascual J, Pepe N, Wang I, Santiago A, Chaggan C, Martinez J, Rivera E, Cota P, Cunha C, Nogaj LA, Moffet DA. Identification of plant extracts that inhibit the formation of diabetes-linked IAPP amyloid. J Herb Med 2016;6:37–41.

Furkan M, Rizvi A, Afsar M, Ajmal MR, Khan RH, Naeem A. In vitro elucidation of the folding intermediates and aggregate formation of hemoglobin induced by acetonitrile: A multispectroscopic approach. Protein and Peptide Letters 2016;23(10):884–91.

Gandy S. The role of cerebral amyloid β accumulation in common forms of Alzheimer disease. J Clin Invest 2005;115:1121–9.

Gazova Z, Siposova K, Kurin E, Mučaji P, Nagy M. Amyloid aggregation of lysozyme: The synergy study of red wine polyphenols. Proteins Struct Funct Bioinforma 2013;81:994–1004.

George RC, Lew J, Graves DJ. Interaction of cinnamaldehyde and epicatechin with tau: implications of beneficial effects in modulating Alzheimer's disease pathogenesis. J Alzheimers Dis 2013;36:21–40.

Ghahghaei A, Bathaie SZ, Kheirkhah H, Bahraminejad E. The protective effect of crocin on the amyloid fibril formation of Aβ42 peptide in vitro. Cell Mol Biol Lett 2013;18:328–39.

Ghosh S, Pandey NK, Dasgupta S. (-)-Epicatechin gallate prevents alkali-salt mediated fibrillogenesis of hen egg white lysozyme. Int J Biol Macromol 2013;54:90–8.

Goate A, Chartier-Harlin M-C, Mullan M, Brown J, Crawford F, Fidani L, et al. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 1991;349:704–6.

Golde TE, Eckman CB, Younkin SG. Biochemical detection of Abeta isoforms: implications for pathogenesis, diagnosis, and treatment of Alzheimer's disease. Biochim Biophys Acta 2000;1502:172–87.

Götz J, Streffer JR, David D, Schild A, Hoerndli F, Pennanen L, et al. Transgenic animal models of Alzheimer's disease and related disorders: histopathology, behavior and therapy. Mol Psychiatry 2004;9:664–83.

Gras SL, Waddington LJ, Goldie KN. Transmission electron microscopy of amyloid fibrils. Methods Mol Biol 2011;752:197–214.

Groenning M. Binding mode of Thioflavin T and other molecular probes in the context of amyloid fibrils—current status. J Chem Biol 2010;3:1–18.

Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β-peptide. Nat Rev Mol Cell Biol 2007;8:101–12.

Hardy J. Amyloid, the presenilins and Alzheimer's disease. Trends Neurosci 1997;20:154–9.

Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 2002;297:353–6.

Harvey A. Natural products in drug discovery. Drug Discov Today 2008;13:894–901.

Hawkes CA, Ng V, McLaurin J. Small molecule inhibitors of Aβ-aggregation and neurotoxicity. Drug Dev Res 2009;70:111–24.

Hendrick JP, Hartl FU. The role of molecular chaperones in protein folding. FASEB J 1995;9(15):1559–69.

Heo HJ, Lee CY. Epicatechin and catechin in cocoa inhibit amyloid beta protein induced apoptosis. J Agric Food Chem 2005;53(5):1445–8.

Hill AF, Desbruslais M, Joiner S, Sidle KCL, Gowland I, Collinge J, et al. The same prion strain causes vCJD and BSE. Nature 1997;389:448–50.

Hippius H, Neundörfer G. The discovery of Alzheimer's disease. Dialogues Clin Neurosci 2003;5:101–8.

Holtzman DM, Morris JC, Goate AM. Alzheimer's Disease: The Challenge of the Second Century. Sci Transl Med 2011;3:77sr1-77sr1.

Hong DP, Fink AL, Uversky VN. Structural characteristics of alpha-synuclein oligomers stabilized by the flavonoid baicalein. J Mol Biol 2008;383:214–23.

Hossain MB, Patras A, Barry-Ryan C, Martin-Diana AB, Brunton NP. Application of principal component and hierarchical cluster analysis to classify different spices based on in vitro antioxidant activity and individual polyphenolic antioxidant compounds. J Funct Foods 2011;3:179–89.

Houghton PJ, Howes MJ. Natural products and derivatives affecting neurotransmission relevant to Alzheimer's and Parkinson's disease. Neurosignals 2005;14:6–22.

Howes MJ, Houghton PJ. Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. Pharmacol Biochem Behav 2003;75:513–27.

Howes MJ, Perry NSL, Houghton PJ. Plants with traditional uses and activities, relevant to the management of Alzheimer's disease and other cognitive disorders. Phyther Res 2003;17:1–18.

Hu H-Y. Editorial: Structural Aspects of Protein Aggregation. Protein Pept Lett 2017;24:280–280.

Hubbard RE. Structure-based drug discovery and protein targets in the CNS. Neuropharmacology 2011;60:7–23.

Iannuzzi C, Irace G, Sirangelo I. Differential effects of glycation on protein aggregation and amyloid formation. Front Mol Biosci 2014;1: 9.

Iriti M, Vitalini S, Fico G, Faoro F. Neuroprotective herbs and foods from different traditional medicines and diets. Molecules 2010;15:3517–55.

Ishii K, Klunk WE, Arawaka S, Debnath ML, Furiya Y, Sahara N, et al. Chrysamine G and its derivative reduce amyloid beta-induced neurotoxicity in mice. Neurosci Lett 2002;333:5–8.

Ittner LM, Götz J. Amyloid-β and tau — a toxic pas de deux in Alzheimer's disease. Nat Rev Neurosci 2011;12:67–72.

Jayamani J, Shanmugam G. Gallic acid, one of the components in many plant tissues, is a potential inhibitor for insulin amyloid fibril formation. Eur J Med Chem 2014;85:352–8.

Jeon WJ, Oh JS, Park MS, Ji GE. Anti-Hyperglycemic Effect of Fermented Ginseng in Type 2 Diabetes Mellitus Mouse Model. Phyther Res 2013;27:166–72.

Jha NN, Ghosh D, Das S, Anoop A, Jacob RS, Singh PK, et al. Effect of curcumin analogs onα-synuclein aggregation and cytotoxicity. Sci Rep 2016;6:28511.

Jung JW, Kang HR, Ji GE, Park MS, Song WJ, Kim MH, et al. Therapeutic effects of fermented red ginseng in allergic rhinitis: a randomized, double-blind, placebo-controlled study. Allergy Asthma Immunol Res 2011;3:103–10.

Justesen U, Knuthsen P. Composition of flavonoids in fresh herbs and calculation of flavonoid intake by use of herbs in traditional Danish dishes. Food Chem 2001;73:245–50.

Karunaweera N, Raju R, Gyengesi E, Münch G. Plant polyphenols as inhibitors of NF-κB induced cytokine production-a potential anti-inflammatory treatment for Alzheimer's disease? Front Mol Neurosci 2015;8:24.

Kayed R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, et al. Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. Science 2003;300:486–9.

Khan E, Mishra SK, Kumar A. Emerging Methods for Structural Analysis of Protein Aggregation. Protein Pept Lett 2017;24(4):331–9.

Kim HJ, Kim P, Shin CY. A comprehensive review of the therapeutic and pharmacological effects of ginseng and ginsenosides in central nervous system. J Ginseng Res 2013;37:8–29.

Kim J, Kim SH, Lee D-S, Lee D-J, Kim S-H, Chung S, et al. Effects of fermented ginseng on memory impairment and β-amyloid reduction in Alzheimer's disease experimental models. J Ginseng Res 2013;37:100–7.

Kim W, Kim Y, Min J, Kim DJ, Chang Y-T, Hecht MH. A high-throughput screen for compounds that inhibit aggregation of the Alzheimer's peptide. ACS Chem Biol 2006;1:461–9.

Klunk WE, Pettegrew JW, Abraham DJ. Quantitative evaluation of congo red binding to

amyloid-like proteins with a beta-pleated sheet conformation. J Histochem Cytochem 1989;37:1273–81.

Knight JD, Hebda JA, Miranker AD. Conserved and cooperative assembly of membrane-bound alpha-helical states of islet amyloid polypeptide. Biochemistry. 2006;45(31):9496-508.

Kodali R, Wetzel R. Polymorphism in the intermediates and products of amyloid assembly. Curr Opin Struct Biol 2007;17:48–57.

Kotormán M, Simon LM, Borics A, Szabó MR, Szabó K, Szögi T, Fülöp L. Amyloid-like fibril formation by trypsin in aqueous ethanol. Inhibition of fibrillation by PEG. Protein Pept Lett 2015;22(12):1104–10.

Kotormán M, Kasi PB, Halász L, Borics A. Inhibition of amyloid-like fibril formation of trypsin by red wines. Protein Pept Lett 2017;24(5):466–70.

Kotormán M, Kelemen Z, Kasi PB, Nemcsók J. Inhibition of the formation of amyloid-like fibrils using herbal extracts. Acta Biol Hung 2018;69(2):125–34.

Kumar-Singh S, Theuns J, Van Broeck B, Pirici D, Vennekens K, Corsmit E, et al. Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Aβ42 and decreased Aβ40. Hum Mutat 2006;27:686–95.

Kwee EM, Niemeyer ED. Variations in phenolic composition and antioxidant properties among 15 basil (Ocimum basilicum L.) cultivars. Food Chem 2011;128:1044–50.

Lakey-Beitia J, Berrocal R, Rao KS, Durant AA. Polyphenols as therapeutic molecules in Alzheimer's disease through modulating amyloid pathways. Mol Neurobiol 2015;51:466–79.

Lakey-Beitia J, Doens D, Jagadeesh Kumar D, Murillo E, Fernandez PL, Rao KS, Durant-Archibold AA. Anti-amyloid aggregation activity of novel carotenoids: implications for Alzheimer's drug discovery. Clin Interv Aging 2017;12:815–822.

Landau M, Sawaya MR, Faull KF, Laganowsky A, Jiang L, Sievers SA, et al. Towards a pharmacophore for amyloid. PLoS Biol 2011;9:e1001080.

Lan X, Wang W, Li Q, Wang J. The Natural Flavonoid Pinocembrin: Molecular Targets and Potential Therapeutic Applications. Mol Neurobiol 2016;53:1794–801.

Lee CW, Won TJ, Kim HR, Lee DH, Hwang KW, Park SY. Protective Effect of Chlorogenic Acid against Aβ-Induced Neurotoxicity. Biomol Ther 2011;19:181–6.

Lemkul JA, Bevan DR. The role of molecular simulations in the development of inhibitors of amyloid β-peptide aggregation for the treatment of Alzheimer's disease. ACS Chem Neurosci 2012;3:845–56.

Lemkul JA, Bevan DR. Morin inhibits the early stages of amyloid β-peptide aggregation by altering tertiary and quaternary interactions to produce "off-pathway" structures. Biochemistry 2012;51:5990–6009.

Lesné S, Koh MT, Kotilinek L, Kayed R, Glabe CG, Yang A, et al. A specific amyloid-β protein assembly in the brain impairs memory. Nature 2006;440:352–7.

Levine H. Small molecule inhibitors of Abeta assembly. Amyloid 2007;14:185–97.

Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, et al. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science 1995;269:973–7.

Li J, Zhu M, Manning-Bog AB, Di Monte DA, Fink AL. Dopamine and L-dopa disaggregate amyloid fibrils: implications for Parkinson's and Alzheimer's disease. FASEB J 2004;18:962–4.

Li W, Sperry JB, Crowe A, Trojanowski JQ, Smith III AB, Lee VM-Y. Inhibition of tau fibrillization by oleocanthal via reaction with the amino groups of tau. J Neurochem 2009;110:1339–51.

Liu J, Chen F, Yin J, Bu F, Zheng B, Yang M, Wang Y, Sun D, Meng Q. The effects of ginsenosides to amyloid fibril formation by RCMκ-casein. Int J Biol Macromol 2015;78:49–55.

Liu R, Meng F, Zhang L, Liu A, Qin H, Lan X, Li L, Du G. Luteolin isolated from the medicinal plant Elsholtzia rugulosa (Labiatae) prevents copper-mediated toxicity in β-amyloid precursor protein Swedish mutation overexpressing SH-SY5Y cells. Molecules 2011;16(3):2084–96.

Liu R, Wu C, Zhou D, Yang F, Tian S, Zhang L, et al. Pinocembrin protects against β-amyloid-induced toxicity in neurons through inhibiting receptor for advanced glycation end products (RAGE)-independent signaling pathways and regulating mitochondrion-mediated apoptosis. BMC Med 2012;10:105.

Lopez del Amo JM, Fink U, Dasari M, Grelle G, Wanker EE, Bieschke J, et al. Structural properties of EGCG-induced, nontoxic Alzheimer's disease Aβ oligomers. J Mol Biol 2012;421:517–24.

Luchsinger JA, Mayeux R. Dietary factors and Alzheimer's disease. Lancet Neurol 2004;3:579–87.

Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y, Sue L, et al. Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am J Pathol 1999;155:853–62.

Ma B, Zhang F, Liu Y, Xie J, Wang X. Resveratrol Induces the Conversion from Amyloid to Amorphous Aggregation of β-lactoglobulin. Protein Pept Lett 2018;24(12):1113–9.

Madeira MH, Boia R, Ambrósio AF, Santiago AR. Having a Coffee Break: The Impact of Caffeine Consumption on Microglia-Mediated Inflammation in Neurodegenerative Diseases. Mediators Inflamm 2017;2017:1–12.

Mahdavimehr M, Meratan AA, Ghobeh M, Ghasemi A, Saboury AA, Nemat-Gorgani M. Inhibition of HEWL fibril formation by taxifolin: Mechanism of action. PLoS One 2017;12(11):e0187841.

Makowska J, Szczesny D, Lichucka A, Giełdoń A, Chmurzyński L, Kaliszan R. Preliminary studies on trigonelline as potential anti-Alzheimer disease agent: Determination by hydrophilic interaction liquid chromatography and modeling of interactions with beta-amyloid. J Chromatogr B 2014;968:101–4.

Marambaud P, Zhao H, Davies P. Resveratrol Promotes Clearance of Alzheimer's Disease Amyloid-β Peptides. J Biol Chem 2005;280:37377–82.

Martino E, Ramaiola I, Urbano M, Bracco F, Collina S. Microwave-assisted extraction of coumarin and related compounds from Melilotus officinalis (L.) Pallas as an alternative to Soxhlet and ultrasound-assisted extraction. J Chromatogr A 2006;1125:147–51.

Martins IC, Kuperstein I, Wilkinson H, Maes E, Vanbrabant M, Jonckheere W, et al. Lipids revert inert Abeta amyloid fibrils to neurotoxic protofibrils that affect learning in mice. EMBO J 2008;27:224–33.

Mayeux R, Stern Y. Epidemiology of Alzheimer disease. Cold Spring Harb Perspect Med 2012;2.

Mazzulli JR, Burbulla LF, Krainc D, Ischiropoulos H. Detection of Free and Protein-Bound *ortho*-Quinones by Near-Infrared Fluorescence. Anal Chem 2016;88:2399–405.

Meng X, Munishkina LA, Fink AL, Uversky VN. Molecular mechanisms underlying the flavonoid-induced inhibition of α-synuclein fibrillation. Biochemistry 2009;48:8206–24.

Menichini F, Tundis R, Bonesi M, Loizzo ML, Conforti F, Statti G, De Cindio B, Houghton PJ, Menichini F. The influence of fruit ripening on the phytochemical content and biological activity of Capsicum chinense Jacq. cv Habanero. Food Chem 2009;114:553–60.

Mirmosayyeb O, Tanhaei A, Sohrabi H, Martins R, Tanhaei M, Najafi M, et al. Possible role of common spices as a preventive and therapeutic agent for Alzheimer′s disease. Int J Prev Med 2017;8:5.

Misharina TA. Antiradical properties of essential oils and extracts from coriander, cardamom, white, red, and black peppers. Appl Biochem Microbiol 2016;52:79–86.

Modi KK, Roy A, Brahmachari S, Rangasamy SB, Pahan K. Cinnamon and Its Metabolite Sodium Benzoate Attenuate the Activation of p21rac and Protect Memory and Learning in an Animal Model of Alzheimer's Disease. PLoS One 2015;10:e0130398.

Moongkarndi P, Srisawat C, Saetun P, Jantaravinid J, Peerapittayamongkol C, Soi-ampornkul R, et al. Protective Effect of Mangosteen Extract against β-Amyloid-Induced Cytotoxicity, Oxidative Stress and Altered Proteome in SK-N-SH Cells. J Proteome Res 2010;9:2076–86.

Mosconi L, Murray J, Tsui WH, Li Y, Davies M, Williams S, et al. Mediterranean Diet and Magnetic Resonance Imaging-Assessed Brain Atrophy in Cognitively Normal Individuals at Risk for Alzheimer's Disease. J Prev Alzheimer's Dis 2014;1:23–32.

Na JY, Song K, Lee JW, Kim S, Kwon J. 6-Shogaol has anti-amyloidogenic activity and ameliorates Alzheimer's disease via CysLT1R-mediated inhibition of cathepsin B. Biochem Biophys Res Commun 2016;477:96–102.

Näslund J, Haroutunian V, Mohs R, Davis KL, Davies P, Greengard P, et al. Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. JAMA 2000;283:1571–7.

Necula M, Kayed R, Milton S, Glabe CG. Small molecule inhibitors of aggregation indicate that amyloid beta oligomerization and fibrillization pathways are independent and distinct. J Biol Chem 2007;282:10311–24.

Nedumpully-Govindan P, Kakinen A, Pilkington EH, Davis TP, Chun Ke P, Ding F. Stabilizing Off-pathway Oligomers by Polyphenol Nanoassemblies for IAPP Aggregation Inhibition. Sci Rep 2016;6:19463.

Nelson KM, Dahlin JL, Bisson J, Graham J, Pauli GF, Walters MA. The Essential Medicinal Chemistry of Curcumin. J Med Chem 2017;60:1620–37.

Nelson R, Sawaya MR, Balbirnie M, Madsen AØ, Riekel C, Grothe R, et al. Structure of the cross-β spine of amyloid-like fibrils. Nature 2005;435:773–8.

Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 2012;75:311–35.

Ng YP, Or TCT, Ip NY. Plant alkaloids as drug leads for Alzheimer's disease. Neurochem Int 2015;89:260–70.

Ngoungoure VLN, Schluesener J, Moundipa PF, Schluesener H. Natural polyphenols binding to amyloid: A broad class of compounds to treat different human amyloid diseases. Mol Nutr Food Res 2015;59:8–20.

Nilsson MR. Techniques to study amyloid fibril formation in vitro. Methods. 2004;34(1):151–60.

Nizhnikov AA, Alexandrov AI, Ryzhova TA, Mitkevich OV, Dergalev AA, Ter-Avanesyan MD, Galkin AP. Proteomic Screening for Amyloid Proteins. PLoS One. 2014;9(12):e116003.

Ochiai R, Sugiura Y, Shioya Y, Otsuka K, Katsuragi Y, Hashiguchi T. Coffee polyphenols improve peripheral endothelial function after glucose loading in healthy male adults. Nutr Res 2014;34:155–9.

Ono K, Hasegawa K, Naiki H, Yamada M. Curcumin has potent anti-amyloidogenic effects for Alzheimer's beta-amyloid fibrils in vitro. J Neurosci Res 2004;75:742–50.

Ono K, Yoshiike Y, Takashima A, Hasegawa K, Naiki H, Yamada M. Vitamin A exhibits potent antiamyloidogenic and fibril-destabilizing effects in vitro. Exp Neurol 2004;189:380–92.

Onozuka H, Nakajima A, Matsuzaki K, Shin RW, Ogino K, Saigusa D, et al. Nobiletin, a citrus flavonoid, improves memory impairment and Abeta pathology in a transgenic mouse model of Alzheimer's disease. J Pharmacol Exp Ther 2008;326:739–44.

Otles S, Yalcin B. Phenolic compounds analysis of root, stalk, and leaves of nettle. ScientificWorldJournal 2012;2012:564367.

Ow SY, Dunstan DE. A brief overview of amyloids and Alzheimer's disease. Protein Sci 2014;23:1315–31.

Pandey N, Strider J, Nolan WC, Yan SX, Galvin JE. Curcumin inhibits aggregation of α-synuclein. Acta Neuropathol 2008;115:479–89.

Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev 2009; 2(5):270–278.

Palhano FL, Lee J, Grimster NP, Kelly JW. Toward the molecular mechanism(s) by which EGCG treatment remodels mature amyloid fibrils. J Am Chem Soc 2013;135:7503–10.

Papandreou MA, Kanakis CD, Polissiou MG, Efthimiopoulos S, Cordopatis P, Margarity M, et al. Inhibitory activity on amyloid-β aggregation and antioxidant properties of *Crocus sativus* stigmas extract and its crocin constituents. J Agric Food Chem 2006;54:8762–8.

Park JW, Lee JC, Ann SR, Seo DW, Choi WS, Yoo YH, et al. A Fermented Ginseng Extract, BST204, Inhibits Proliferation and Motility of Human Colon Cancer Cells. Biomol Ther 2011;19:211–7.

Peng L, Sun S, Xie LH, Wicks SM, Xie JT. Ginsenoside Re: Pharmacological Effects on Cardiovascular System. Cardiovasc Ther 2012;30:e183–8.

Pepys MB. Amyloidosis. Annu Rev Med 2006;57:223–41.

Petkova AT, Ishii Y, Balbach JJ, Antzutkin ON, Leapman RD, Delaglio F, et al. A structural model for Alzheimer's beta -amyloid fibrils based on experimental constraints from solid state NMR. Proc Natl Acad Sci U S A 2002;99:16742–7.

Petkova AT, Leapman RD, Guo Z, Yau WM, Mattson MP, Tycko R. Self-propagating, molecular-level polymorphism in Alzheimer's beta-amyloid fibrils. Science 2005;307(5707):262–5.

Petkova AT, Yau WM, Tycko R. Experimental constraints on quaternary structure in Alzheimer's beta-amyloid fibrils. Biochemistry 2006;45(2):498–512.

Peterson DW, George RC, Scaramozzino F, LaPointe NE, Anderson RA, Graves DJ, et al. Cinnamon extract inhibits tau aggregation associated with Alzheimer's disease in vitro. J Alzheimer's Dis 2009;17:585–97.

Pithadia A, Brender JR, Fierke CA, Ramamoorthy A. Inhibition of IAPP Aggregation and Toxicity by Natural Products and Derivatives. J Diabetes Res 2016;2016:1–12.

Popovych N, Brender JR, Soong R, Vivekanandan S, Hartman K, Basrur V, et al. Site specific interaction of the polyphenol EGCG with the SEVI amyloid precursor peptide PAP(248–286). J Phys Chem B 2012;116:3650–8.

Porat Y, Abramowitz A, Gazit E. Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism. Chem Biol Drug Des 2006;67:27–37.

Porzoor A, Alford B, Hügel H, Grando D, Caine J, Macreadie I. Anti-amyloidogenic properties of some phenolic compounds. Biomolecules 2015;5:505–27.

Pourshahidi LK, Navarini L, Petracco M, Strain JJ. A Comprehensive Overview of the Risks and Benefits of Coffee Consumption. Compr Rev Food Sci Food Saf 2016;15:671–84.

Raimondi D, Orlando G, Pancsa R, Khan T, Vranken WF. Exploring the Sequence-based Prediction of Folding Initiation Sites in Proteins. Sci Rep 2017;7(1):8826.

Ramazzotti M, Melani F, Marchi L, Mulinacci N, Gestri S, Tiribilli B, et al. Mechanisms for the inhibition of amyloid aggregation by small ligands 2016;0:1–14.

Ramshini H, Ebrahim-Habibi A, Aryanejad S, Rad A. Effect of Cinnamomum Verum Extract on the Amyloid Formation of Hen Egg-white Lysozyme and Study of its Possible Role in Alzheimer's Disease. Basic Clin Neurosci 2015;6:29–37.

Rao PPN, Mohamed T, Teckwani K, Tin G. Curcumin Binding to Beta Amyloid: A Computational Study. Chem Biol Drug Des 2015;86:813–20.

Reinke AA, Gestwicki JE. Structure-activity relationships of amyloid beta-aggregation inhibitors based on curcumin: influence of linker length and flexibility. Chem Biol Drug Des 2007;70:206–15.

Rezai-Zadeh K, Arendash GW, Hou H, Fernandez F, Jensen M, Runfeldt M, Shytle RD, Tan J. Green tea epigallocatechin-3-gallate (EGCG) reduces beta-amyloid mediated cognitive impairment and modulates tau pathology in Alzheimer transgenic mice. Brain Res 2008;1214:177–87.

Rigacci S, Guidotti V, Bucciantini M, Parri M, Nediani C, Cerbai E, et al. Oleuropein aglycon prevents cytotoxic amyloid aggregation of human amylin. J Nutr Biochem 2010;21:726–35.

Ryan TM, Friedhuber A, Lind M, Howlett GJ, Masters C, Roberts BR. Small amphipathic molecules modulate secondary structure and amyloid fibril-forming kinetics of Alzheimer disease peptide Aβ(1–42). J Biol Chem 2012;287:16947–54.

Santos GM, Maia GA, Sousa PH, Costa JM, Figueiredo RW, Prado GM. [Correlation between antioxidant activity and bioactive compounds of açaí (*Euterpe oleracea* Mart) comercial pulps]. Arch Latinoam Nutr 2008;58:187–192.

Sarroukh R, Goormaghtigh E, Ruysschaert JM, Raussens V. ATR-FTIR: a "rejuvenated" tool to investigate amyloid proteins. Biochim Biophys Acta 2013;1828(10):2328–38.

Sato M, Murakami K, Uno M, Nakagawa Y, Katayama S, Akagi K, et al. Site-specific inhibitory mechanism for amyloid β42 aggregation by catechol-type flavonoids targeting the Lys residues. J Biol Chem 2013;288:23212–24.

Scarmeas N, Luchsinger JA, Schupf N, Brickman AM, Cosentino S, Tang MX, et al. Physical activity, diet, and risk of Alzheimer disease. JAMA 2009;302:627.

Scarmeas N, Stern Y, Mayeux R, Luchsinger JA. Mediterranean diet, Alzheimer disease, and vascular mediation. Arch Neurol 2006;63:1709.

Scholey AB, Tildesley NTJ, Ballard CG, Wesnes KA, Tasker A, Perry EK, et al. An extract of Salvia (sage) with anticholinesterase properties improves memory and attention in healthy older volunteers. Psychopharmacology (Berl) 2008;198:127–39.

Schwert GW, Takenaka Y. A spectrophotometric determination of trypsin and chymotrypsin. Biochim Biophys Acta 1955;16:570–5.

Scott MR, Will R, Ironside J, Nguyen HO, Tremblay P, DeArmond SJ, et al. Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. Proc Natl Acad Sci U S A 1999;96:15137–42.

Seo JY, Lee JH, Kim NW, Her E, Chang SH, Ko NY, et al. Effect of a fermented ginseng extract, BST204, on the expression of cyclooxygenase-2 in murine macrophages. Int Immunopharmacol 2005;5:929–36.

Seraj Z, Seyedarabi A, Saboury AA, Habibi-Rezaei M, Ahmadian S, Ghasemi A. Unraveling the novel effects of aroma from small molecules in preventing hen egg white lysozyme amyloid fibril formation. PLoS One 2018;13:e0189754.

Sgarbossa A, Antonella. Natural Biomolecules and Protein Aggregation: Emerging Strategies against Amyloidogenesis. Int J Mol Sci 2012;13:17121–37.

Shariatizi S, Meratan AA, Ghasemi A, Nemat-Gorgani M. Inhibition of amyloid fibrillation and cytotoxicity of lysozyme fibrillation products by polyphenols. Int J Biol Macromol 2015;80:95–106.

Sharma B, Kalita S, Paul A, Mandal B, Paul S, Selkoe DJ, et al. The role of caffeine as an inhibitor in the aggregation of amyloid forming peptides: a unified molecular dynamics simulation and experimental study. RSC Adv 2016;6:78548–58.

Shoval H, Lichtenberg D, Gazit E. The molecular mechanisms of the anti-amyloid effects of phenols. Amyloid 2007;14:73–87.

Shukitt-Hale B, Miller MG, Chu YF, Lyle BJ, Joseph JA. Coffee, but not caffeine, has positive effects on cognition and psychomotor behavior in aging. Age (Omaha) 2013;35:2183–92.

Siddiqi MK, Alam P, Chaturvedi SK, Nusrat S, Shahein YE, Khan RH. Attenuation of amyloid fibrillation in presence of Warfarin: A biophysical investigation. Int J Biol Macromol 2017;95:713–8.

Simon LM, Laczkó I, Demcsák A, Tóth D, Kotormán M, Fülöp L. The formation of amyloid-like fibrils of α-chymotrypsin in different aqueous organic solvents. Protein Pept Lett 2012;19:544–50.

Singh BN, Shankar S, Srivastava RK. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochem Pharmacol 2011;82:1807–21.

Singh PK, Kotia V, Ghosh D, Mohite GM, Kumar A, Maji SK. Curcumin modulates α-synuclein aggregation and toxicity. ACS Chem Neurosci 2013;4:393–407.

Sipe JD, Cohen AS. Review: History of the Amyloid Fibril. J Struct Biol 2000;130:88–98.

Siqueira-Filho AG, Cunha CL, Tajik AJ, Seward JB, Schattenberg TT, Giuliani ER. M-mode and two-dimensional echocardiographic features in cardiac amyloidosis. Circulation 1981;63:188–96.

Šneideris T, Baranauskienė L, Cannon JG, Rutkienė R, Meškys R, Smirnovas V. Looking for a generic inhibitor of amyloid-like fibril formation among flavone derivatives. PeerJ 2015;3:e1271.

Soto-Ortega DD, Murphy BP, Gonzalez-Velasquez FJ, Wilson KA, Xie F, Wang Q, et al. Inhibition of amyloid-β aggregation by coumarin analogs can be manipulated by functionalization of the aromatic center. Bioorg Med Chem 2011;19:2596–602.

Stefani M. Protein misfolding and aggregation: new examples in medicine and biology of the dark side of the protein world. Biochim Biophys Acta - Mol Basis Dis 2004;1739:5–25.

Stefani M, Rigacci S. Protein Folding and Aggregation into Amyloid: The Interference by Natural Phenolic Compounds. Int J Mol Sci 2013;14:12411–57.

Stefani M, Rigacci S. Beneficial properties of natural phenols: Highlight on protection against pathological conditions associated with amyloid aggregation. BioFactors 2014;40:482–93.

Stroud JC, Liu C, Teng PK, Eisenberg D. Toxic fibrillar oligomers of amyloid-β have cross-β structure. Proc Natl Acad Sci 2012;109:7717–22.

Suantawee T, Wesarachanon K, Anantsuphasak K, Daenphetploy T, Thien-Ngern S, Thilavech T, et al. Protein glycation inhibitory activity and antioxidant capacity of clove extract. J Food Sci Technol 2014;52:3843–50.

Sumner IL, Edwards RA, Asuni AA, Teeling JL. Antibody Engineering for Optimized Immunotherapy in Alzheimer's Disease. Front Neurosci. 2018;12:254.

Sunde M, Serpell LC, Bartlam M, Fraser PE, Pepys MB, Blake CC. Common core structure of amyloid fibrils by synchrotron X-ray diffraction. J Mol Biol 1997;273:729–39.

Takasaki J, Ono K, Yoshiike Y, Hirohata M, Ikeda T, Morinaga A, et al. Vitamin A has anti-oligomerization effects on amyloid-β in vitro. J Alzheimers Dis 2011;27:271–80.

Tam JHK, Pasternak SH. Amyloid and Alzheimer's disease: inside and out. Can J Neurol Sci 2012;39:286–98.

Teixeira J, Silva T, Andrade PB, Borges F. Alzheimer's disease and antioxidant therapy: how long how far? Curr Med Chem 2013;20:2939–52.

Thapa A, Jett SD, Chi EY. Curcumin Attenuates Amyloid-β Aggregate Toxicity and Modulates Amyloid-β Aggregation Pathway. ACS Chem Neurosci 2016;7:56–68.

Tildesley NTJ, Kennedy DO, Perry EK, Ballard CG, Savelev S, Wesnes KA, et al. Salvia lavandulaefolia (Spanish sage) enhances memory in healthy young volunteers. Pharmacol Biochem Behav 2003;75:669–74.

Tohda C, Matsumoto N, Zou K, Meselhy MR, Komatsu K. Aβ(25–35)-induced memory impairment, axonal atrophy, and synaptic loss are ameliorated by M1, A metabolite of protopanaxadiol-type saponins. Neuropsychopharmacology 2004;29:860–8.

Trinh HT, Han SJ, Kim SW, Lee YC, Kim DH. Bifidus fermentation increases hypolipidemic and hypoglycemic effects of red ginseng. J Microbiol Biotechnol 2007;17:1127–33.

Tu LH, Young LM, Wong AG, Ashcroft AE, Radford SE, Raleigh DP. Mutational analysis of the ability of resveratrol to inhibit amyloid formation by islet amyloid polypeptide: critical evaluation of the importance of aromatic–inhibitor and histidine–inhibitor interactions. Biochemistry 2015;54:666–76.

Tundis R, Menichini F. Bonesi M, Conforti F, Statti G, Menichini F, Loizzo MR. Antioxidant and hypoglycaemic activities and their relationship to phytochemicals in Capsicum annuum cultivars during fruit development. LWT - Food Sci Technol 2013;53:370–7.

Uribe E, Marín D, Vega-Gálvez A, Quispe-Fuentes I, Rodríguez A. Assessment of vacuum-dried peppermint (Mentha piperita L.) as a source of natural antioxidants. Food Chem 2016;190:559–65.

Vallverdú-Queralt A, Regueiro J, Martínez-Huélamo M, Rinaldi Alvarenga JF, Leal LN, Lamuela-Raventos RM. A comprehensive study on the phenolic profile of widely used culinary herbs and spices: Rosemary, thyme, oregano, cinnamon, cumin and bay. Food Chem 2014;154:299–307.

Velander P, Wu L, Ray WK, Helm RF, Xu B. Amylin Amyloid Inhibition by Flavonoid Baicalein: Key Roles of Its Vicinal Dihydroxyl Groups of the Catechol Moiety. Biochemistry 2016;55:4255–8.

Velander P, Wu L, Henderson F, Zhang S, Bevan DR, Xu B. Natural product-based amyloid inhibitors. Biochem Pharmacol 2017;139:40–55.

Venkatesan R, Ji E, Kim SY. Phytochemicals that regulate neurodegenerative disease by targeting neurotrophins: a comprehensive review. BioMed Res Int 2015;2015:814068.

Vickers A, Zollman C. ABC of complementary medicine: herbal medicine. BMJ 1999;319:1050–3.

Villareal MO, Sasaki K, Margout D, Savry C, Almaksour Z, Larroque M, Isoda H. Neuroprotective effect of Picholine virgin olive oil and its hydroxycinnamic acids component against β-amyloid-induced toxicity in SH-SY5Y neurotypic cells. Cytotechnology 2016;68(6):2567–78.

Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, Condron MM, Lomakin A, et al. Amyloid beta-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. J Biol Chem 1999;274:25945–52.

Walsh P, Neudecker P, Sharpe S. Structural Properties and Dynamic Behavior of Nonfibrillar Oligomers Formed by PrP(106−126). J Am Chem Soc 2010;132:7684–95.

Wang J, Ho L, Zhao W, Ono K, Rosensweig C, Chen L, et al. Grape-derived polyphenolics prevent Abeta oligomerization and attenuate cognitive deterioration in a mouse model of Alzheimer's disease. J Neurosci 2008;28:6388–92.

Wang SH, Liu FF, Dong XY, Sun Y. Thermodynamic analysis of the molecular interactions between amyloid β-peptide 42 and (−)-epigallocatechin-3-gallate. J Phys Chem B 2010;114:11576–83.

Waterhouse AL. Determination of Total Phenolics. In: *Current Protocols in Food Analytical Chemistry*; John Wiley and Sons, 2002; I1.1.1-I1.1.8.

Williams AR, Ramsay A, Hansen TVA, Ropiak HM, Mejer H, Nejsum P, et al. Anthelmintic activity of trans-cinnamaldehyde and A- and B-type proanthocyanidins derived from cinnamon (Cinnamomum verum). Sci Rep 2015;5:14791.

Wiltzius JJW, Sievers SA, Sawaya MR, Cascio D, Popov D, Riekel C, et al. Atomic structure of the cross-beta spine of islet amyloid polypeptide (amylin). Protein Sci 2008;17:1467–74.

Xie H, Wang JR, Yau LF, Liu Y, Liu L, Han QB, et al. Quantitative analysis of the flavonoid glycosides and terpene trilactones in the extract of Ginkgo biloba and evaluation of their inhibitory activity towards fibril formation of β-amyloid peptide. Molecules 2014;19:4466–78.

Xie H, Wang JR, Yau LF, Liu Y, Liu L, Han QB, et al. Catechins and procyanidins of Ginkgo biloba show potent activities towards the inhibition of β-amyloid peptide aggregation and destabilization of preformed fibrils. Molecules 2014;19:5119–34.

Xue WF, Hellewell AL, Gosal WS, Homans SW, Hewitt EW, Radford SE. Fibril fragmentation enhances amyloid cytotoxicity. J Biol Chem 2009;284:34272–82.

Yamada M, Ono K, Hamaguchi T, Noguchi-Shinohara M. Natural Phenolic Compounds as Therapeutic and Preventive Agents for Cerebral Amyloidosis. Adv. Exp. Med. Biol., vol. 863, 2015, p. 79–94.

Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, et al. Curcumin inhibits formation of amyloid β oligomers and fibrils, binds plaques, and reduces amyloid *in vivo*. J Biol Chem 2005;280:5892–901.

Yoon JS, Yang H, Kim SH, Sung SH, Kim YC. Limonoids from *Dictamnus dasycarpus* protect against glutamate-induced toxicity in primary cultured rat cortical cells. J Mol Neurosci 2010;42:9–16.

Young LM, Saunders JC, Mahood RA, Revill CH, Foster RJ, Tu L-H, et al. Screening and classifying small-molecule inhibitors of amyloid formation using ion mobility spectrometry–mass spectrometry. Nat Chem 2015;7:73–81.

Yu SY, Lee YJ, Kim JD, Kang SN, Lee SK, Jang JY, et al. Phenolic composition, antioxidant activity and anti-adipogenic effect of hot water extract from safflower (Carthamus tinctorius L.) seed. Nutrients 2013;5:4894–907.

Yuan HD, Chung SH. Fermented ginseng protects streptozotocin-induced damage in rat pancreas by inhibiting nuclear factor-κB. Phyther Res 2010;24:S190–5.

Zhang SQ, Obregon D, Ehrhart J, Deng J, Tian J, Hou H, Giunta B, Sawmiller D, Tan J. Baicalein reduces β-amyloid and promotes nonamyloidogenic amyloid precursor protein processing in an Alzheimer's disease transgenic mouse model. J Neurosci Res 2013;91(9):1239-46.

Zhao H, Li Q, Zhang Z, Pei X, Wang J, Li Y. Long-term ginsenoside consumption prevents memory loss in aged SAMP8 mice by decreasing oxidative stress and up-regulating the plasticity-related proteins in hippocampus. Brain Res 2009;1256:111–22.

Zhao L, Wang J-L, Liu R, Li X-X, Li J-F, Zhang L. Neuroprotective, Anti-Amyloidogenic and Neurotrophic Effects of Apigenin in an Alzheimer's Disease Mouse Model. Molecules 2013;18:9949–65.

Zhao R, So M, Maat H, Ray NJ, Arisaka F, Goto Y, Carver JA, Hall D. Measurement of amyloid formation by turbidity assay-seeing through the cloud. Biophys Rev 2016;8(4), 445–71.

Zhu M, Rajamani S, Kaylor J, Han S, Zhou F, Fink AL. The flavonoid baicalein inhibits fibrillation of alpha-synuclein and disaggregates existing fibrils. J Biol Chem 2004;279:26846–57.

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8. Summary

Background

Amyloid aggregation is a hallmark of several central nervous system neurological disorders including neuro development diseases affecting brain and peripheral tissues. The formation of insoluble amyloid is associated with a number of fatal neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's diseases, or type II diabetes mellitus, transmissible spongiform encephalopaty, cerebellar ataxias and primary and secondary systemic amyloidosis. Amyloid formation is a generic form of a polypeptide conformation and most proteins have a potential to form amyloid-like structure under appropriate conditions. The core structure of the fibrils is stabilized by hydrogen bonding between atoms of the polypeptide backbone. Despite different proteins being responsible for each disease, all of them share similar features including beta-sheet-rich secondary structures and fibril-like protein aggregates.

Prevention is a more effective strategy than the treatment of chronic diseases. Functional foods that contain significant amounts of bioactive components play important roles in the prevention of chronic diseases. Unfolded or partially unfolded proteins associate with each other to form small, soluble aggregates that undergo further assembly into protofibrils or protofilaments and then into mature fibrils, which is associated with increasingly common and highly debilitating diseases. The amyloid diseases involve predominantly the aggregation of specific proteins, but fibrils can be formed by many other peptides and proteins too. Natural phenolic compounds, a wide panel of plant molecules, are one of the most actively investigated categories of potential amyloid inhibitors. One therapeutic approach is the screening or development of natural bioactive molecule inhibitors of Aβ aggregation. Dietary natural products are a rich source of phytochemicals and active compounds, which have the potential for future drug development for AD. In

the last decade, researchers have reported that certain fruits, vegetables, spices, drinks and beverages can potentially counteract AD pathogenesis. These natural products are effective at inhibiting oxidative stress, Aβ accumulation and toxicity, tau phosphorylation, and neuro-inflammation and exhibit antioxidant and anti-inflammatory properties. Researchers are currently searching for new, safer and more effective therapies that can target the pathophysiology of AD more precisely and inhibit the Aβ accumulation and toxicity.

Aims

Our primary interest here was to discover novel molecules possessing anti-neurodegenerative and anti-amyloidogenic properties through the screening of a diverse library of natural compounds. By considering these main objectives, our aims were:

- Collection, extraction of bioactive compounds and total phenolic compound analysis from screened categories or groups of natural products.
- Modification of trypsin with phenylmethylsulfonyl fluoride (PMSF) for the formation of amyloid-like fibrils in aqueous organic solvents.
- *In vitro* inhibitory screening by turbidity, size exclusion chromatography, aggregation kinetics, CR binding, FTIR, HPLC-MS, CD and TEM on amyloid-like fibril formation of trypsin by natural product extracts in aqueous organic ethanol.
- Selection of suitable therapeutic agents for amyloid related neurodegenerative diseases.

Materials and methods

To address the above questions we chose the following specific methods like preparation of the extracts, assay of enzyme activity, modification of trypsin with phenylmethylsulfonyl fluoride (PMSF), turbidity measurements, determination of the total

phenolic content, aggregation kinetics, CR binding, HPLC-MS, ECD measurements, size exclusion chromatography, FTIR spectroscopy analysis, transmission electron microscopy, which can address the inhibitory activity of natural product extracts on amyloid-like fibril formation of trypsin in aqueous organic solvents like in 60% ethanol at pH 7.0.

Results and discussion

Recent studies on Aβ amyloid formation indicate that several natural small aromatic compounds and flavonoids interfere with aggregation pathways possibly by remodelling the amyloid intermediates through the different mechanisms or interactions. The effects of different inhibitory agents on PMS-trypsin aggregation can also be monitored *via* turbidity measurements. The total phenolic content of the different inhibitory agents was determined, and it varied between 163.7 ± 12.6 and 7,702 ± 528.5 mg GAE/l. By using HPLC-MS, we determined that the limonin concentration of the grapefruit seed extract was 16.8 ng/ml. The aggregation kinetic assays were used to monitor the time course of aggregation by PMS-trypsin through monitoring the increase of absorption at 350 nm in the absence and presence of various inhibitory agents at different concentrations.

The Congo red absorption difference at 550 nm was found to decrease with incubation in the presence of inhibitory agents. With the size-exclusion chromatography analysis experiment we demonstrated that the inhibitory effect of the Eduscho coffee extract on the formation of amyloid-like fibrils is due to its capacity to stabilize the oligomeric form of the protein. FTIR result indicates that the chilli extract inhibited amyloid aggregation by stabilizing the native form of the protein by aggregating and remodelling or degrading toxic amyloid oligomers. Electronic circular dichroism analysis concludes that the addition of some natural product extract is effective in preventing PMS-trypsin from undergoing helix-to-coil transition. TEM images demonstrated, that the

Eduscho coffee, chili, *P. ginseng*, grapefruit seed and peppermint extracts had inhibitory effect on the formation of amyloid-like fibrils of PMS-trypsin. The extent of fibril formation was reduced at a significant level in their presence.

The present study concludes that the natural product extracts like the coffee, chili, *P. ginseng*, grapefruit seed and peppermint extracts may be efficient anti-amyloidogenic agents, as they arrest PMS-trypsin fibrillation at 60% ethanol at pH 7.0 in a concentration dependent manner.

9. Összefoglalás

Az amiloid fibrillumok jelenléte számos nem kívánatos betegségre jellemző. Ilyenek pl. az Alzheimer-, a Parkinson-, a Hungtinton kór, a prion betegségek és a 2-es típusú cukorbetegség (*diabetes mellitus*) is, melyek világszerte milliók életét keserítik meg. Az amiloid fibrillumok olyan fibrilláris fehérje aggregátumok, melyek a β-lemez szerkezetű polipeptidláncok egymás mellé rendeződése révén jönnek létre. A fibrillumokban a β-lemezek a fibrillumok hossztengelyeire merőlegesen orientálódva helyezkednek el. A fibrillumokat az egymás mellett lévő peptid-gerincek megfelelő atomjai között kialakuló hidrogénhíd kötések sokasága stabilizálja. Fibrillumképzésre bizonyos enyhe denaturáló körülmények mellett valamennyi fehérje képes lehet. Az amiloid fibrillumok hosszú, vízben nem oldódó, elágazások nélküli képződmények. Keletkezésük szigmoid alakú görbével jellemezhető, melyben a kezdeti lassú lag fázist gyors aggregáció követ, majd a folyamat telítési szakasszal zárul. A lag fáziban olyan magok jönnek létre, melyek monomerek hozzáadásával fibrillumokká alakulnak. Némely festékek, mint pl. a kongóvörös vagy a tioflavin T, nagy affinitással képesek az amyloid fibrillumokhoz kötődni, melyet jellemző spektrális változások kisérnek, így lehetővé téve a fibrillumok jelenlétének érzékeny detektálását.

Mivel a fehérje konformációs betegségekben szenvedők száma évről-évre folyamatosan emelkedik, rendkívül fontos a megelőzésük. A krónikus betegségek megelőzése eredményesebb lehet, mint a kezelésük. Napjainkban számos olyan törekvés van, mely különböző, növényekben előforduló biomolekulák felhasználásával igyekszik kivédeni az amiloid fibrillumok keletkezését. A már igen rég óta széles körben használt illetve fogyasztott fűszerekben, étrend kiegészítőkben, kávékban, teákban, borokban, gyümölcsökben és zöldségekben jelen lévő bioaktív vegyületek fontos szerepet játszhatnak (nem kívánatos mellékhatások nélkül) a különböző amiloidózisok megelőzésében, illetve a

tüneteik enyhítésében. A bennük jelen lévő polifenolos vegyületek jótékony hatása részben annak köszönhető, hogy képesek stabilizálni a fehérjék natív szerkezetét, illetve aromás gyűrűik révén olyan speciális aromás-aromás kölcsönhatások kialakítására képesek, melyek segítségével gátolni tudják a fibrillumok létrejöttét. Jelenleg több, mint 8000 növényi polifenolt ismerünk. Közülük számos vegyület, mint pl. a kurkumin, a galluszsav és a rezveratrol anti-amiloidogén hatását már leírták. Az amiloid fibrillumok gátlásában nem polifenolos vegyületek is részt vehetnek.

Kísérleteinkben különböző növényi kivonatok amiloid-szerű fibrillum képződést gátló hatását vizsgáltuk modell fehérjeként fenil-metil-szulfonil fluoriddal (PMSF) kémiailag irreverzibilisen módosított, inaktivált tripszint használva, 60 %-os etanolos közegben pH 7,0-nél. Munkánk célja volt a kereskedelmi forgalomban lévő különböző gátló ágensek hatékonyságának összehasonlítása. Az általunk megvizsgált fűszerek közül a chili, a teák közül a borsmenta, az étrend kiegészítők közül a grapefruit mag kivonat, valamint a kávék közül az Eduscho kávé bizonyult a leghatékonyabb inhibitornak. A kávék és teák esetében korrelációt találtunk az összes polifenol tartalmuk és az aggregátum képződést gátló hatásuk között. Kongó vörös kötődési kísérletekkel kimutattuk a grapefruit mag-, a chili-, a borsmenta- és az Eduscho kávé kivonatainak koncentrációtól függő mértékű gátló hatását. A grapefruit mag kivonatban is jelen lévő limoninról először írtuk le, hogy anti-amiloidogén hatással rendelkezik. Az Eduscho kávé és a ginseng kivonatokról megállapítottuk, hogy nincsen versengés a kongóvörös és a gátló ágensek biomolekulái között a fibrillumokhoz való kötődésben, illetve lehetséges az is, hogy más-más felületi helyekhez kötődnek. Méret kizárásos kromatográfia felhasználásával kimutattuk, hogy a *Panax ginseng* kivonat a PMS-tripszin monomer formáját, míg az Eduscho kávé az oligomer formát stabilizálja. Fourier-transzformációs infravörös spektroszkópiai analízissel bizonyítottuk, hogy a chili kivonat a natív forma stabilizálása

révén gátolja a PMS-tripszin aggregációját. Elektronikus cirkuláris dikroizmus spektroszkópiai elemzéssel fény derült arra, hogy az Euscho kávé kivonat megakadályozza a fehérjében lévő α-hélix szerkezeteknek a rendezetlen szerkezetűekké alakulását ez által gátolva a fibrillációt. Kimutattuk, hogy a chili extraktum jelenléte stabilizálta a PMS-tripszin oldott állapotát. Feltételezhető, hogy közben a fehérje szerkezetete kis mértékben megváltozott ugyan, de az aggregátumok egyáltalán nem, vagy csak nagyon lassan tudnak kialakulni. A különböző gátló ágensek fibrillum képződésre kifejtett jótékony hatását transzmissziós elektron mikroszkópos felvételekkel is alátámasztottuk.

Kísérleti eredményeink alapján megállapítható, hogy az Eduscho kávé, a chili, a *P. ginseng*, a grapefruit mag és a borsmenta kivonatai hatékony anti-amiloidogén ágensek a PMS-tripszin aggregációjában pH 7,0-nél.

10. List of publications

Publications related to thesis

MTMT number: 10053006

1. **Kasi PB**, Borics A, Molnár K, László L, Kotormán M. Eduscho Coffee Extract Effectively Inhibits the Formation of Amyloid-like Fibrils by Trypsin in Aqueous Ethanol. Nat Prod Commun 2018;13(12):1695–1698. Impact Factor: 0.809
2. **Kasi PB**, Borics A, Varga M, Endre G, Molnár K, László L, Kotormán M. Grapefruit Seed Extract Inhibits the Formation of Amyloid-like Fibrils by Trypsin in Aqueous Ethanol. Nat Prod Commun 2018;13(11):1437–1440. Impact Factor: 0.809
3. **Kasi PB**, Kotormán M, Borics A, Hervay BG, Molnár K, László L. The Inhibitory Effect of Panax Ginseng Extract on Amyloid-like Fibril Formation of Trypsin in Aqueous Ethanol. Protein Pept Lett. 2018;25(3):253–259.
   doi: 10.2174/0929866525666171229231226. Impact Factor: 1.039

Publications not directly related to thesis

1. Kotormán M, **Kasi PB**, Halász L, Borics A. Inhibition of Amyloid-like Fibril Formation of Trypsin by Red Wines. Protein Pept Lett. 2017;24(5):466–470.
   doi: 10.2174/0929866524666170214125847. Impact Factor: 1.039
2. Kotormán M, Kelemen Z, **Kasi PB**, Nemcsók J. Inhibition of the formation of amyloid-like fibrils using herbal extracts. Acta Biol Hung. 2018 Jun;69(2):125–134. doi: 10.1556/018.69.2018.2.2. Impact Factor: 0.439
3. Kotormán M, Varga A, **Kasi PB**, Nemcsók J. Inhibition of the formation of amyloid-like fibrils with spices, especially cloves. Acta Biol Hung. 2018;69(4):385–394. doi: 10.1556/018.69.2018.4.2. Impact Factor: 0.439