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The influence of plant extracts on β-Amyloid induced pathologies in an APP/PS1 mouse model of Alzheimer's disease

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„Nur wenige wissen, wie viel man wissen muss, um zu wissen, wie wenig man weiß."

Werner Heisenberg

Contents

Summary

Alzheimer's disease (AD) is defined as a progressive loss of memory and degeneration of cognitive functions, which first manifest as dysfunctions of social, orientational and occupational skills. Changes like morphological lesions and neuronal dysfunction are associated with extracellular beta-amyloid (Aβ) and its deposits developing from excessive Aβ generation and accumulation. These are accompanied by inflammatory processes, which result in neuronal degeneration. Among other Aβ variants, $A\beta_{42}$ is the neurotoxic form and the main component of senile plaques. Currently, the most used symptomatic treatment opportunities are acetylcholinesterase inhibitors, which are used to compensate the loss of cholinergic neurons, and α-secretase activators that shift the Aβ cascade towards the non-amyloidogenic pathway for generating more of the non-toxic Aβ species.

In this study, herbal extracts were investigated in an amyloid precursor protein (APP) / presenilin 1 (PS1) mouse model for their ability to intervene AD typical pathological processes and to counteract the cognitive decline. Promising candidates were identified for follow-up studies by using an explorative screening approach comprising characteristic parameters such as (i) intracerebral Aβ load, (ii) deposition rate, and (iii) cognitive performance. Therefor, mice were daily orally administered herbal extracts for 50 days, starting after AD onset. Out of twelve, the extracts of *Hypericum perforatum* (Saint John's wort, SJW) and *Sideritis scardica* met the above mentioned criteria. Both reduced the intracerebral levels of toxic $A\beta_{42}$ and plaque load, and significantly alleviated memory impairments.

A follow-up study then analysed various extraction forms of SJW that varied in their hyperforin and hypericin concentrations as well as extracts of *Sideritis* spp. species (*Sideritis euboea* and *Sideritis scardica*). In addition, their mode of action was explored. For this purpose, mice were classified in two clinics, namely (i) the AD initiation group, starting low dosed with the onset of first pathological mechanisms and (ii) the post AD onset group, treated with the ten-fold dosage after the beginning of AD onset. Two extracts containing only minimal to virtually no amounts of hyperforin showed the highest impact on Aβ burden in APP/PS1 mice as determined by ELISA measurements and histological plaque quantification. These data are in contrast to previous reports that hyperforin represents the most important of two bioactive constituents of SJW. Both extracts also showed an improved memory performance in Morris water maze experiments and counteracted the neurodegeneration *in vivo*, indicating that hyperforin is not the relevant compound of SJW extracts with respect to AD aspects. A highly enhanced blood-brain barrier ABC transporter activity of ABCC1 (MRP1) was identified as the underlying molecular mechanism. This finding points to $A\beta_{42}$ reduction as the result of a highly increased protein export mediated by ABCC1.

In contrast to SJW, Sideritis species are not the centre of attention of AD research although they are well-known for their anti-inflammatory and antioxidative properties. Treatment with extracts of both *S. scardica* and *S. euboea* strongly reduced intracerebral $A\beta_{42}$ concentration as well as the plaque load, and highly enhanced spatial memory of APP/PS1 mice. Moreover, when applied in combination, the extracts were able to fully rescue neuronal loss. Mechanistically, this study revealed that the reduction of soluble and insoluble $A\beta_{42}$ species is the consequence of a significantly enhanced expression of the metalloprotease ADAM10 which generates non-toxic Aβ species and a highly increased phagocytic microglia activity.

In summary, this study revealed promising beneficial effects of herbal extracts as novel options in AD treatment.

Abbreviations

ABC	ATP-binding-cassette
Aβ	beta amyloid
AD	Alzheimer's disease
ADAM10	A disintegrin and metalloproteinase domain-containing protein 10
ALS	Amyotrophic Lateral Sclerosis
APH1	anterior pharynx-defective 1
apoE	apolipoprotein E
APP	amyloid precursor protein
APS	ammonium peroxydisulphate
ATP	adenosine 5'-triphosphate
BACE1	beta-site APP cleaving enzyme 1, beta-secretase 1
BBB	blood-brain barrier
BCA	bicinchoninic acid
BCRP1	breast cancer resistant protein 1 / ABCG2
Bp	base pairs
°C	degree Celsius
C-terminal	carboxy terminal
CJD	Creutzfeldt-Jacob Disease
CSF	cerebrospinal fluid
D	Days
DER	drug extract ratio
dest.	Destillata
DLB	dementia with Lewy bodies
DNA	desoxyribonucleic acid
EDTA	ethylene-diamine tetra-acetate
EGCG	epigallocatechin-3-gallat
ELISA	enzyme-linked immuno sorbent assay
FTLD	frontotemporal lobe degeneration/dementia
GSH	γ-L-Glutamyl-L-cysteinyl-glycin
GuaHCl	guanidine-hydrochloride
HD	Huntington's disease
HRP	horseradish peroxidase
IHC	Immunohistochemistry
IL-1β	interleukin-1β
IL-6	interleukin-6
kDa	kilo Dalton

MCI	mild cognitive impairment
MDR	multidrug-resistance (ABCB1)
MWM	Morris water maze
MRP	multidrug resistance-associated protein (ABCC1)
ND	neurodegenerative disease
NMDA	N-methyl-D-aspartic acid
PAGE	polyacryl amide gel electrophoresis
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
PD	Parkinson's disease
PEN-2	presenilin enhancer 2
PFA	Paraformaldehyde
P-gp	P-glycoprotein / ABCB1
PHF	paired helical filaments
PMSF	phenylmethanesulphonyl fluoride
Prp	prion protein
PS1 & 2	presenilin 1 & 2
PVDF	polyvinylidene fluoride
RAM	radial arm maze
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SJW	St. John's wort
SNP	single nucleotide polymorphism
TAE	tris-acetate-ethylene-diamine tetra-acetate
Taq	Thermus aquaticus
TBST	tris-buffered saline tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
Tris	tris-(hydroxymethyl)-aminomethane

1 Introduction

1.1 Neurodegeneration

Neurodegenerative disease (ND) is the general term for a group of slowly progressing, hereditary or sporadic diseases of the central nervous system. It refers to the gradual loss of structure and augmented dysfunction of specific neuronal populations, ultimately resulting in their death. Still, age is the most consistent risk factor for developing a ND, e.g. Alzheimer's disease (AD) or Parkinson's disease (PD). Oxidative stress as well as DNA mutations of mitochondria – the critical regulators of cell death - both contribute to aging (Lin and Beal, 2006, Schaffer et al., 2012). Due to the worldwide increase of live expectancy and thus rising numbers of elderly, NDs have become the most prevalent diseases of elderly in the Western world. While the causes of most neurodegenerative diseases are mainly unknown, there are some presumptions: malnutrition such as vitamin deficiency; toxins (e.g. alcohol); virus infections; inflammatory conditions such as Multiple Sclerosis, vascular arteriosclerosis, cerebrovascular disease (CVD) (Honjo et al., 2012); and protein depositions as seen in the most common forms of dementia, AD and PD (Sulkava et al., 1983, Bredesen et al., 2006, Rubinsztein, 2006). ND's are roughly divided into different groups according to their phenotypic effects, which are characterised by loss of functions such as motor control, processing sensory information, decision making, and later on effects on memory, and dementia (Przedborski et al., 2003). ND's of the central nervous system (CNS) are grouped according to the region into diseases of the cerebral cortex, the basal ganglia, the brainstem and the cerebellum, and the spinal cord. Within each group, a given disease may be further classified based on its main clinical features. For example, the group of diseases that predominantly affect the cerebral cortex is divided into dementing (e.g. AD) and non-dementing conditions (Przedborski et al., 2003). A large subset of ND's shows intra- and/or extracellular deposits of filamentous and toxic proteins or protein fragments (see Table 1). Diseases such AD, PD and Creutzfeldt-Jakob disease (CJD) are characterised by depositions of soluble proteins which form abnormal protein fibrils (Drzezga, 2008).

Table 1. Forms of neurodegenerative diseases, intracellular deposits and involved proteins (adapted fromDrzezga (2008))

Disease	Proteins involved	Examples
Amyloidoses	β-amyloid (Aβ)	Alzheimer's disease (AD)
Prionoses	prion protein (Prp)	Creutzfeldt-Jakob disease (CJD)
Tauopathies	tau (τ)	Alzheimer's disease (AD)
		Progressive supranuclear palsy (PSP)
		Pick's disease (PiD)
α-Synucleinopathies	α-synuclein (α-syn)	Parkinson's disease (PD)
		Dementia with Lewy bodies (DLB)
		Multiple Systemic Atrophy (MSA)
Ubiquitin diseases	Ubiquin, TDP43	Amyotrophic lateral sclerosis (ALS)
Polyglutamin-expansion diseases	(Gln)x-Proteins	Chorea Huntington (HD)
		Kennedy disease (SMBA)

1.2 Dementia

Dementia, as a consequence of ND's, is an irreversible loss of at least two global cognitive abilities involving the cerebral cortex, and occurrence of behavioural abnormalities. Dementia may affect adults of all ages, but the risk increases with age hence it is far more common in the elderly population. According to European epidemiological studies, dementia affects 6–7% of the population older than 65 (Di Luca et al., 2011). The frequency of dementia increases with rising age from less than 2% for the 65-69 year-olds, to 5% for the 75-79 year-olds and to more than 20% for the 85-89 year-olds. Every third person over 90 years of age suffers from moderate to severe dementia. About 55% of those affected by dementia suffer from AD (Bickel, 2001). In Europe, the estimated number of patients aged 65 and over who have dementia is 4.9 million, with an estimated annual incidence approaching one million, thus these numbers are expected to increase dramatically. Although uncommon, it can occur before the age of 65, termed "early onset dementia" (Fadil et al., 2009).

Unlike stable brain damages as seen in stroke or head injury, ND's often affect multiple regions of the brain (see Table 2). It is a non-specific illness syndrome characterised by a variety of cognitive dysfunctions and abiding symptoms reflecting the impairment of memory, attention, language, and problem solving (Cummings et al., 1980). To be classified as dementia, the syndrome must achieve deficits in following criteria: (i) the ability to generate speech and understand spoken or written language; (ii) to recognize or identify objects, the execution of motor activities, sensory function and comprehension of the required task; (iii)

and the ability to think abstractly, to plan and carry out complex tasks. In later stages, higher mental functions are affected; hence patients may be disoriented in time, in place, and in person (Maslow, 2008).

Dementias can be classified into primary dementia (90% of all dementia cases) including neurodegenerative and vascular origins, and secondary dementias (10% of all dementia cases). While the causative changes of a primary dementia are to be found in the brain and underlie primary neuronal changes, the origins of secondary dementias are to be found outside the brain in the form of e.g. infections, tumours, metabolic disturbances, brain injury and toxins like alcohol (Kastner and Löbach, 2007). Most dementias are incurable, although scientists show respectable progress in developing new medications that can slow down the disease progress. Especially cholinesterase inhibitors are often used early in the disease course of AD. Cognitive and behavioural interventions may also be appropriate (Cummings et al., 1980).

Table 2. Types of dementia, characteristic hallmarks and clinical symptoms (Rubinsztein, 2006, Imarisio et al., 2008, Alzheimer's-Association, 2012, Rosenmann and Meiner, 2013, Overk and Masliah, 2014)

Type	Dementia	Clinical symptoms	Hallmarks
cortical	Alzheimer's disease (AD)	early state: problems in remembering names and recent events, mood swings moderate state: local disorientation, confusion, behavioural disorder, impaired judgement severe state: vulnerable to infections, bedridden	Aβ plaques neurofibrillary tangles (NTF´s) Hirano bodies neuronal cell death brain atrophy
	Dementia of Lewy bodies (DLB)	see also symptoms of AD, in addition to visual hallucinations, muscle rigidity, tremor	intraneuronal abnormal deposits of alpha-synuclein (Lewy bodies)
	Frontotemporal dementia (FTLD)	personality and behavioural changes and language difficulties	neuronal cell damage, especially in frontal and lateral brain regions
	Vascular dementia	can overlap with symptoms of AD but memory is less affected than in AD	impaired blood flow into the CNS Strokes that block vessels
subcortical	Parkinson's disease (PD)	abnormalities of motor control, tremors, rigidity, loss of postural reflexes	abnormal alpha-synuclein deposits
	Huntington's disease (HD)	uncoordinated, jerky body movements, decline in mental abilities (chorea)	deposition of the mutated Huntingtin, neurotoxicity leads to apoptosis

1.3 Alzheimer's disease

By far the most frequently cited cause of dementing cerebral cortex pathology is the Alzheimer's disease (AD). Already in 1906, the Bavarian neurologist Alois Alzheimer depicted a „strengthen disease of the cerebral cortex". In the year 1901, Auguste Deter (Figure 1), a 51 years old patient, has first attracted attention because of outbursts of jealousy against her husband. "I have lost myself, as it were" she said repeatedly during the interview by Alois Alzheimer (in excerpts see below). Later on, she was afflicted with intense and rapidly increasing memory loss. Five years later she died. While Alzheimer was performing a brain autopsy, he noted the presence of dense deposits surrounding the nerve cells: neuritic or amyloid plaques. Inside the nerve cells, he observed twisted bands of fibres, the neurofibrillary tangles. The case of Auguste D. is the first known and documented case of AD (Suh and Checler, 2002).

"What is your name?" - "Auguste."
"Sur name?" - "Auguste."
"What is your husband's name?" - "I think, Auguste."
"Your husband?" - "I see, my husband."
"Are you married?" - "To Auguste."
"How old are you?" - "Fifty-one."
"Where do you live?" - "Oh, you have been to our place"
"Are you married?" - "Oh, I am so confused."
"Where are you right now?" - "Here and everywhere, here and now, you must not think badly of me."

Figure 1. The abridged dialogue between Dr. Alois Alzheimer and Auguste Deter after her hospitalisation in 1901. Auguste Deter was the first person diagnosed with Alzheimer's disease in the year 1906 (Alzheimer, 1907).

Commonly, AD is slowly progressing, and the pathogenesis starts years before any of the clinical symptoms are being observed. The progress of the disease can be classified into three stages of severity, whereby rate and intensity differ between patients. (i) In the early stage, the patients initially recognise their own disturbances in short-term memory, followed by unexplainable mood swings, language difficulties and mild spatial disorientation. (ii) The moderate stage is characterised by the difficulty to perform routine tasks like washing and dressing independently. Patients are emotionally instable and often change their personality. Memories of the distant past may be confused with the present, and affect the person's ability to comprehend the current situation, date and time. They may have trouble recognizing familiar people; they may become depressed, irritable and restless, or apathetic and

withdrawn. (iii) In the severe stage, patients become bedridden and vulnerable to infections (pneumonia, influenza, nephritis etc.), which mostly leads to death (from www.alzheimer.de).

Aging is the major risk factor in this form of neurodegenerative disorder, and the number of affected people is increasing rapidly (Alloul et al., 1998). Today, there are about 1 million demented patients in Germany, and 2/3 out of these present AD patients. In 2001 there were about 24.3 million AD patients worldwide, and it is assumed that until 2040 the estimated number will reach 81.1 million patients (Ferri et al., 2005) while the number of people older than 80 years will approach 370 million by the year 2050.

1.3.1 Molecular fundamentals

Alzheimer's dementia is a ubiquitous and one of the most destructive and feared human diseases of the central nervous system that affects aging populations. AD's pathology is a consequence of a conglomeration of pathological mechanism resulting in (i) augmented toxic protein generation, (ii) protein folding and (iii) neuronal network dysfunction and neuronal death (Marchesi, 2012, Nelson et al., 2012). Under all pathological phenomena, the most characteristic hallmark of AD are neuritic plaques composed of toxic Amyloid beta protein species. Central to the disease is the sequential proteolytic processing of the amyloid precursor protein (APP) by three membrane-associated proteases, α-, β- and γ-secretase, leading either to the amyloidogenic or the non-amyloidogenic pathway (Anderson et al., 1992, De Strooper and Annaert, 2000). Cleavages by the β-secretase and the γ-secretase complexes generate highly neurotoxic beta-amyloid peptides (Aβ) which are prone to aggregate to these dense neuritic protein formations.

The human APP gene is highly expressed in the brain and is encoded by 19 exons located on chromosome 21 (Goldgaber et al., 1987), which is a crucial aspect in trisomy 21 (Millan Sanchez et al., 2012). APP consists of a single transmembrane region, a large extracellular domain, and a short cytoplasmic tail. It is a house keeping gene and its function depends on the cell types in which it is expressed (Pollwein et al., 1992). APP is able to affect neuronal survival (Mattson et al., 1993), neurite outgrowth and synaptogenesis (Small et al., 1994), and cell adhesion. This protein also provides some benefits in neuroprotection associated with soluble APP alpha (sAPPα), which *in vitro* can protect cells against death due to glucose deficiency (Mattson et al., 1993). Nevertheless, studies of the different variants of AD (i) autosomal dominant, (ii) late-onset AD, and (iii) the rare genetic mutation of APP indicate a critical role of amyloid in AD pathogenesis (Gilbert, 2013). The Aβ domain is located within this type I transmembrane protein at the junction between the intraluminal and transmembrane domains. The production of neurotoxic Aβ is mediated by the concerted action of β-secretase (β-site APP cleaving enzyme, BACE1) (Vassar et al., 1999) and γ-secretase, a multiprotein complex thought to be made up of following transmembrane proteins: presenilin 1 (PS1), presenilin 2 (PS2), nicastrin, APH1 and PEN2 (Edbauer et al.,

2003, Haass, 2004). Cleavage by β-secretases generates a soluble sAPPβ fragment and a membrane bound 99 amino acid fragment (C-terminal) containing Aβ. Further proteolysis by the γ-secretase within the transmembrane domain results in the production of the 4kDa Aβ peptides. Proteolysis by β-secretases can result in a variety of different Aβ fragments: $Aβ_{38}$, $Aβ_{40}$, and $Aβ_{42}$ (Figure 2). Additionally, Lesné et al. reported of a specific 56kDA soluble form of Aβ called Aβ*56 (Lesné et al., 2006). The resulting Aβ proteins are prone to aggregate, thereby giving rise to plenty of Aβ species of different neurotoxicities (Suh and Checler, 2002, Wang et al., 2006a, Thinakaran and Koo, 2008). However, the hydrophobic $Aβ_{42}$ is highly prone to fibril formation – more than $Aβ_{40}$ – hence, $Aβ_{42}$ is the major Aβ species found in amyloid plaques (Suh and Checler, 2002). In contrast, APP cleavage by one of the α-secretases ADAM 9, 10, and 17 results in the production of soluble fragments (sAPPα) without producing toxic Aβ species. A critical event in the pathology of AD is the aggregation of soluble Aβ peptides to β-sheet conformation. Aβ accumulates with increasing age from monomers to oligomers, still soluble protofibrils, insoluble fibrils, and finally diffuse or dense-cored Aβ plaques.

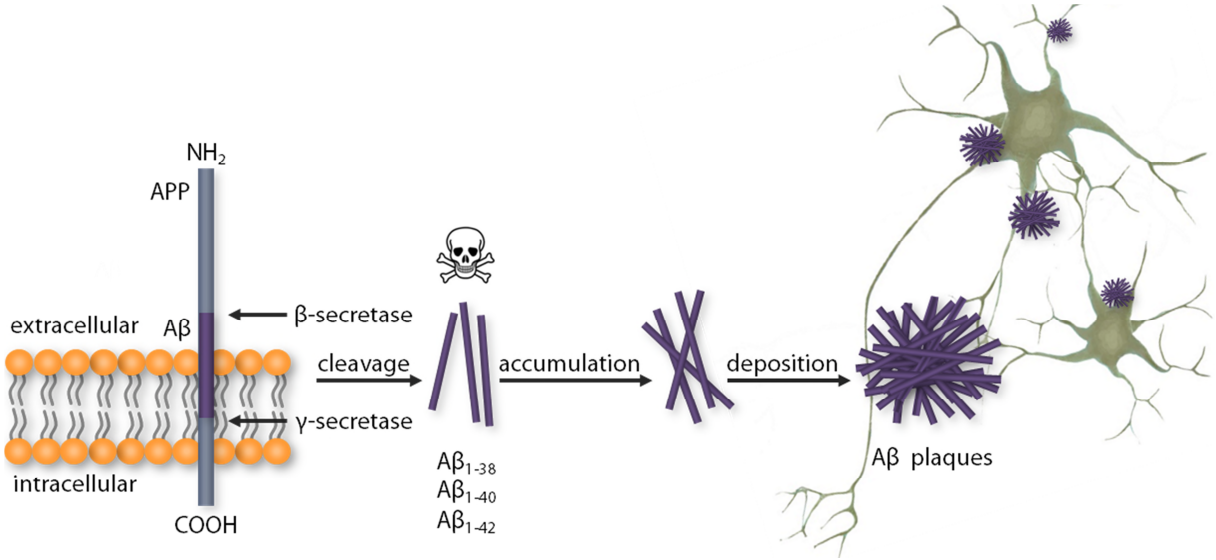

Figure 2. Amyloidogenic processing of the membranous amyloid precursor protein (APP) by γ- and β-secretase generates various Aβ peptide species. These peptides are neurotoxic and prone to aggregate to protofibrils, insoluble fibrils and finally to senile plaques.

Aβ oligomers are now widely recognised as the primary neurotoxic structures leading to AD (Gilbert, 2013). Today, different research groups try to find suitable mathematical models to predict Aβ oligomerisation. Kelley and colleagues established a computational approach which allows the prediction of peptide folding at a longer timescale of seconds and much lower and realistic micromolar concentrations. With their approach, they could predict the formation of an Aβ trimer within tens of microseconds while tetramers would form at least 1000 times slower, which has yet to be described experimentally. By using more detailed computational Aβ folding prediction models, a lot more understanding in peptide folding and

function will be gained (Kelley et al., 2008). More importantly, Aβ peptides may begin their toxic actions even before fibril formation. To date, it is known that soluble Aβ levels, not Aβ aggregates account for cognitive decline in AD (Nicoll et al., 2006, Lesné et al., 2008, Gilbert, 2013).

1.3.2 Pathology

Alzheimer's pathology enfolds a variety of morphological changes resulting in neuronal degeneration. Affected brain regions include the temporal and parietal lobes as well as specific regions within the frontal cortex, hippocampus and cingulate gyrus (Figure 3A) (Wenk, 2003). This is accompanied by disturbance of different cortical functions, including memory, judgment, orientation, comprehension, learning capacity, as well as language (Ellis, 2005). An AD affected brain is among other features morphologically characterised by extracellular beta-amyloid formations (plaques), developing from an abnormal accumulation of the highly neurotoxic Aβ and neurofibrillary tangles (NFT) (Figure 3B, C) (Jarrett et al., 1993, Roychaudhuri et al., 2009).

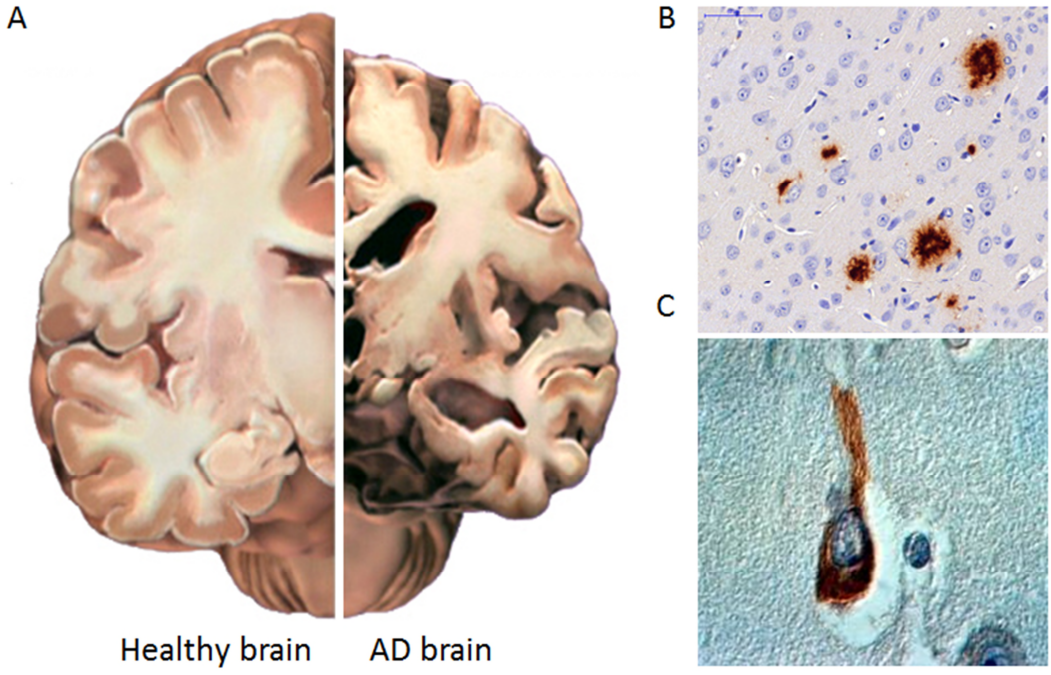

Figure 3. (A) Neuronal decline causes pathological changes in brain tissue of AD patients, in consequence leading to a characteristically brain atrophy (adapted form Alzheimer.org). (B) β-amyloid depositions as well as (C) neurofibrillary tangles (NFT) consisting of hyperphosphorylated stabilizing protein tau are morphological hallmarks of AD (microphotographs with kind permission from J. Pahnke).

Patients affected with AD often also have cholinergic deficits in association with the disease, i.e. the cognitive deterioration is associated with a progressive loss of cholinergic neurons and a subsequent decline in levels of acetylcholine (ACh) in the brain, particularly in the temporal and parietal neocortex and hippocampus (Davies and Maloney, 1976, Whitehouse et al.,

1982). This major impact also includes the reduced activity of choline acetyltransferase especially in the cerebral cortex and hippocampus resulting in a disrupted interneuronal connectivity (Wenk, 2003).

Intracellular, neurofibrillary tangles (NTF's) are a typical hallmark of AD. They primarily consist of hyperphosphorylated tau, a microtubule associated, stabilizing protein (Glenner et al., 1984). During abnormal phosphorylation, tau adopts an altered conformation and becomes delocalised from axonal to somatodendritic compartments (Goedert et al., 1988, Lichtenberg et al., 1988). In addition to neurotoxic Aβ, this leads to a reduced synaptic density, inflammatory reactions and neuronal cell death, which result in neuronal degeneration and brain atrophy (Figure 3**Figure** C) (Ghiso and Frangione, 2002, Klucken et al., 2003, Castellani et al., 2006). Another pathological sign for AD are Hirano bodies, which were first described in 1965 by Hirano (Fechheimer et al., 2002). Hirano bodies are intracellular aggregates of actin and actin-associated proteins in neurons and also lead to neuronal destabilisation and decline (Fechheimer et al., 2002, Maselli et al., 2002).

AD is also associated with morphological changes in capillary networks. In most of the cases, AD coincides with cerebral amyloid angiopathy (CAA), and there is evidence that Aβ itself and in the form vascular beta-amyloid depositions leads to blood vessel dysfunction in animal and human studies (Smith and Greenberg, 2009). Aβ may remain in solution and enter the plasma via perivascular drainage pathways or efflux across the blood-brain barrier (Shibata et al., 2000, Weller et al., 2008). Aβ depositions in the vascular media and adventitia lead to impaired blood vessel morphology and function, and finally to loss of integrity of the vessel wall resulting in brain haemorrhages. Hence, CAA is increasingly recognised as a probable cause of brain ischemia and cognitive impairment independent of stroke (Greenberg et al., 2004).

1.3.3 Beta-Amyloid burden and cognitive decline

Over the course of time a theory was developed that plaque formation and the number of neuritic plaques are weakly correlated to the degree of cognitive dysfunctions. Since the publication of an immunisation study against plaques in the year 2001 it is known that Aβ plaques may not be the critical pathogenic entity (Selkoe, 2001, Smith et al., 2006, Holmes et al., 2008, Larson and Lesne, 2012). Aβ peptides, especially $A\beta_{42}$, are toxic to neurons, and as the result of degeneration of synapses and neurons, brains of AD patients become atrophic and patients suffer from cognitive impairment (Lesné et al., 2008, Varela-Nallar et al., 2010). Hence it is hypothesised that the level of intracerebral $A\beta_{42}$ highly is correlated to the degree of cognitive dysfunctions. Concluding, different processes lead to neuronal decline such as (i) toxicity of $A\beta_{42}$ itself, (ii) destruction of the cytoskeleton by hyperphosphorylated tau proteins and its intraneuronal formations called 'neurofibrillary tangles', and (iii) neuronal

inflammation (Lue et al., 1999, Wenk, 2003, Simi et al., 2007, Wang et al., 2011). There are also various reasons for cognitive decline, namely (i) neuronal death, (ii) reduced synaptic plasticity of especially acetylcholinergic synapses and (iii) plaque formation associated with disrupted neuronal network integrity (Naslund et al., 2000, Mattson, 2004, Xia, 2010).

ABC transporter in AD

ABC transporters (ATP-binding cassette transporters) are the substrate-specialised transport systems of the CNS. They exert numerous important functions at the blood brain barrier (BBB), which is a physical and metabolic diffusion barrier between brain and blood (Algotsson and Winblad, 2007). ABC transporters are a superfamily of large transmembrane proteins that utilise the energy of ATP hydrolysis for translocation of substrates like metabolic products, drugs, lipids and sterols across phospholipid bilayers, widely distributed in eukaryotic cells (Jones and George, 2004). The transporter superfamily consists of 7 subfamilies named ABCA to ABCG which all share one important similarity: their function is based on the highly conserved ATP-binding cassette, the most characteristic feature of the superfamily (Borst and Elferink, 2002). They characteristically build a functional entity via four domains: (i) two transmembrane domains, which form the translocation pathway for its substrates across the membrane, and (ii) two nucleotide-binding domains (NBD's, ATP-binding cassettes domains) for the binding and hydrolysis of (Seeger and van Veen, 2009).

Besides their importance for brain homeostasis and numerous neurogenic functions, in recent years it became evident that an insufficient clearance of Aβ might be the main reason for its slow, but unstoppable accumulation within the brain (Mawuenyega et al., 2010, Schumacher et al., 2012). Several studies have shown that the BBB transporter ABCB1 contributes to this clearance in mouse models and cell culture studies (Lam et al., 2001, Cirrito et al., 2005, Kuhnke et al., 2007, Miller et al., 2008, Krohn et al., 2011, Vogelgesang et al., 2011). Moreover, studies of Krohn and colleagues recently have shown that another ABC transporter (ABCC1) has a vast impact on Aβ-pathology in different mouse models of AD and is a promising therapeutic target (Krohn et al., 2011). These data further support the hypothesis of an impaired clearance as a prominent reason for Aβ accumulation in the brain of sporadic AD patients. Summarising, the importance of the ABC transporters in the BBB for the excretion of neurotoxic Aβ peptides is being intensely discussed, and there are important natural substances such as plant extracts which may be able to modulate rather than activate the ABC transporter in different ways.

Microglia in AD

Brain inflammation caused by neuritic plaques and necrotic cells is a pathological hallmark of AD. Within the Alzheimer aetiopathology, microglia, the macrophages of the central nervous system, are of growing importance. With a portion of about 10% of the cells in the nervous system, they represent the first line of defence in case of toxins, pathogens, other tissue injury, and clearing cellular debris. Different studies indicate neuroprotective abilities e.g. they are associated with Aβ aggregates, which they degrade. Amyloid peptides and their precursor protein APP are potent glial activators (Barger and Harmon, 1997, Paranjape et al., 2012). Hence, inflammation first occurs in pathologically vulnerable regions within an affected AD brain, resulting in increased expression of acute phase proteins and proinflammatory cytokines such as IL-1β, TNFα and IL-6 as well as chemokines like TGFβ (Rubio-Perez and Morillas-Ruiz, 2012).

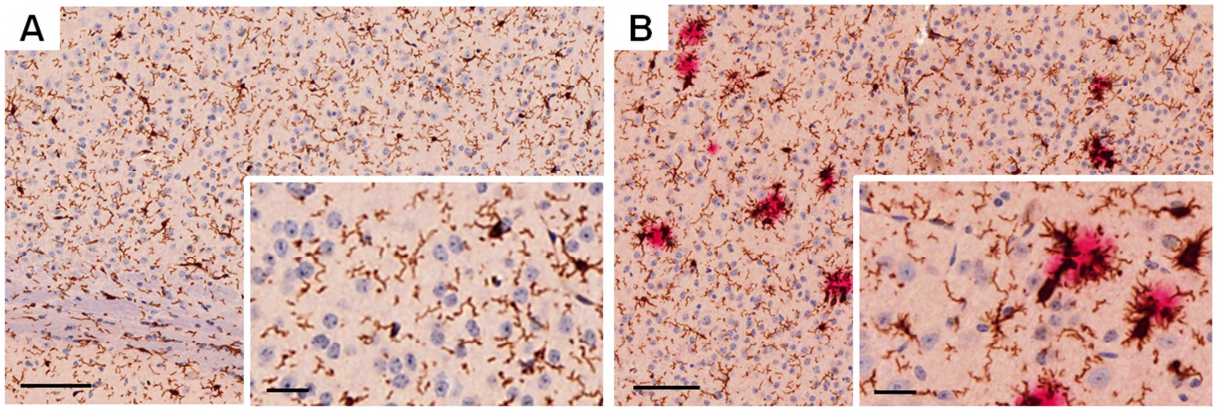

Figure 4. Microglia cells (brown), the macrophages of the CNS act as first line of defense. In healthy brains (A), microglia remain in an inert stage. AD affected brains shows characteristic beta amyloid depositions (red) accompanied by microgliosis (B). Microglia typically surround and degrade these plaques by phagocytic activity (scale bars: 100 µm, 50 µm).

Common causes and genetic risk factors of AD

Within Alzheimer's disease, there are two different recognised variants. One is the so-called familial form based on genetic alterations. In 1991, causes for the pathogenesis of AD have been found in different mutations in the APP-caused autosomal-dominant early-onset form of AD (Suh and Checler, 2002). Only a small percentage of all AD cases, probably less than 5%, is caused by such genetic variations, which were found only in 496 families worldwide (Maurer and Hoyer, 2006). To date, mutations in three genes serve to transmit AD. Mutations of the presenilin gene 1 (PS1) on chromosome 14 and the presenilin gene 2 (PS2) on chromosome 1 both lead to an enhanced production of $A\beta_{42}$ peptides (Selkoe, 2001). The third is a missense mutation in the APP gene on chromosome 21 which increases the probability of amyloidogenic cleavage of APP by the secretase complex (Selkoe, 2001, Haass and Selkoe, 2007). All these mutations share common features of enhancing the β-secretase

cleavage of APP to increase the production of the amyloidogenic $A\beta_{42}$ as the primary component of plaques. The fourth mutation is found on chromosome 19 within the ε4 allele of the apolipoprotein E (ApoE4) gene. Its overexpression influences the density of plaques and increases vascular deposits and thereby provides an important genetic risk factor for the disorder in the typical late-onset period (Strittmatter et al., 1993, Poirier, 1996). There are also other genes that are considered susceptibility or risk factors for AD such as β2-macroglobulin (Blacker and Tanzi, 1998), a gene for a component of β-ketoglutarate dehydrogenase (Ali et al., 1994) as well as several mitochondrial genes. In this familial, inherited AD forms, the disease tends to develop before the age of 65, sometimes in individuals as young as 30 (Law et al., 2001).

The second variant of AD is the sporadic form which includes >95% of all cases. Causes for this form are mainly environmental factors and are not yet sufficiently known. Epidemiological studies have demonstrated risk factors for AD that include age, gender (females are at greater risk), low education, previous head injury, diet, and cardiovascular disease (Law et al., 2001). For the last decade, two major hypotheses on the cause of AD have been proposed. On one hand, the "amyloid cascade hypothesis" proposes that the neurodegenerative process is a series of events triggered by the abnormal processing of the APP (Hardy and Higgins, 1992). The "neuronal cytoskeletal degeneration hypothesis" (Braak and Braak, 1991) on the other hand suggests that cytoskeletal changes are the triggering events. The most frequent sporadic forms of AD are associated with an abnormal accumulation of Aβ (Spillantini et al., 1997, Selkoe, 2001).

1.4 Current therapeutic strategies

To date, there is no cure for AD, only several available pharmacologic treatment options that slightly reduce the symptoms of cognitive impairment in order to slow down the aetiopathology (Ellis, 2005). Typical currently used AD therapeutics comprise different acetylcholinesterase inhibitors: tacrine, rivastigmine, galanthamine and donepezil. These therapeutics share one aim, to buffer the acetylcholine decrease which is caused by the decrease of cholinergic neurons. They act as acetylcholinesterase blockers leading to an increase of the postsynaptic acetylcholine with arguable efficacy in mild to moderate AD (Stahl, 2000). For the symptomatic treatment of dementia in the advanced stages of AD, donepezil is a temperate therapeutic (Ellis, 2005). These therapeutics share partially strong side effects like nausea and vomiting, muscle cramps, decreased bradycardia, anorexia, weight loss, and pyrosis (Howard et al., 2012, Tariot et al., 2012).

A second group of therapeutics comprises memantine, a non-competitive NMDA receptor antagonist. With increased excitotoxicity by glutamate overstimulation, memantine acts as an inhibitor. As an acetylcholinesterase blocker, memantine has also been shown to have minor

effects in the treatment of mild to severe AD (Reisberg et al., 2003). In contrast, Schneider and colleagues studied clinical trials including patients with mild AD and finally reported a meagre effectiveness of memantine notably in the case of moderate AD (Schneider et al., 2011). In addition, this therapeutic shows a wide range of adverse effects including hallucinations, confusion, dizziness, headache, and fatigue (Cummings, 2004, Howard et al., 2012).

Other therapy options in research target the intervention within the Aβ cascade. Main pursuits comprise (i) the inhibition of APP processing, Aβ production and aggregation pathologies, (ii) modulating inflammatory responses and microglial activation as well as oxidative stress and the age-related damaging agents ROS (reactive oxygen species), (iii) the inhibition of Aβ induced neurotoxicity and (iv) countering specific neurotransmitter abnormalities (Pike et al., 1992, Klein, 2002, Cummings, 2004, Dinamarca et al., 2006, Huber et al., 2006, He et al., 2010, Choi et al., 2012). However, all these strategies share at least two major shortcomings. First, to be effective, a significant amount of a therapeutic has to pass the blood brain-barrier (BBB) and second, none of them targets the causative event or mechanism which is still elusive that initially leads to the accumulation of Aβ in the brain of sporadic AD patients.

1.5 Phytopharmacy in the field of AD

Beside previous clinical therapy approaches, to date, a small group of natural substances is under consideration to have beneficial effects on AD. Studies of extracts of *Humulus lupulus*, or hops, showed a variety of positive effects against anxiety; as a herbal sedative it acts against insomnia and bears an emphasis on estrogenic properties (Chadwick et al., 2006, Salter and Brownie, 2010). The hop cone constituent xanthohumol, belonging to the polyphenol family, holds anti-inflammatory and antioxidative properties (Reichling, 2010). Related AD studies indicated that its β-hop acid colupulone binds to the PX-receptor thereby increasing the expression of P-gp (ABCB1), an assumed Aβ transporter (Teotico et al., 2008). Another traditional herb used especially in the field of sleeping disorders and insomnia is *Valeriana officinalis*. It acts as a sleep-inducing aid although its efficacy is controversial (Brattstrom, 2007, Taibi et al., 2007). The green tea plant *Camellia sinensis,* whose consumption started 5000 years ago, bears a lot of positive effects on health. Due to high antioxidative properties it also shows potential in the treatment of cancer and cancer chemoprevention as well as in protecting from cardiovascular diseases (Mak, 2012, Trudel et al., 2012). Polyphenols of the green tea are beneficial agents against psychiatric and cognitive disorders and support a healthy brain (Gomez-Pinilla and Nguyen, 2012). Regarding AD research, the prominent agent Epigallocatechin-3-gallat (EGCG) of the green tea plant *Camellia sinensis* is able to enhance the activity of the α-secretase ADAM10 as well as able to avert the genesis of $A\beta_{42}$

(Obregon et al., 2006). *Ginkgo biloba* has long been used in Asian regions as traditional medicine and became also a very popular traditional herb in the Western world, especially in the treatment of concentration problems, cognitive dysfunction, confusion, anxiety, tinnitus and dizziness. Terpene and flavonoid rich extracts of *Ginkgo biloba* also showed antioxidative and anti-apoptotic effects on cardiac injury in rats (El Boghdady, 2012). Nowadays it has become famous for memory enhancing properties, hence it is in the focus of science especially in the field of neurodegeneration and cognitive aging (Birks and Grimley Evans, 2009, Daffner, 2010). Yet various randomised studies with Ginkgo extracts in AD patients showed in contrast no significant differences between Ginkgo treatment groups and placebo groups (Weinmann et al., 2010). In 2008, a study with a cohort of 482 volunteers aged 75 years or older with mild cognitive impairments (MCI) were assessed every 6 months after twice-daily dose of *Ginkgo biloba* extracts. The study detected no effect in reducing either the overall incidence rate of dementia or AD incidence in individuals with MCI (DeKosky et al., 2008, Vellas et al., 2012). *Rhodiola rosea* showed beneficial effects in a wide range of diseases like depression and anxiety as well as in reducing fatigue (Chan, 2012). It bears cognition improving properties for example in stress induced fatigue due to antioxidative and neuroprotective effects (Fan et al., 2001, Fintelmann and Gruenwald, 2007, Qu et al., 2009). *Aloysia citrodora* (Lemon Aloysia) is a deciduous shrub, widely cultivated in Portuguese home gardens. Its aerial parts are mainly used as stomachic, digestive, sedative, febrifuge and antispasmodic. *In vitro* studies regarding bioactive compounds showed antioxidative properties which also play an important role in AD (Guimaraes et al., 2011). Entirely unknown in the field of AD research are members of the Greek herb *Sideritis* spp., although there are some studies indicating strong anti-inflammatory and antioxidative properties due to their abundance of secondary plant metabolites (Gonzalez-Burgos et al., 2011, Linardaki et al., 2011). Already in the focus of neurodegenerative disorders is the ubiquitous herb *Hypericum perforatum.* Numerous studies attest beneficial indications in pathological aspects of AD like Aβ caused neurotoxicity, disassembling of Aβ depositions and spatial memory decline (Dinamarca et al., 2006, Griffith et al., 2010).

Hypericum perforatum as exploratory focus in AD research

Hypericum perforatum (Saint John's wort, SJW) has a long history of herbal use and has been utilised as a medical plant since the ancient world. It is a yellow-flowering plant with traditional flowering and harvesting on St John's day, 24th June, hence it is also called Saint John's wort (SJW). This herb belongs to the large family of Hypericaceae with approximately 370 species of the genus Hypericum. This herb is distributed worldwide and typically grows wild in many meadows. The species name perforatum refers to the presence of small oil glands in the leaves (Figure 5) (Marjorie Blamey, 2008).

Besides hypericin, hyperforin is one of the main active constituents of SJW and effective in the CNS (Butterweck and Schmidt, 2007, Teotico et al., 2008, Park, 2010). Nowadays, the herb is used in treating a wide range of disorders; it acts as mood lifter in herbal treatment of mild to moderate depression and nervous discomposure (Chatterjee et al., 1998, Cervo et al., 2002, Mennini and Gobbi, 2004, Dinamarca et al., 2006, Linde et al., 2008, Griffith et al., 2010).

Figure 5. *Hypericum perforatum* (Saint John's wort, SJW) is a member of the Hypericaceae family, a ubiquitary herb, which is essentially used to treat mild forms of depression (figures by Prof. Dr. Thomé, and Otto Wilhelm, H. Zell and M. Gasperl).

However, the role of hyperforin with regard to AD is a matter of on-going discussion. Investigations indicate that hyperforin bears memory enhancing properties in rodents (Klusa et al., 2001, Cerpa et al., 2010). Interestingly, hyperforin is also a potent PXR ligand that leads to increased expression of the ABCB1 transporter *in vitro* (Watkins et al., 2003, Ott et al., 2009). Similar results have been shown using colupulone which indirectly increased the expression of ABCB1 at the human blood brain barrier (Miller et al., 2008, Teotico et al., 2008). It is assumed that different extracts of SJW, especially hyperforin-rich ones, enhance the export activity of ABC transporters of the blood brain barrier and thus reduce the concentration of intracerebral monomeric Aβ (Vogelgesang et al., 2011). Afterwards the exported Aβ is removed through the blood stream.

Of particular importance are the results of Dinamarca and colleagues, who detected important effects of intracerebrally injected hyperforin, since it was able to reduce the activation of microglia and to enhance the export function of BBB ABC transporters. It also protects from neurotoxicity in hippocampal neuronal cultures, and is able to disassemble fibrils into amorphous material and protofibrils *in vitro* (Dinamarca et al., 2006). With all its beneficial effects on the molecular mechanisms of AD, hyperforin is able to protect from neuronal damage and memory loss after intracerebral injection of pure hyperforin (Dinamarca et al., 2006). Microglia, the immune effector cells in the brain (Streit et al., 2004), are able to counteract the damaging effects of neurotoxic Aβ in AD by phagocytosis-mediated clearance of protein aggregates. Studies evidenced that hyperforin is a modulator of phagocytic activity of microglia (Kraus et al., 2007, Bateman et al., 2009). Therefore, the survival and health of microglia are critical for preventing neurodegenerative diseases (Streit, 2004). It was shown that flavonoids of SJW reduced the formation of amyloid-induced reactive oxygen species in microglia, which can be activated in response to injury. Indirect neurotoxicity in the presence of Aβ involves activated microglia that produce inflammatory mediators such as cytokines, nitric oxide, and reactive oxygen species (Cotter et al., 1999), further promoting inflammatory processes that contribute to cell loss as well (Akiyama et al., 2000). Investigations of individual single compounds in extracts of SJW revealed that the flavanols, catechin and epicatechin slightly increase cell viability while hyperforin as well as hypericin and pseudohypericin influence cell survival.

In general, SJW is well tolerated when administered in normal dosages. Most common adverse effects are related to hyperforin and some to hypericin. Health problems are allergic reactions, gastrointestinal symptoms, dizziness, confusion, restlessness, lethargy, and xerostomia (Klemow et al., 2011). In addition, SJW may cause photosensitivity and augmented sunburns (Ernst et al., 1998), whereas normal doses of SJW taken for mild depression do not have significant associated phototoxic effects and are well tolerated (Gastpar et al., 2005). *In vitro* studies with human lens epithelial cells and retinal pigment epithelial cells suggest that SJW may have phototoxic effects on the eye. Cells were damaged by exposure to light after hypericin treatment (Wielgus et al., 2007). Hyperforin highly interacts with cytochrome P450 (CYP), an enzyme super family involved in drug metabolism and synthesis of steroid hormones. SJW in some cases increases the effectiveness of other compounds when taken together which may in turn increase the compound's effects to toxic levels. Conversely, the herb may decrease or even cancel effects of other drug constituents (Parker et al., 2001), e.g. it interacts negatively and nullifies the effect of steroid contraceptives (Thummel and Wilkinson, 1998).

Sideritis spp., a traditional Greek herb

Sideritis spp., also known as ironwort, mountain tea and shepherd's tea, is a member of the Laminaceae family. This genus consists of about 150 species and is primarily distributed throughout the Mediterranean region (Gonzalez-Burgos et al., 2011). Its name Sideritis derives from ancient times from the Greek word “sideros” which means “iron” in reference to its properties to heal wounds caused by weapons made of this metal.

Figure 6. *Sideritis* spp. is a member of the Lamiaceae family and distributed throughout the Mediterranean region. There, it is well known as mountain tea and valued for its medicinal properties. Left: exemplarily *Sideritis montana* is shown, right: *Sideritis euboea* (figures by Johann G. Sturm and Deyan Vasilev).

There are many chemical constituents which have been identified in this genus such as (i) terpenes, (ii) flavonoids, and (iii) essential oils (Barberan et al., 1987, Kirimer et al., 2000, Fraga, 2012). These compounds are the main pharmacological effectors and occur in almost every species. Further active compounds are iridoids, coumarins, lignanes, and sterols (Gonzalez-Burgos et al., 2011).

Especially the Greek species *Sideritis scardica* and *Sideritis euboea*, known as mountain tea, have a long history of herbal use in traditional Mediterranean medicine because of their anti-inflammatory, anti-ulcerogenic, and digestive properties (González-Burgos, 2009). Nowadays, the herb is known to enhance the antioxidative defence of the adult rodent brain and to act antimicrobially (Tsaknis and Lalas, 2005). Yet most of the medicinal uses of *Sideritis* spp. are

limited to folk medicine (Gonzalez-Burgos et al., 2011). Members of this genus find also application in the field of common colds, burns and wounds, gastrointestinal disorders, and as a stimulant in general which may explain the popular use of these herbs in traditional medicine (Alipieva et al., 2010). Currently it is largely unknown in academic medicine. However, different scientific approaches detected strong anti-inflammatory effects and antioxidant activities thus preventing oxidative stress which is believed to be one of the causes of disorders affecting the central nervous system (Melo et al., 2011, Grosso et al., 2013, Vasilopoulou et al., 2013). Neurobiological impacts of herbal components, especially flavonoid-rich ones, on cognition and behaviour have been indicated (Vasilopoulou et al., 2011, Rafnsson et al., 2013, Rendeiro et al., 2013, Sokolov et al., 2013).

Sideritis spp. is also renowned to be a rich source of a variety of flavonoids (Gonzalez-Burgos et al., 2011, Linardaki et al., 2011). Studies suggest that these secondary metabolites are able to improve memory capacity by enhancing the efficiency of information storage and retrieval (Spencer, 2010, Rendeiro et al., 2012). There is also increasing evidence that flavonoid-rich foods such as fruit juices and red wine or supplements might delay the initiation of neurodegenerative disorders such as AD or slow down their progression (Williams and Spencer, 2012). Other studies also showed that flavonoids bear anti-inflammatory abilities accompanied by increased Aβ phagocytosis by microglia and macrophages (Kraus et al., 2007). Thus, these properties appeared to be even useful for a possible treatment option in the field of neurodegenerative disorders such as AD in order to counteract neuroinflammation which plays an elementary role neuronal decline (Rao et al., 2012). Moreover, flavonoids function as multi-target botanical agents or drugs and can modulate enzymes and receptor activities (Williams and Spencer, 2012, Grosso et al., 2013). If crossing the BBB as shown for phenolic compounds including nobiletin (Nakajima et al., 2007), EGCG (Cheng et al., 2013), tangeritin (Datla et al., 2007) and quercetin (Kawabata et al., 2010), amongst others a compound of *Sideritis lycia* (Dinçer C et al., 2010), they take effect on brain regions, which are involved in learning and memory, especially the hippocampus (Grosso et al., 2013). Neurobiological actions of flavonoids are believed to be based on (i) direct interactions with cellular cascades enhancing the expression of neuroprotective and neuromodulatory proteins that promote neurogenesis, neuronal function and neuronal connectivity, and (ii) improvement of blood-flow and brain angiogenesis (Sokolov et al., 2013). Studies of Vasilopoulou et al. showed that oral administration of the *Sideritis* species *S. clandestina* prevents anxiety-related behaviours in rodents (Vasilopoulou et al., 2011).

2 Aims of the study

AD is the most common type of neurodegenerative dementias, and also one of the most significant challenges for future health care systems and even for our society. Until today the pathology of AD is only partially understood and apart from symptomatic treatments, no cure is in sight. The use of herbal extracts, partially in medical use since ancient times, might harbour the potential to become a mild treatment option against Alzheimer pathology. In recent times several studies indicated positive effects of herbal components such as SJW's hyperforin, catechine and caffeine of *Camellia sinensis* on pathological changes similar to those seen in dementia. Even though most of the positive results were observed *in vitro,* they are very encouraging and motivated the implementation of the present *in vivo* study in the APP/PS1 mouse model, which aimed to answer the following questions.

1. Using an explorative screening approach, which herbal extracts are promising candidates harbouring effective traits against characteristic morphological Alzheimer's pathologies such as:
    i. intracerebral and neurotoxic $A\beta_{42}$ level,
    ii. plaque load (number, size and cortical coverage)?
2. Can the extract administration of those candidates even significantly counteract the cognitive decline in APP/PS1 mice which is observable during AD pathogenesis?

Follow-up studies on the most promising candidates of the explorative screening which meet predefined criteria should answer the following questions:

3. Which one of two treatment strategies is most potent in affecting AD pathologies:
    i. low dose treatment during AD initiation,
    ii. high dose treatment post AD onset?
4. Do effects on AD pathology in APP/PS1 mice vary between different extraction forms of certain plants presumably due to differing concentrations of specific known bio-active constituents, e.g. hyperforin in Saint John's Wort?
5. Might some of the tested genera also harbour other active species, potentially similarly or even more active against AD pathologies?

In order to characterise the influence of the tested herbal extracts on hallmarks of AD pathology, the mice were observed in histological and molecular biological studies, as well as comparatively analysed in relation to changes in the cognitive capacity during behavioural studies.

3 Material and methods

3.1 Chemicals

2-Mercaptoethanol	Carl Roth GmbH
Agarose	Biozym Diagnostik GmbH
Acetic acid	Carl Roth
Bromphenol blue	Carl Roth
$CaCl_2*(2H_2O)$	Carl Roth
D-Glucose	Sigma
EDTA	MERCK
Ethanol 96%	Carl Roth
Ethidium bromide	Invitrogen
Glacial acetic acid	Carl Roth
Glycerol	Carl Roth
Guanidinhydrochloride	Carl Roth
HCl	Carl Roth
Heparin	Sigma-Aldrich
Igepal CA630	Fluka Biochemika
KCl	Carl Roth
Methanol	J.T. Baker
Milk powder	Carl Roth
$MgCl_2*(6H_2O)$	Carl Roth
NaCl	Carl Roth
NaH_3	Carl Roth
$NaHCO_3$	Carl Roth
Paraffin Paraplast	Leica Biosystems /Menarini
PBS (10x)	Sigma-Aldrich
Paraformaldehyde (4% in PBS)	Formafix Switzerland AG
Proteinase K	Carl Roth
PVDF membrane	Carl Roth
RNAlater	Applied Biosystems
TEMED	Carl Roth
Tris-HCl	Carl Roth
Triton X-100	Carl Roth
Trypan blue	Carl Roth
Tween 20	Merck
Xylene	Carl Roth

3.2 Equipment

Bond-Max staining system	Leica Biosystems
Centrifuge Universal 320R	Hettich
CO_2 incubator	Binder
Embedding system Leica EG 1160	Leica Biosystems
Histokinette STP 120	Mikrom
LICOR Odyssey Imaging scanner	Odyssey LI-COR
NanoDrop 2000 spectrophotometer	Thermo Scientific
Stereo-Mikroskop STEMI DV4 32Z -fach	Zeiss
Microplateshaker – TITRAMAX100	Heidolph
Microtitration plate reader Tecan Sunrise ™	Tecan
Microtome RM 2155	Leica Biosystems
Mini-PROTEAN® Tetra Cell	BIO-RAD
Mirax Desk	Zeiss
Morris water maze	Custom-made
Orbital Shaker OS-20	Lab4you
Plate reader ParadigmTM detection platform	Beckman-Coulter
PerfectBlue Wet-Blot	Peqlab
PreCellys®24 Homogenisator	Peqlab
Spectrophotometer ND-1000	Nanodrop Technologies, Inc.
Thermocycler	Biometra

3.3 Kits

Kit	Company
hAmyloid β_{42} Elisa (high sense)	The Genetics Company
BLUEextended 100bp	Bioron
BCA™ Protein Assay Kit	PIERCE
ECL PLUS Western Blotting Detection System	Amersham Bioscience
ABCB1 PREDEASY™	Solvo Biotechnology
ABCC1 PREDEASY™	Solvo Biotechnology
Page Ruler™ Prestained Protein Ladder	Fermentas
Complete Mini protease inhibitor cocktail	Roche Diagnostics
Bond-MaxTM Bond Polymer Refine Detection-Kit	Leica
Odyssey™ Blocking Buffer	Odyssey LI-COR
Mini-PROTEAN TGX Any kD Stain-Free Gel	Bio-Rad

3.4 Software

AxioVision	Zeiss Microimaging GmbH
MS Office	Microsoft
Prism 5	GraphPad Software, Inc.
Odyssey® 3.0 Application Software	Odyssey LI-COR

3.5 Mice and diets

3.5.1 Animal procedures

Mouse model

Transgenic B6 mice expressing the human Amyloid precursor and Presenilin1 genes under control of the Thy1 promoter (APPswe, KM670/671NL, PS1-L166P) (Radde et al., 2006) were provided by R. Radde and M. Jucker (University of Tübingen, Germany) and are hereafter referred to as APP/PS1. This mouse model is characterised by a fast pathology starting with an early Aβ deposition within the first 6 weeks of age (see Figure 7). All animals were bred in the animal care facility of the Neurodegeneration Research Lab (NRL) at the University of Rostock, Germany. The mice were housed under a 12 h/12 h light/dark cycle under maintenance of a mean temperature of 22 °C. The animals had access to food (Sniff, Germany) and water ad libitum. All protocols involving the use of animals were approved by the local authorities.

Genotyping of mice

Tissue samples of mice tails were used for genotyping. They were digested overnight at 55 °C in mouse tail lysis buffer with solved Proteinase K (see Table 3), afterwards denatured at 95 °C for 30min. To detect the transgenic status of the APPPS1 transgenic mice, a specific primer pair was used, which lead to the amplification of a 250 bp sized DNA in case of a present APPPS1 transgene in the sample genome. The used primers were APPswe-forward 5' GAATTCCGACATGACTCAGG 3' and APPswe-reverse 5' GTTCTGCTGCATCTTGGACA 3'.

Table 3. DNA extraction buffer

Compound	Concentration
Tris-HCl	100 mM
KCl	1 M
Tween 20	0.1% v/v
Igepal CA-630	0.4% v/v
dH_2O	appropriate volume

To amplify selected DNA fragments, different steps are performed by the thermocycler: First, the heat denaturation step that separates both DNA strands at 95 °C. Second, in the annealing step, the primers attach to their specific DNA sequence and finally, in the elongation step the DNA polymerase (Taq-Polymerase) elongates the primers with the appropriate nucleotides to produce exact copies of each single-stranded DNA. Denaturation, annealing and elongation by Taq-polymerase at 72 °C steps are repeated up to 40 times. The resulting DNA amplificates are separated by size using agarose gel electrophoresis resulting in products of distinct size that are visualised by binding fluorescing ethidiumbromide.

Table 4. TAE buffer (50x)

Compound	Concentration
Tris	242 g
HCl	51.7 ml
EDTA (pH 8.0)	60 mM
dH_2O	appropriate volume

Table 5. DNA loading dye (6x)

Compound	Concentration
TrisHCl	10 mM
Bromphenol blue	0.03% v/v
Xylene cyanol FF	0.03% v/v
Glycerol	60% v/v
EDTA (pH 8.0)	60 mM
dH_2O	appropriate volume

3.5.2 Extracts

For analyses, different herbal extracts were applied at appropriate dosages (see Table 6). All extracts were industrially compounded by Finzelberg GmbH & Co KG and were quality and purity proved. The extraction medium was fully evaporated so that the extract resulted in an ethanol-free and water soluble powder. For the purpose of administration, extracts were dissolved in water only. Extracts dispersions were provided freshly each day right before administration.

Table 6. Herbal extracts used for an exploratory screening in the APP/PS1 mouse model: extract codes, solvents, drug/extract-ratio (DER), daily dosages and animal number used for investigations.

Herb	Charge	Extract code	Extraction medium	DER native	Dose	Animals (n)
Hypericum perforatum	TPA 51-08	SJW80low	80% EtOH	3-6:1	4 g/kg	11
Valeriana officinalis	08006114	Valerian	35% EtOH	3-6:1	9 g/kg	7
Humulus lupulus	TPA 136-08-2	Hops/c (Colupulone)	40% EtOH	4-8:1	1.5 g/kg	5
Humulus lupulus	TPA 136-08-1	Hops/x (Xantohumol)	Aqua dest.	4-8:1	1.5 g/kg	4
Camellia sinensis	18804/09	Camellia/ct (catechine)	Aqua dest.	n/a	4 g/kg	6
Camellia sinensis	18808/09	Camellia/tn (theanine)	Aqua dest.	n/a	4 g/kg	6
Sideritis euboea	UB 2009-23-2	Sideritis	20% EtOH	6:1	12 g/kg	10
Rhodiola roseae	TPA 145-10	Rhodiola	Aqua dest.	3:1	2 g/kg	6
Ginkgo biloba	TPA 127-11	Ginkgo/T (Tebonin® CRG 0110211)	60% acetone	35-67:1	2.4 g/kg	6
Ginkgo biloba	09011698	Ginkgo/TA (Tebonin analogue)	60% acetone	35-67:1	2.4 g/kg	8
Aloysia citrodora	UB 2010-91	Aloysia/A	Aqua dest.	4:1	4 g/kg	6
Aloysia citrodora	UB 2010-97	Aloysia/E	20% EtOH	4:1	4 g/kg	6

For additional analyses of SJW, different extraction forms characterised by varying hyperforin concentrations were provided by Finzelberg GmbH & Co KG and applied for the study (AD initiation: 400 mg/kg; post AD onset: 4 g/kg bodyweight; typical human dosages). Aerial parts of SJW were extracted using water, 60% ethanol, and 80% ethanol to yield extracts with different contents of hyperforin (Hf) and hypericin (Hi). The amount of extractable substances was strongly determined by the selected strength of the solvent (see Table **7**). To verify the SJW80high extract, LAIF®900 (Steigerwald Arzneimittelwerk GmbH) was tested as an analogue.

Table 7. SJW extracts used for treatments in the APP/PS1 mouse model: St. John's wort extracts, solvents, hyperforin and hypericin contents, drug/extract-ratio (DER), and animal number used for investigations. Extracts with a relative content of hyperforin with less than 1.5% are denoted as 'low', and with more than 2.5% as 'high'.

Extract	Charge	Extraction medium	Hyperforin content (Hf)	Hypericin content (Hi)	DER native	Animals (n) pre/post onset
SJWwater	UB 2008-66	Aqua dest.	0.00% (-)	0.002%	8-12:1	6 / 4
SJW60low	UB 2008-67	60% EtOH	1.45%	0.215%	3-6:1	7 / 5
SJW60high	UB 2008-68	60% EtOH	6.08%	0.191%	3-6:1	6 / 4
SJW80low	TPA 51-08	80% EtOH	0.32%	0.090%	3-6:1	7 / 11
SJW80high (LAIF®900)	TPA 170-09	80% EtOH	2.88%	0.165%	3-6:1	5 / 6

Within the genus Sideritis, the species *Sideritis euboea,* the species *Sideritis scardica* and a 1:1 combination of both were applied (AD initiation: 1.2 g/kg; post AD onset: 12 g/kg body-weight) for additional analyses of effects on the AD mouse model by *Sideritis* spp. (Table 8).

Table 8. *Sideritis* spp. extracts used for treatments in the APP/PS1 mouse model: Sideritis species, solvents, drug/extract-ratio (DER), and animal number used for investigations.

Name	Charge	Extraction medium	DER native	Animals (n) pre/post onset
Sideritis euboea	UB 2009-23-2	20% ethanol	6:1	6 / 6
Sideritis scardica	UB 2009-22-2	20% ethanol	6:1	5 / 6
Sideritis extract combination	UB 2009-38 UB 2010-22	20% ethanol	6:1	10 / 6

Extract administration

Extracts dispensed in water and water as vehicle control where daily applied in female mice until an age of 100 days. Solutions were applied orally by gavage (20 gauge).

3.5.3 Study design

Prescreening

To determine promising extracts on AD pathological hallmarks in APP/PS1 mouse model, mice were first subjected to a general screening containing various methods including (i) Morris water maze to detect memory impairments, (ii) changes in the intracerebral beta amyloid load, especially of soluble species via ELISA as well (iii) its depositions in terms of plaques via quantification of immunohistochemical stainings. Mice were treated daily with high dosages of extracts of various herbal species similar to human dosages from day 50 on, i.e. a few days after onset of AD pathology, for further 50 consecutive days until the age of 100 days. To pinpoint promising extracts for further studies five behavioural and morphological parameters were analysed and indicate the quality of the herbs' effect on the severity of the disease in an APP/PS1 mouse model: (i) memory, (ii) intracerebral $A\beta_{42}$ level, (iii) plaque number, (iv) plaque size and (v) the cortical coverage by plaques. No less than four parameters had to be fulfilled, whereby an increase in the cognitive performance in comparison to the vehicle treated control mice was mandatory.

In-depth study

For further analyses of screening favourites, two different experimental strategies with treatments before and after the onset of AD characteristics were pursued:

(i) in the AD initiation paradigm, the mice were treated daily from an age of 40d, simultaneously with the initiation of Aβ deposition, until an age of 100 days with a dosage corresponding to the human dosage,

(ii) the post AD onset treatment strategy mimicked a "therapeutic" approach with a 10 times higher extract dosage starting at the age of 50 d, when initial amyloid deposits were already present.

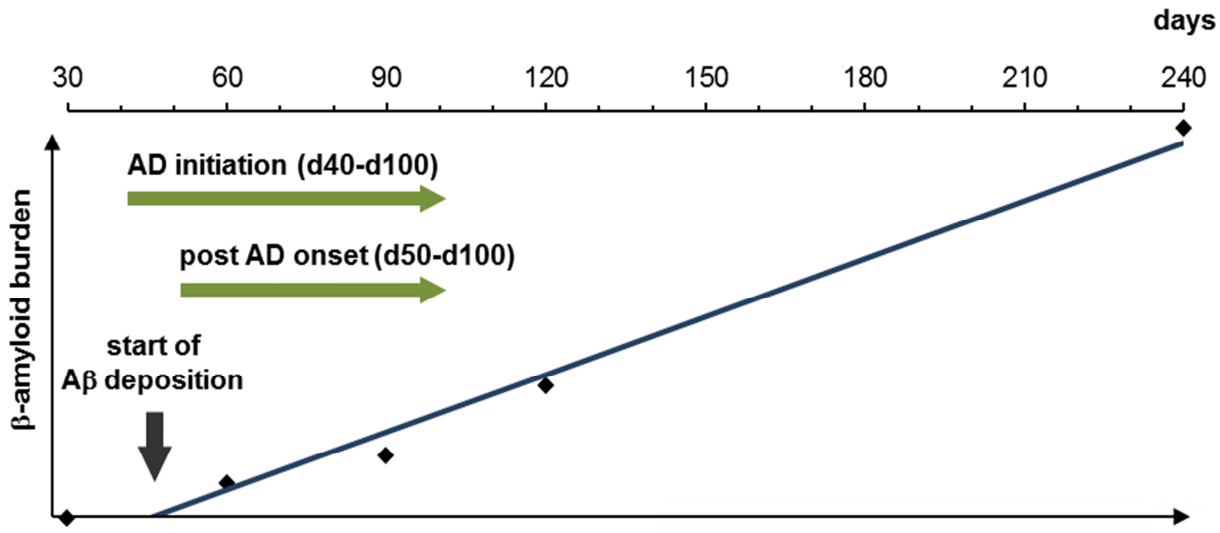

Figure 7. Schematic illustration of the increasing Aβ burden within the aetiopathology in APP/PS1 mice. Test procedures of SJW and Sideritis extracts were composed of two experimental strategies (i) AD initiation treatment and (ii) post AD onset treatment 10 days after onset of Aβ deposition until an age of 100 days. The tissue was recovered at day 100 after behavioural testing procedure (data points adapted from (Radde et al., 2006)).

3.6 Morris water maze

Experiments were carried out according to the protocol of the Johns Hopkins Neurogenetics and Behavior Center (Morris, 1984, Sumit Narwal, 2012) with minor adaptations. At the age of 95-100 days the animals' ability to learn and remember the spatial location of a hidden platform in a circular pool of water (Ø 130 cm, maintained at 19-21 °C) was tested. The water was made opaque by addition of low-fat milk, and the platform was hidden 1 cm below the water surface. Several landmarks were placed on the walls surrounding the pool. The water maze was conceptually divided into 4 quadrants, and each animal's trace was recorded by a camera during the experiment. Mice were trained and tested in two sessions per day over a period of 4 consecutive days. The two sessions were separated by 4 hours and consisted of 4 trials per session. The platform was centrally located in the southwest quadrant. At the beginning of every training day, trials without the platform were given for free swimming, lasting 30 sec, followed by four platform trials. The start position of the first trial of each session was south, while the start position was changed counter-clockwise in the subsequent trials to east, north and west. The animals were allowed to move freely until they reached the platform or until 1 min had elapsed. In case the animals did not reach the platform, they were put on the platform for 15 sec before returning them to the home cage until the next trial.

3.7 Tissue preparation

For the tissue preparation, mice were sacrificed by cervical dislocation on the last day of treatment and behavioural testing, and transcardially perfused with PBS. Brains were removed, thereof one hemisphere was stored in 4% PFA for paraffin embedding and immunohistochemistry, the other half was snap frozen in liquid nitrogen and stored at -80 °C. Frozen hemispheres were thawed in 500 µl RNAlater® (Ambion) for one hour on ice and afterwards homogenised for 12 s at 6000 rpm with a Homogenizer (PreCellys®24) for immediate biochemical analysis or stored for later use at -80 °C.

3.8 Protein biochemistry

3.8.1 Total protein quantification via BCA

The BCA™ Protein Assay (formulation based on bicinchoninic acid) was used for detection and quantification of total protein. The protein concentrations of brain homogenates are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). The samples (stored at -80 °C) were thawed on ice for at least 30 minutes and intensely vortexed. Assays were performed according to the manufacturer's instructions using appropriate dilutions.

3.8.2 Total protein quantification via spectrophotometry

For the protein quantification via spectrophotometry, a sample volume of 2 µl was measured in duplicate using a spectrophotometer. The extinction was taken at 280 nm for protein content estimation with 1 OD representing 0.6 respectively 0.5 mg protein per ml within the GuaHCl or the Carbonate fraction. All spectra were normalised to the extinction at 320 nm.

3.8.3 Enzyme-Linked-Immunosorbent-Assay

To detect the effects of orally given herbal extracts on highly neurotoxic intracerebral $A\beta_{42}$ levels, the Enzyme-Linked-Immunosorbent-Assay (ELISA) method was used. To determine the content of both soluble and insoluble $A\beta_{42}$ species an ELISA Kit (TK42HS, The Genetics Company Schlieren, Switzerland) was used. A defined assemblage of Carbonate buffer was added to 20 mg brain homogenates, homogenised (PreCellys®24, 12 s, 6500 rpm), and separated into carbonate-soluble supernatant and insoluble pellet by centrifugation (20 min, 14,000 rpm, 4 °C). The resulting supernatant (buffer-soluble fraction) was mixed with 8 M guanidine HCl (1:1.6) and represents the buffer-soluble $A\beta_{42}$ species. Subsequently the pellet was dissolved and shaken in 5 M guanidine hydrochloride buffer (1300 rpm for 3 h at room temperature), and then centrifuged (20 min, 14,000 rpm, 4 °C) to extract the aggregated $A\beta_{42}$ species. The resulting supernatant represents the guanidine-soluble fraction. Protein contents

of all samples were measured in duplicate using a spectrophotometer. ELISAs were performed according to the manufacturer's instructions using appropriate dilutions.

Table 9. Carbonate buffer (pH 11.5)

Compound	Concentration
Na_2CO_3	100 mM
$NaCl_2$	50 mM
Protease-Inhibitor (Complete-mini, Roche)	1 tablet per 100 mL
dH_2O	appropriate volume

Table 10. 5 M Guanidin-hydrochloride buffer (pH 8.0)

Compound	Concentration
Guanidin-HCl	5 M
Tris-HCl	50 mM
dH_2O	appropriate volume

Table 11. 8 M Guanidin buffer (pH 8.0)

Compound	Concentration
Guanidin-HCl	8.2 M
Tris-HCl	82 mM
dH_2O	appropriate volume

3.8.4 Western blot

For Western blotting, tissue homogenates were prepared into three protein fractions as described by Lesné et al. (Lesné et al., 2006). For protein detection, samples from the choroid plexus were homogenised in RIPA buffer. Total protein concentrations of protein samples were determined using a BCA™ protein assay kit (Pierce, part of Thermo Fisher Scientific, Rockford, USA). After SDS-PAGE of 10 µg total protein per lane, proteins were blotted onto a PVDF membrane. Blots were probed for either MDR1 (D11, 1:500, Santa Cruz), MRP1 (1:200; Alexis Biochemicals), ADAM10 (1:1000, Calbiochem), BACE1 (1:1000, Abcam), or β-actin (1:20,000, Sigma) dissolved in Odyssey® blocking buffer (LI-COR). As detection antibodies, IRDye® secondary anti-mouse, anti-rat and anti-rabbit antibodies of LI-COR (all diluted 1:10,000) were used. The Odyssey® two-channel IR direct detection system (LI-COR) was used for visualisation and for relative quantification of the target proteins.

Table 12. Protein extraction buffer I (pH 7.6)

Compound	Concentration
Tris-HCl	100 mM
NaCl	150 mM
EDTA	2 mM
PMSF	1 mM
SDS	20% v/v
Igepal CA-630	0.01% v/v
Protease inhibitor (Complete-mini, Roche)	1 tablet per 100 mL
dH_2O	appropriate volume

Table 13. Protein extraction buffer II (pH 7.6)

Compound	Concentration
Tris-HCl	100 mM
NaCl	150 mM
Triton X-100	0.1% v/v
dH_2O	appropriate volume

Table 14. Protein extraction buffer III (pH 7.6)

Compound	Concentration
Tris-HCl	50 mM
NaCl	137.5 mM
EDTA	5 mM
PMSF	1 mM
NP40	1% w/v
Glycerol	10% w/v
Protease inhibitor (Complete-mini, Roche)	1 tablet per 100 mL
dH_2O	appropriate volume

Table 15. SDS-PAGE running buffer

Compound	Concentration
Tris	25 mM
Glycin	192 mM
SDS	0.1% w/v
dH_2O	appropriate volume

Table 16. SDS-PAGE transfer buffer

Compound	Concentration
Tris	25 mM
Glycin	192 mM
Methanol	20% v/v
dH_2O	appropriate volume

Table 17. 10x Tris buffered saline (TBS, pH 7.4)

Compound	Concentration
Tris	500 mM
NaCl	1.5 M
dH_2O	appropriate volume

Table 18. Tris buffered saline Tween20 (TBST)

Compound	Concentration
10x TBS	10% v/v
Tween20	0.01% v/v
dH_2O	appropriate volume

Table 19. 10x PBS (pH 7.4)

Compound	Concentration
NaCl	1.37 M
KCl	27 mM
Na_2HPO_4	120 mM
KH_2PO_4	20 mM
dH_2O	appropriate volume

Table 20. 4x protein sample buffer

Compound	Concentration
Tris/HCl	200 mM (pH 6.8)
Glycerol	40% v/v
SDS	16% v/v
Mercaptoethanol	4% v/v
Bromphenol blue	0.25% v/v
dH_2O	appropriate volume

3.9 Immunohistochemistry

Brains were prepared as described before in section 6.7. In brief, brain hemispheres were post-fixed for at least 24 hours in 4% paraformaldehyde prior embedding the samples in paraffin (see Table 21). For immunohistochemistry, 4 µm thick coronal sections were stained using a BondMax™ (Menarini/Leica) automated immunostaining system. Sections were pretreated with 98% formic acid for 5 min and immunostained for Aβ using the anti-human Aβ clone 6F3D (1:200, Dako) and the Bond™ Polymer Refine Detection kit (Menarini/Leica). For double-stained slides, microglia were immunostained on the same sections using anti-Iba1 (1:1000, Wako) and the Bond™ Polymer AP-Red Detection kit (Menarini/Leica). Neurons were stained with anti-NeuN antibody (1:1000, Millipore). Whole tissue sections were digitised at high resolution (230 nm) using the MiraxDesk scanner and finally semi-automatically analysed using the AxioVision software package (Zeiss, Germany).

Table 21. Dehydration protocol for PFA fixed tissues before slicing and staining

Step	Reagent	Time (min)
1	Formalin 4% buffered	5′
2	Ethanol 70%	180′
3	Ethanol 80%	60′
4	Ethanol 80%	120′
5	Ethanol 90%	60′
6	Ethanol 90%	60′
7	Ethanol abs.	120′
8	Ethanol abs.	120′
9	Xylol	120′
10	Xylol	120′
11	Paraffin 60 °C	240′
12	Paraffin 60 °C	240′

3.10 $A\beta_{42}$ phagocytose assay

In vitro microglia activity was analysed by the Aβ-phagocytose assay according to Floden and colleagues (Floden and Combs, 2006). Briefly, neonatal animals were killed by decapitation, and brains were removed under sterile conditions. Cortices were mechanically dissociated using a scalpel followed by chemical dissociation in trypsin/EDTA (Merck KgaA) for 15 min at 37 °C. Cells were placed on ice in 25 ml DMEM for 15 min to allow larger tissue chunks sink to the ground, afterwards the supernatant was transferred into fresh medium. This step was repeated 3 times. After centrifugation (315 rpm, 15 min), the pellet was resuspended in 1

ml medium, and cells were seeded in 75 cm^2 flasks ($2x10^6$). Cells were incubated at 37 °C and 5% CO_2 for 21 d (Moussaud and Draheim, 2010). Afterwards, cells were shaken at RT for 45 min at 300 rpm. The medium was transferred into a 50 ml tube, centrifuged (315 rpm, 15 min) and cells seeded in 100 µl medium in wells of a 96 well plate (30.000 per well). After incubating for 1 hour at 37 °C, cells were incubated with water solved extracts (400 µg/ml) for 18 hours at 37 °C and afterwards incubated with 500 nM fluorescent-labelled $A\beta_{42}$ for additional 6 h. Cells were washed with PBS, transferred into a black 96 well plate and measured with the PARADIGM™ spectrophotometer (Beckman-Coulter) at excitation of 485 nm and emission at 535 nm.

3.11 ABC transporter activity assay

In vitro ABCB1 and ABCC1 activity was measured using the cell free SB MDR1 and MRP1 PREDEASY™ ATPase Kit (Solvo Biotechnology) according to manufacturer instructions. In brief, a provided membrane preparation of Sf9 cells containing human ABCB1 or ABCC1 was diluted in assay buffer to give 8 µg membrane per well. Assay mixtures were prepared either with or without standard substrate (ABCB1 substrate: verapamil, ABCC1 substrate: NEM-GS) and with or without vanadate as control for nonspecific ATPase activities. Powdered extracts were diluted in DMSO (Sigma) and applied in final concentration of 0.05 µg/ml. Samples were measured using the PARADIGM™ spectrophotometer (Beckman-Coulter) at 610 nm.

3.12 Statistical analysis

Results were presented as mean ± standard error of the mean (SEM). A statistical analysis for value distribution revealed logarithmic-normal (base e) distributed values. For significance calculation, a non-parametric Mann-Whitney test was performed after the values were transformed into normal distribution. A probability level just as or less than 0.05 was accepted as significant.

4 Results

4.1 Influence of herbal extracts on APP/PS1 mice by an explorative screening

To detect and select traditional herbs that are potentially beneficial in counteracting AD characteristic hallmarks, a comprehensive methodical screening was performed with different extracts of various herbal species. This screening was composed of quantifications of (i) the level of cognitive decline via Morris water maze, (ii) the toxic intracerebral $A\beta_{42}$ level via ELISA measurements and (iii) its deposits via immunohistochemistry.

The screening's principal was the application of high dosed endemic herbs like *Hypericum perforatum, Valeriana officinalis* and *Aloysia citrodora* as well as herbs from traditional Chinese and Greek medicine including *Sideritis euboea* and *Rhodiola roseae*. Herbs known from literature with positive effects regarding the Alzheimer pathology like the tee plant *Camellia sinensis* and *Humulus lupulus* as well as extracts of *Ginkgo biloba*, used in the field of memory impairments, were also tested (see Table 6).

4.1.1 Reduction of the intracerebral $A\beta_{42}$ level

Fundamental neuronal decline typical for AD is the toxically elevated brain Aβ level. ELISA measurements of the most toxic species $A\beta_{42}$ are an indicator for the efficacy of the extract in the treatment of APP/PS1 mice.

The treatment for 50 days with different herbal extracts at high dosages led in some cases to Aβ decreasing effects. The application of SJW80low led to a significant reduction of intracerebral $A\beta_{42}$ by 52.1% (Figure 8A). Both, the Greek herb *Sideritis euboea* and *Rhodiola roseae* highly reduced the $A\beta_{42}$ concentration by 55.0% (Sideritis) and 32.0% (Rhodiola, Figure 8F). Studies of two *Ginkgo biloba* extracts indicated also significantly decreased $A\beta_{42}$ levels by 35.8% (Ginkgo/T) and 26.4% (Ginkgo/TA, Figure 8D). Mice treated with a catechin-rich *Camellia sinensi* extracts significantly reduced the Aβ level by 24.3%. In contrast, the theanine-rich Camellia extract did not change the $A\beta_{42}$ levels in any way (Figure 8C). The screening of Valerian, both extracts of *Humulus lupulus* and both Aloysia extracts showed that an application for 50 days did not affect soluble $A\beta_{42}$ species (Figure 8A, B, E).

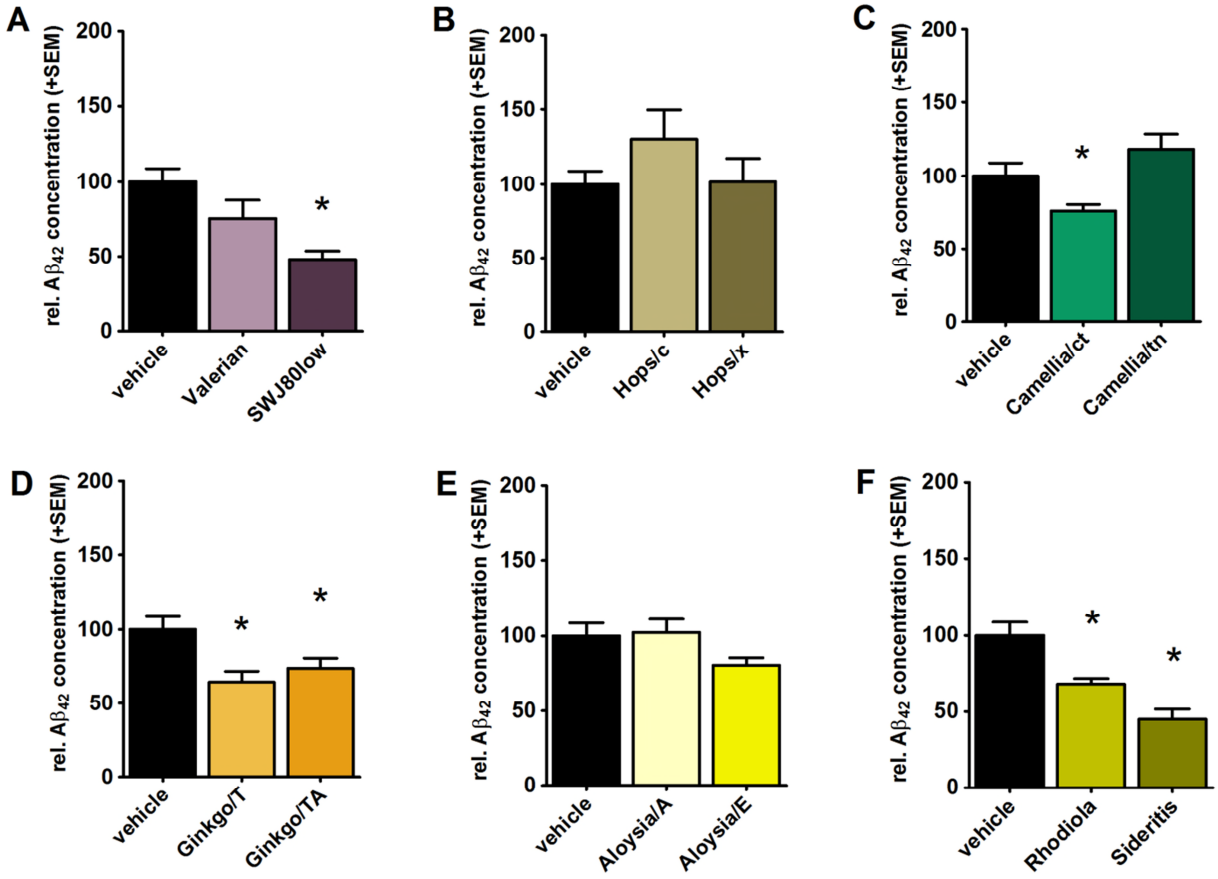

Figure 8. Influence of herbal extracts on intracerebral $A\beta_{42}$ concentration: $A\beta_{42}$-ELISA measurements of brain homogenates, normalised to vehicle treated littermates. Extracts were applied according to the post AD onset treatment strategy. Application of extracts reduced $A\beta_{42}$ concentration in APP/PS1 mice after treatment with (A) SJW80low, (C) Camellia/ct, (D) Ginkgo/T and Ginkgo/TA, as well as (F) Rhodiola and Sideritis (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.1.2 Decrement of cortical Aβ depositions

Effects of extract treatment on plaque number

With increasing age, soluble $A\beta_{42}$ species are prone to aggregate to insoluble oligomers and to fibrils, and finally to deposit as extracellular Aβ plaques. Immunohistological analyses of brain sections of mice stained for Aβ after 50 d treatment at an age of 100 days indicated effects on the most prominent pathological hallmark of AD, the senile plaques.

An application of SJW significantly reduced the plaque number by 35.7% (Figure 9A). Mice treated with hops/c even showed an increase in the plaque number by 42.4%, while administration of hops/x did not affect the deposition number at all (Figure 9B). The green tea plant extract Camellia/ct also was able to decrease the deposition rate by 30.0% (Figure 9C). In addition to reducing soluble Aβ levels, Sideritis application caused a reduction in the plaque number by 40.5% (Figure 9F). The extract Ginkgo/TA lowered the plaque number significantly by 49.6%. Ginkgo/T on the other hand had no significant effect on plaque number (Figure 9D). Hops/x, Camellia/tn, both Aloysia extracts as well as Rhodiola treatment did not significantly change the deposition rate (Figure 9B, C, E, F).

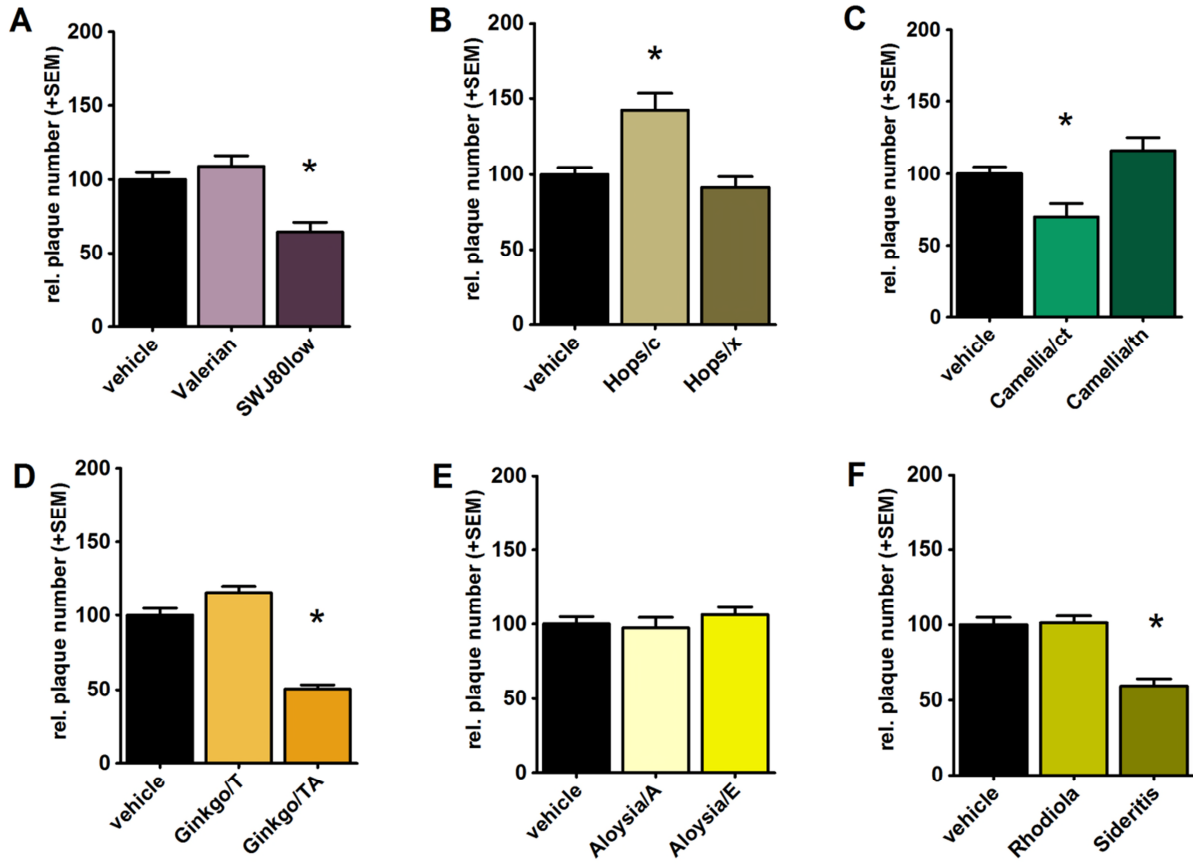

Figure 9. Reduction of neocortical plaque number: Quantification of Aβ-deposits immunohistochemically stained with 6F3D in cortical microphotographs. Post AD onset treatment led to a significant decrease in plaque number in (A) SJW, (C) Camellia/ct, (D) Ginkgo/TA, and (F) Sideritis scardica treatment group. Only an application of Hops/c led to a significant increase in the Aβ-deposition rate (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

Influence of extract application on plaque size

In addition to the number of plaques, also their size was analysed. As seen in the plaque number parameter, the size is also significantly reduced after treatment with high dosed SJW extract by 21.5% (Figure 10A). In contrast, application of both green tea extracts led to a significant reduction in size by 36.1% (Camellia/ct) and 49.2% (Camellia/tn, Figure 10C). Depositions of mice treated with either Ginkgo extract showed plaque sizes reduced by 31.3% (Ginkgo/T) and 27.9% (Ginkgo/TA, Figure 10D). Similarly, Sideritis treated mice exhibited smaller plaques, reduced in size by 35.7% (Figure 10F). The plaque size in mice of the Valerian and Rhodiola group did not change significantly (Figure 10A, F). Treatment with neither Hops nor Aloysia extracts led to reductions of plaque size (Figure 10B, E).

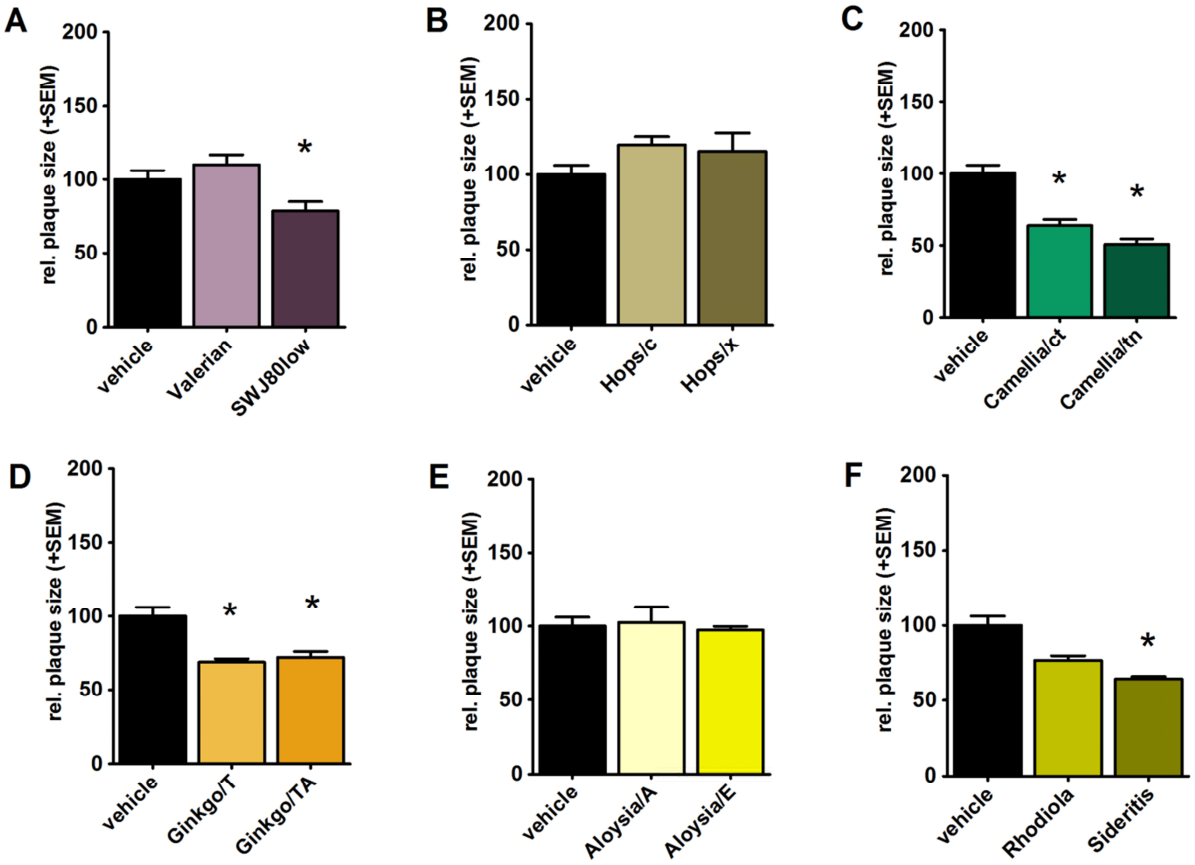

Figure 10. Influence on plaque morphology: Size analyses of Aβ-deposits based on cortical brain regions immunohistochemically stained with 6F3D. Extract application in post AD onset manner resulted in a significant diminution of plaque size after application of (A) SJW80low, (C) both Camellia extracts as well as (D) both Ginkgo extracts and (F) Sideritis (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

Effects of herbal extracts on cerebral plaque coverage

Both plaque number as well as size provide information on the overall plaque load in the APP/PS1 mouse model, represented by the coverage of brains with Aβ deposits relative to the cortex area referred here as plaque coverage. Referring to the results above, immunohistochemical analyses of brain sections against Aβ indicated no changes in plaque coverage after Valerian treatment but a significantly reduced plaque load by 47.0% after SJW therapy (Figure 11A). Treatments with different extracts of Camellia indicated a reduction of the overall plaque coverage by 57.2% for Camellia/ct and in contrast no effects on plaque morphology and thus on plaque burden for Camellia/tn (Figure 11C). Extract application of Sideritis led to a significant decrease in plaque number, size, and hence plaque burden by 62.3% compared to mice treated with the vehicle solution (Figure 11F). Within the Ginkgo extract group, only Ginkgo/TA showed a reduced plaque load by 66.7% (Figure 11D). The Ginkgo/TA and Rhodiola extract were not able to affect the overall plaque load (Figure 11D, F). Mice treated with the Hops/c and Aloysia showed no changes in plaque burden compared to vehicle treated controls (Figure 11B, E).

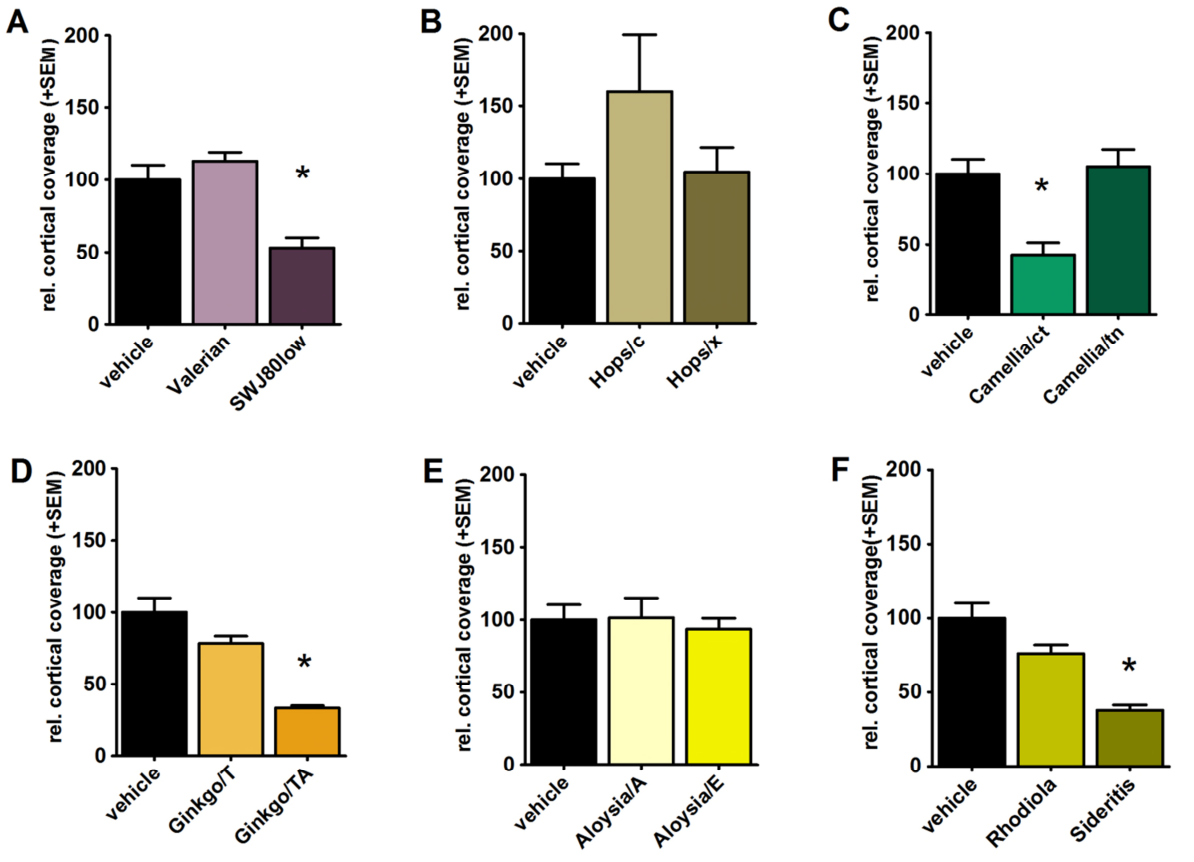

Figure 11. Reduction of the plaque burden: Combination of both parameters, number and size of Aβ deposits, in cortical areas immunohistochemically stained with 6F3D. The reduction of plaque number and size in APP/PS1 mice treated with herbal extracts according to the post AD onset strategy finally resulted in a lowered Aβ burden in the following groups: (A) SJW80low, (C) Camellia/ct, (D) Ginkgo/TA and (F) Sideritis (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.1.3 Memory enhancing properties of herbal extracts

To evaluate whether different plant extracts have a positive effect on cognitive functions in the case of an AD symptomatology, the screening first started with the behavioural testing procedure using a Morris water maze at an age of 100 d to compare spatial memory function of vehicle treated and extract treated mice. At this age, untreated animals of the APP/PS1 mouse model develop high levels of neurotoxic $A\beta_{42}$ and its depositions, resulting in a high-grade neuronal degeneration and memory decline. Measurements of the escape latency significantly indicated an intense memory decline of this 100 d old APP/PS1 mouse model. In comparison to their non-transgenic littermates they showed significantly increased escape latency values at the second and third day of testing (day 3: +54.4%, day 4: +37.5%). Two of twelve extracts had beneficial effects in the AD mouse model. The low hyperforin extract of *Hypericum perforatum* (SJW80low) as well as the extract of *Sideritis euboea* strongly improved cognition after 50 d treatment as shown by constantly reduced escape latency values from the second test day on (day 3: -37.8% SJW80low, -51.2% Sideritis) and especially at the third test day (day 4: -52.6% SJW80low, -39.9% Sideritis) compared to their vehicle treated littermates (Figure 12A, F).

Camellia/ct led to inconsistently decreased escape latencies already at the first test day (day 2: -40.3%), but no changes in memory performance in comparison to vehicle treated littermates at day two and three of testing (Figure 12C). Valerian and Ginkgo/T led to an inconsistently improved memory performance only at the second test day (reduction of escape latency at day 3: -38.3% Valerian, -38.1% Ginkgo/T, Figure 12A, C, D). In contrast, Hops/c led to a deteriotated memory performance, shown by increased escape latencies by 64.8% (Figure 12B). Hops/x, Camellia/tn as well as Ginkgo/TA, both Aloysia extracts, and Rhodiola did not change the memory performance of mice in comparison to their vehicle treated littermates (Figure 12B, D, E, F).

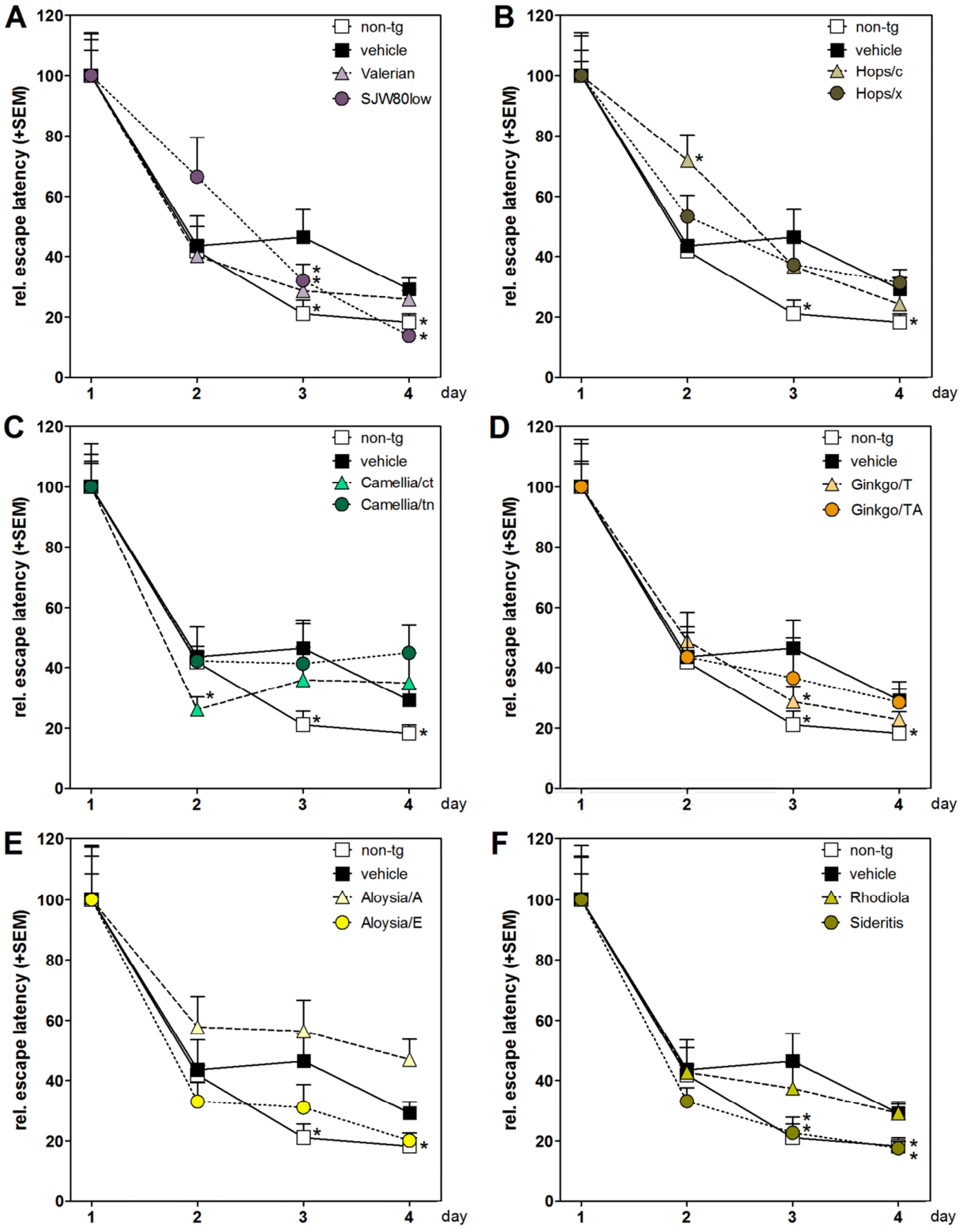

Figure 12. Improvement of spatial memory performance: Morris water maze test of APP/PS1 mice treated with herbal extracts in comparison to vehicle treated and non-transgenic littermates. Mean escape latencies were normalised to their day one performances. Post onset treatment with (A) SJW80low and (F) Sideritis led to a steadily improved memory performance as shown by significantly decreased escape latencies comparable to that of non-transgenic littermates. In contrast, only short-termed and inconsistently reduced escape latencies were found in the (A) Valerian, (C) both Camellia extract and (D) Ginkgo/T groups (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.1.4 Selection of pre-screened extracts for further in-depth studies

The explorative screening provided various information in which way different herbal extracts exert influence on AD pathological hallmarks: (i) the impaired memory, (ii) the level of toxic intracerebral $A\beta_{42}$ and (iii) the plaque load. Taken together, one behavioural and four morphological parameters indicate the quality of the herbs' effect on the severity of the disease in an APP/PS1 mouse model (Table 22). To pinpoint promising extracts for further studies, no less than three morphological parameters had to be complied, while an increase in the memory performance at day 3 and 4 in comparison to the vehicle treated control mice was mandatory.

Analyses indicated only extracts of *Hypericum perforatum* and *Sideritis* spp. as promising candidates for further studies. Daily application of both extracts led to increased memory performance, to highly decreased toxic $A\beta_{42}$ level as well as significantly reduced plaque load in APP/PS1 mice.

Table 22. Results of the exploratory extract screening in the APP/PS1 mouse model regarding five analysed parameters: (i) the influence on memory, (ii) the level of toxic intracerebral $A\beta_{42}$ level and (iii) the plaque load. Significantly increased memory performance and reduced $A\beta_{42}$ and plaque load are indicated by a plus sign. 'plus' signs denote significant beneficial effects.

Extracts	Memory	Intracerebral $A\beta_{42}$ level	Plaque number	Plaque size	Cortical coverage
SJW80low	+	+	+	+	+
Valerian					
Hops/c					
Hops/x					
Camellia/ct		+	+	+	+
Camellia/tn				+	
Ginkgo/T		+		+	
Ginkgo/TA		+	+	+	+
Aloysia/A					
Aloysia/E					
Rhodiola		+			
Sideritis	+	+	+	+	+

As a consequence of the promising results of the explorative extract screening, which indicated extracts of *Hypericum perforatum* and *Sideritis* spp. as favourites in AD treatment in an APP/PS1 mouse model, detailed analyses were performed.

4.2 The role of the extraction form of *Hypericum perforatum* extracts in the effectiveness in the APP/PS1 mouse model

The SJW extract was analysed regarding its extract specificity in dependency of the main active component hyperforin. Five different extraction forms varying in their hyperforin content from zero to high concentrations (see material and methods section) were investigated to elucidate the efficiency of SJW extracts based on their specific compositions. In addition, two treatment paradigms were pursued: (i) an "AD initiation" treatment paradigm, starting simultaneously with the onset of first Aβ accumulations and (ii) a post onset therapy strategy starting after Aβ deposits were already present.

4.2.1 Reduction of intracerebral $A\beta_{42}$ levels by hyperforin low extracts

To determine the treatment effects of different extracts of SJW containing low or high levels of hyperforin on pathological hallmarks in an APP/PS1 mouse model, two different experimental strategies with treatment before and after the onset of the β-amyloid pathology were investigated. These studies also include two different extract dosages (see materials and methods section).

To investigate to which extend the different extracts of SJW are able to lower $A\beta_{42}$ burden, brain homogenates were analysed via ELISA measurements of separated buffer-soluble (soluble Aβ, small oligomers) and guanidine-soluble $A\beta_{42}$ fractions (insoluble Aβ, oligomers and fibrils of higher order). Analyses showed that daily treatment with specific low hyperforin containing extracts of SJW lead to a strong decrease of intracerebral $A\beta_{42}$. Both extracts made using 80% EtOH and characterised by low and high hyperforin contents (0.32% SJW80low, 2.88% SJW80high) were able to reduce the intracerebral level of buffer-soluble $A\beta_{42}$ significantly by 38.9% (SJW80low) and 37.7% (SJW80high) in the AD initiation treatment, and 52.5% (SJW80low) and 48.5% (SJW80high) in the post onset treatment. In contrast, the water extract (no hyperforin content) and the 60% EtOH extracts (hyperforin contents: 6.08% SJW60high; 1.42% SJW60low) showed no significant changes of buffer-soluble $A\beta_{42}$ fractions following both treatment paradigms (Figure 13A, B). Guanidine-soluble Aβ species did not significantly decrease after any of the 1-fold AD initiation treatments (Figure 13C). However, application of the post onset, 10-fold extract treatment led to decreased $A\beta_{42}$ amounts with SJW80low (-50.2%) and SJW80high (-30.7%). SJWaqua, SJW60low, and SJW60high did not change the guanidine-soluble Aβ levels (Figure 13D).

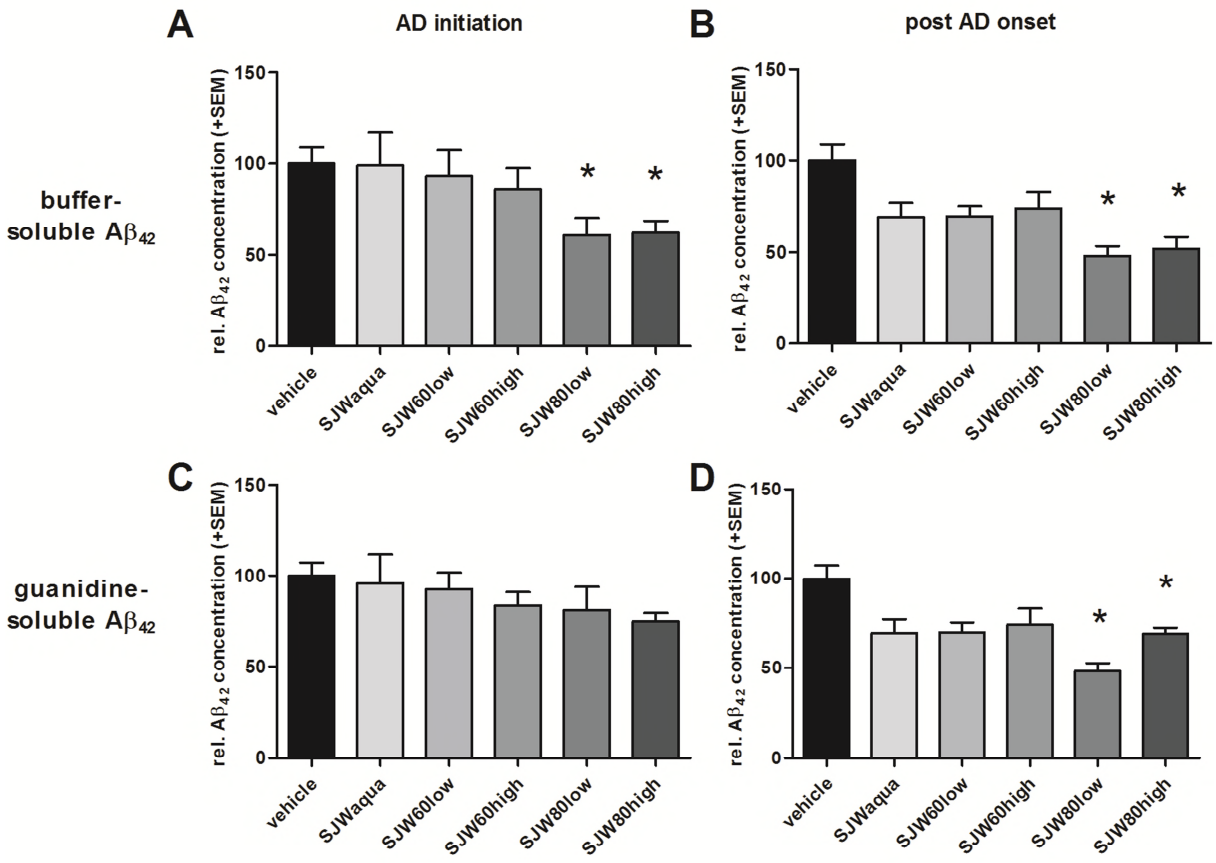

Figure 13. Reduction of brain Aβ$_{42}$ levels: ELISA-measurements of buffer- and guanidine-soluble Aβ$_{42}$ in brain homogenates of SJW treated mice in comparison to vehicle treated littermates. 80%-ethanol hyperforin low extracts reduced buffer-soluble Aβ$_{42}$ concentrations significantly in the AD initiation (A) and post-onset treatment paradigms (B). Guanidine-soluble Aβ$_{42}$ was affected by the post-onset treatment paradigm only (D). Here, the low-hyperforin extract (SJW80low) was even more potent than the SJW80high extract (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.2.2 Hyperforin low extracts diminish the cortical Aβ deposition rate

After the evaluation of soluble Aβ species via ELISA, immunohistochemical analyses of brain sections stained for β-amyloid were carried out to detect effects of various SJW extracts on the morphological hallmark of AD and the $A\beta_{42}$ plaque development. Comparing morphological changes of plaques and overall amyloid pathology between control (Figure 14A) and treated groups (B-F) revealed a significant decrease of Aβ-deposits in the low hyperforin treatment groups (Figure 14G, H). Daily treatment with either of both 80% EtOH extracts led to a strong decrease in plaque number by 29.4% (SJW80low) and 39.3% (SJW80high) with AD initiation treatment and 19.8% (SJW60low), 35.7% (SJW80low) and 39.8% (SJW80high) within the post onset treatment groups, respectively (Figure 14G, H).

Additionally, the plaque size was also reduced in these groups, which resulted in significantly decreased cortical plaque coverage by 53.1% (SJW60low), 47.0% (SJW80low) and 49.2% (SJW80high) in the AD initiation treatment group. The post onset treatment strategy also led to a significant reduction in the cortical plaque coverage in these extract groups by 45.9% (SJW60low), 49.1% (SJW80low) and 62.4% (SJW80high) (Figure 14H). This effect is observable in both the AD initiation as well as the post onset treatment compared to the vehicle control group.

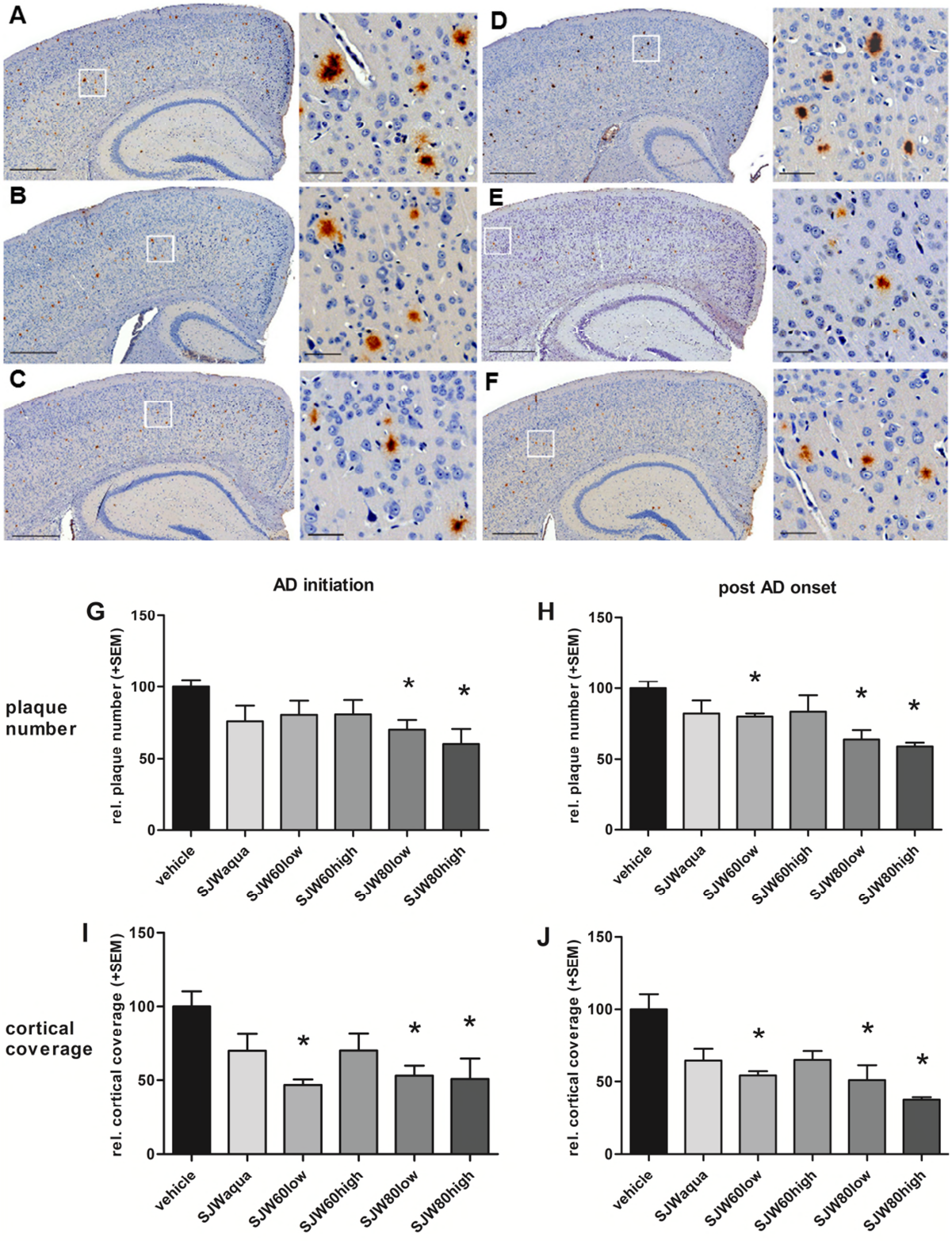

Figure 14. Lowering of neocortical plaque burden: Histological presentation of cortical Aβ deposits (6F3D): (A) vehicle, (B) SJWwater, (C) SJW60low, (D) SJW60high, (E) SJW80low, (F) SJW80high (scale bars: 500 µm, 50 µm). (G, H). Quantification of plaque number indicated a significant lowering by hyperforin-low SJW extracts; within that group, the 80%-ethanol extracts elicited the strongest reduction. (I, J) The cortical coverage by plaques was significantly decreased independent of the hyperforin content. Coverage was reduced by 80%- and 60%-ethanol, low hyperforin extract treatment (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

Subsequent analyses of plaque accretion and size showed that extract application led to a reduction of large deposits. Therefore, plaques were classified into three groups: (i) plaques smaller than 400 µm², (ii) 400 – 700 µm² and (iii) plaques larger than 700 µm². While the fraction of plaques larger than 700 µm² in diameter represented 30% of the overall plaque number in the control group, this plaque fraction is reduced to less than 10% in certain low and high hyperforin concentrated extract groups (SJW60low, SJW80low and SJW80 high) after AD initiation treatment. The SJW60high group, treated with the highest hyperforin amount showed neither reduced number of large nor middle-sized plaques. Hence, the number of small plaques is as well unaffected. Less drastic yet significant effects on the number of large plaques were also observed in the SJWwater group (Figure 15A).

In the post onset study on the other hand, the water extract showed a strong, significant reduction in large and medium plaque classes and a significant shift to small plaques. This effect was also seen in the SJW60high group after treatment with high extract dosage. Again, strongest effects on plaque growth could be observed for all three lowest hyperforin groups. Using the therapeutically high extract dosage inhibited the growth of plaques larger than 700 µm² down to 3% (Figure 15B). While medium-sized plaques were only significantly decreased in the SJW80high group, this class remained unchanged in both SJW80 groups. Contrary, the category of small plaques was significantly higher by around 40% in the lowest hyperforin groups. Summarizing, treatments with low hyperforin extracts highly influenced plaque growth. These extracts led to a significant reduction of plaques and to a shift to smaller plaques. Although an effect is seen in the other treatment groups as well, it only reached significance for the overall cortical plaque coverage in the SJW80low groups (Figure 15A, B).

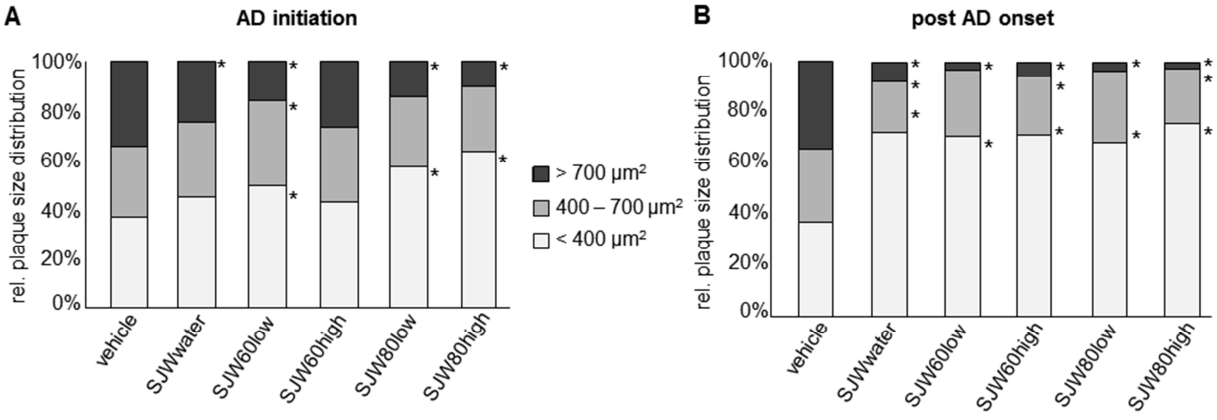

Figure 15. Changes in plaque morphology by SJW: SJW treatment highly influenced plaque growth resulting in a size shift from large (>700 µm²) to small plaques (<400 µm²). (A) With AD initiation treatment, low hyperforin extracts significantly reduced the number of plaques larger than 700 µm². (B) The post AD onset treatment paradigm reduced the development of large deposits even more potently. Changes were observable in all extract groups (Mann-Whitney vs. vehicle, *p≤0.05).

4.2.3 Hyperforin low extracts stimulate the memory performance in APP/PS1 transgenic mice

Due to the positive effect of the 80% EtOH extracts on beta-amyloid accumulation and neuroprotection, it was consequently investigated whether the extracts have an effect on cognitive functions as well. To do so, the Morris water maze test was used to compare the spatial memory function of vehicle treated and extract treated mice.

Indeed, already on the third test day, strong improvements of cognition after AD initiation with both low-hyperforin 80% EtOH extracts were detected. Daily treatment for 60 days led to reduced escape latencies at day three by 53.3% (SJW80low) and 48.2% (SJW80high) in comparison with the control group. Likewise, at day four the escape latency values were diminished by 48.5% (SJW80low) and 41.4% (SJW80high) compared to controls (Figure 16A). At both days, mice treated with SJW80 extracts reached a comparable performance level of non-transgenic mice of 54.4% (day 3) and 38.0% (day 4) similar to the vehicle treated group. In contrast, there are no significant differences after treatment with the 60% high-hyperforin, 60% low-hyperforin and water extracts in comparison with the vehicle group, indicating that these extracts were not able to reduce memory loss.

During the post onset therapy, the SJW80high extract reduced the escape latency by 40.0% at the first test day (day 2). A total of four extracts were able to significantly enhance the cognitive abilities on the third test day as seen by reduced escape latencies (SJWwater: -36.8%, SJW60high: -39.9%, SJW80low: -30.7% and SJW80high: -33.5%) (Figure 16B). Out of this four extracts, only two, SJW80low and SJW80high were able to abide this cognition enhancing effect until the last test day shown by escape latencies reduced by 52.9% SJW80low and 53.2% SJW80high in comparison to the vehicle treated control group on this last test day.

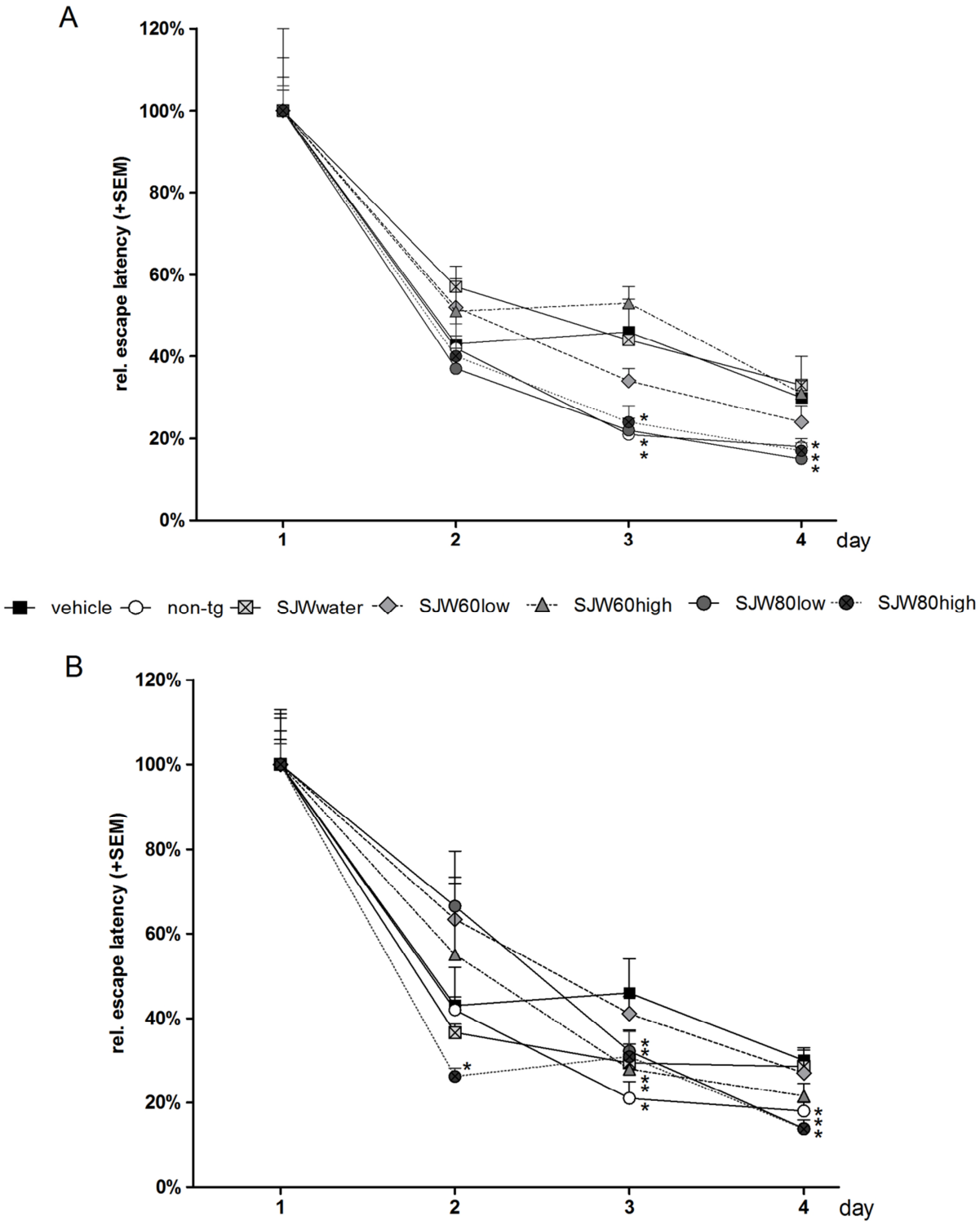

Figure 16. Improvement of spatial memory performance: Morris water maze performance of APP/PS1 mice treated with SJW extracts normalised to their day one performance. (A) 80%-ethanol, low-hyperforin extracts applied in the AD initiation paradigm led to significantly reduced escape latencies at day three and four comparable to non-transgenic littermates. (B) The post AD onset extract application led to significantly reduced escape latencies in all extract groups except SJW60low at day three. At day four, SJW80 extracts showed significantly improved cognition performance even though not containing significant amounts of hyperforin (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.2.4 *Hypericum perforatum* protects against neuron loss

As a consequence of the previous biochemical and morphological results, the influence of the extracts on neuronal cell loss in the AD mouse model was analysed. Immunohistochemical stainings of the neuronal marker NeuN were analysed whereby the area occupied by neurons within the cortex was measured and comparatively analysed (Figure 17A, B). Quantification of the NeuN positive area significantly indicated a neuronal loss in 100 d old APP/PS1 mice. In comparison to their non-transgenic littermates they showed a significantly reduced neuronal area by 16.4%. Intriguingly, treatments with one 60% EtOH and both 80% EtOH extracts fully rescued neuronal cell loss indicated by a significantly increased neuronal area in comparison to the vehicle treated APP/PS1 mice after both AD initiation (+24.0% SJW60low, +29.3% SJW80low and +24.9% SJW80high) and post onset extract application (+21.0% SJW80low and +27.0% SJW80high) (Figure 17C, D).

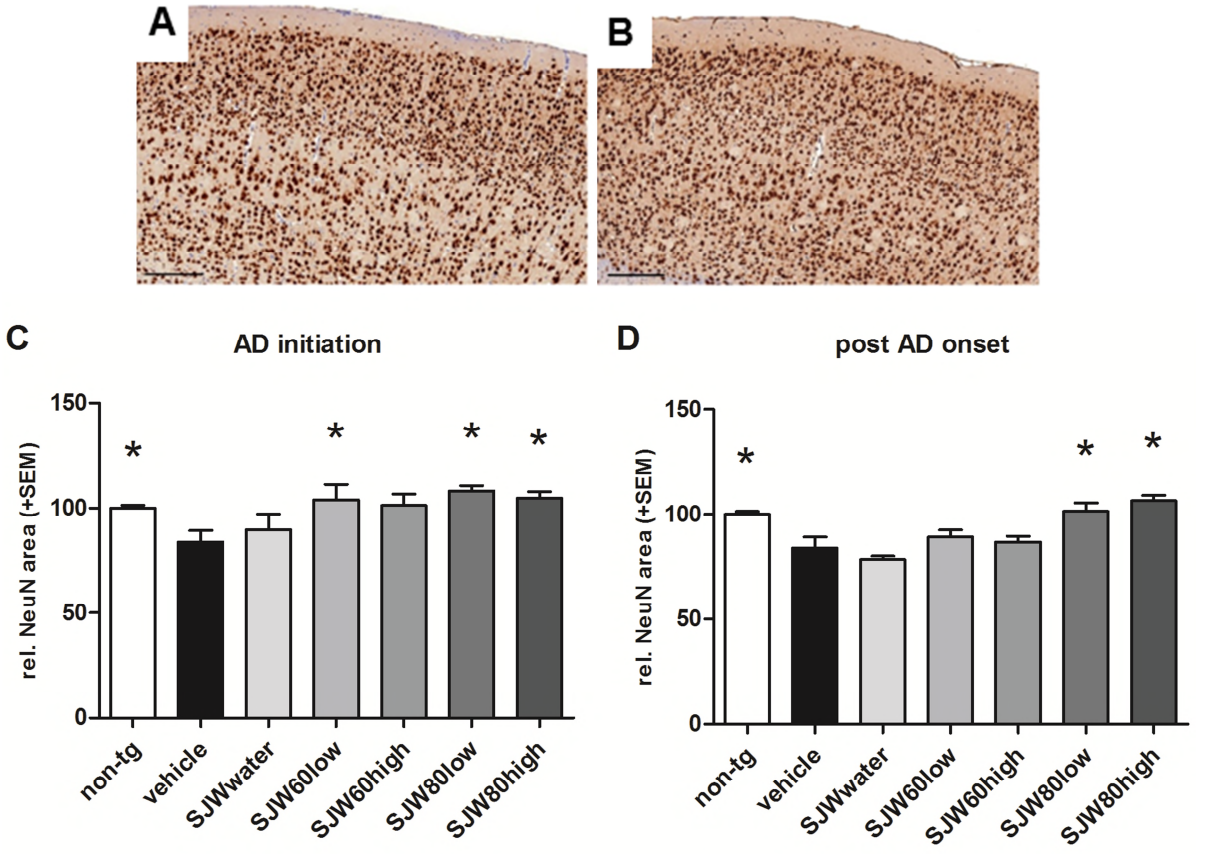

Figure 17. Prevention from neuronal loss: (A, B) Brain sections of (A) APP/PS1 vehicle treated mice and (B) SJW treated littermates were immunohistologically stained against neuronal protein NeuN (scale bars: 50 µm). (C, D) Quantification of cortical neuronal area of transgenic mice normalised to non-transgenic littermates unveiled neuroprotective effects. The neuronal area was significantly increased after (C) AD initiation and (D) post AD onset application to a level comparable with non-transgenic mice (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.2.5 Extracts of *Hypericum perforatum* stimulate the phagocytic activity of microglia *in vitro* and *in vivo*

Mechanisms by which the extracts take effect should be elucidated. It has been reported that hyperforin is a modulator of phagocytic activity of microglia (Kraus et al., 2007). Therefore, the microglia activating properties of the five different SJW extracts *in vitro* were investigated. Primary microglial cells were treated with 400 µg extract for 18 hours and co-incubated with fluorescent labelled $A\beta_{42}$ to evaluate microglial phagocytic activity (Figure 18A, B). This treatment resulted in a significantly increased phagocytic activity for all extracts, regardless of their hyperforin concentration, whereby the effect was strongest after treatment with the SJW80low and SJW80high extracts (SJWwater +59.6%; SJW60low +62.8%; SJW60high +46.1%; SJW80low +77.6%; SJW80high +77.8%). The low hyperforin containing SJW80low extract even elicited a significantly higher Aβ-phagocytosis activity than the SJW60high extract with a high hyperforin concentration (Figure 18C).

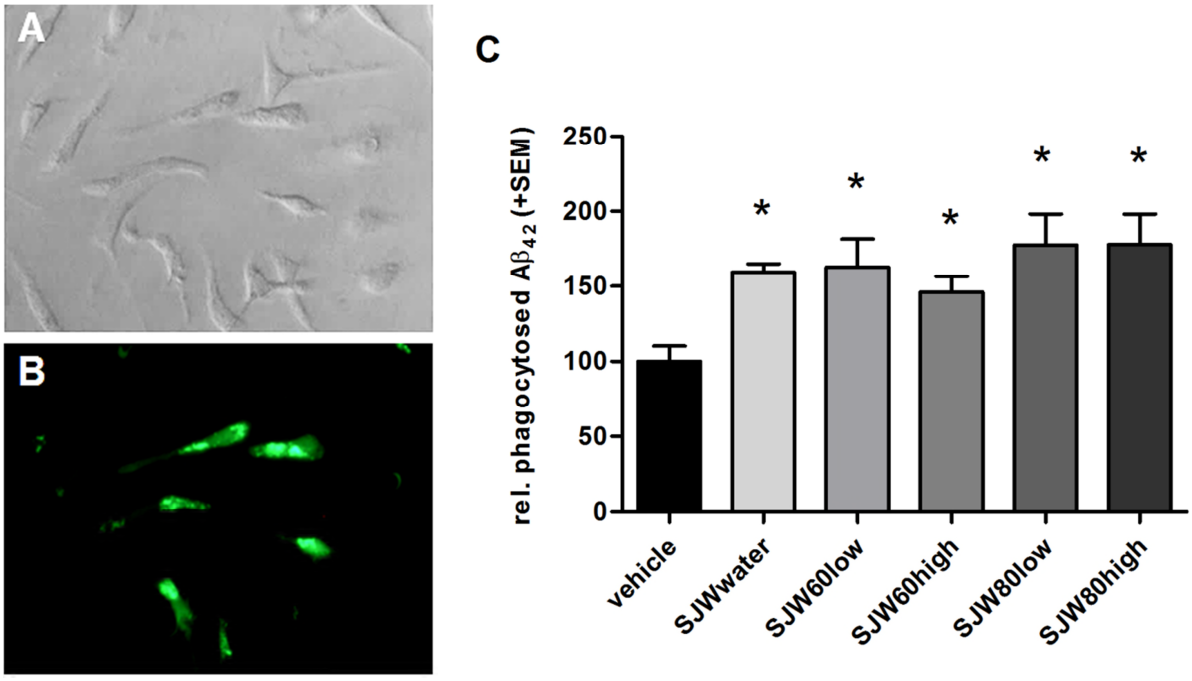

Figure 18. Activation of phagocytic microglia *in vitro*: (A) Bright-field and (B) fluorescence microphotographs of a microglia culture treated with SJW extracts and FITC-labeled $A\beta_{42}$. (C) Quantification revealed significantly activating effects by all SJW extracts obviously independent of hyperforin content (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

To analyse the effects of SJW extracts on microglia activity *in vivo*, a co-staining against both β-amyloid peptides (6F3D) and microglial cells (Iba-1) was performed on paraffin embedded brain sections (Figure 19A-F) (Scheffler et al., 2010). This double staining showed, in contrast to the *in vitro* results, an augmentation of the cortical microglial area by 22.7% (SJW80low) and 42.4% (SJW80high), respectively, only after treatment with either of both SJW80 extracts

(Figure 19G, H). Furthermore, the augmenting effect of low-hyperforin extracts on microglial activity was only observable in the post-onset treatment groups.

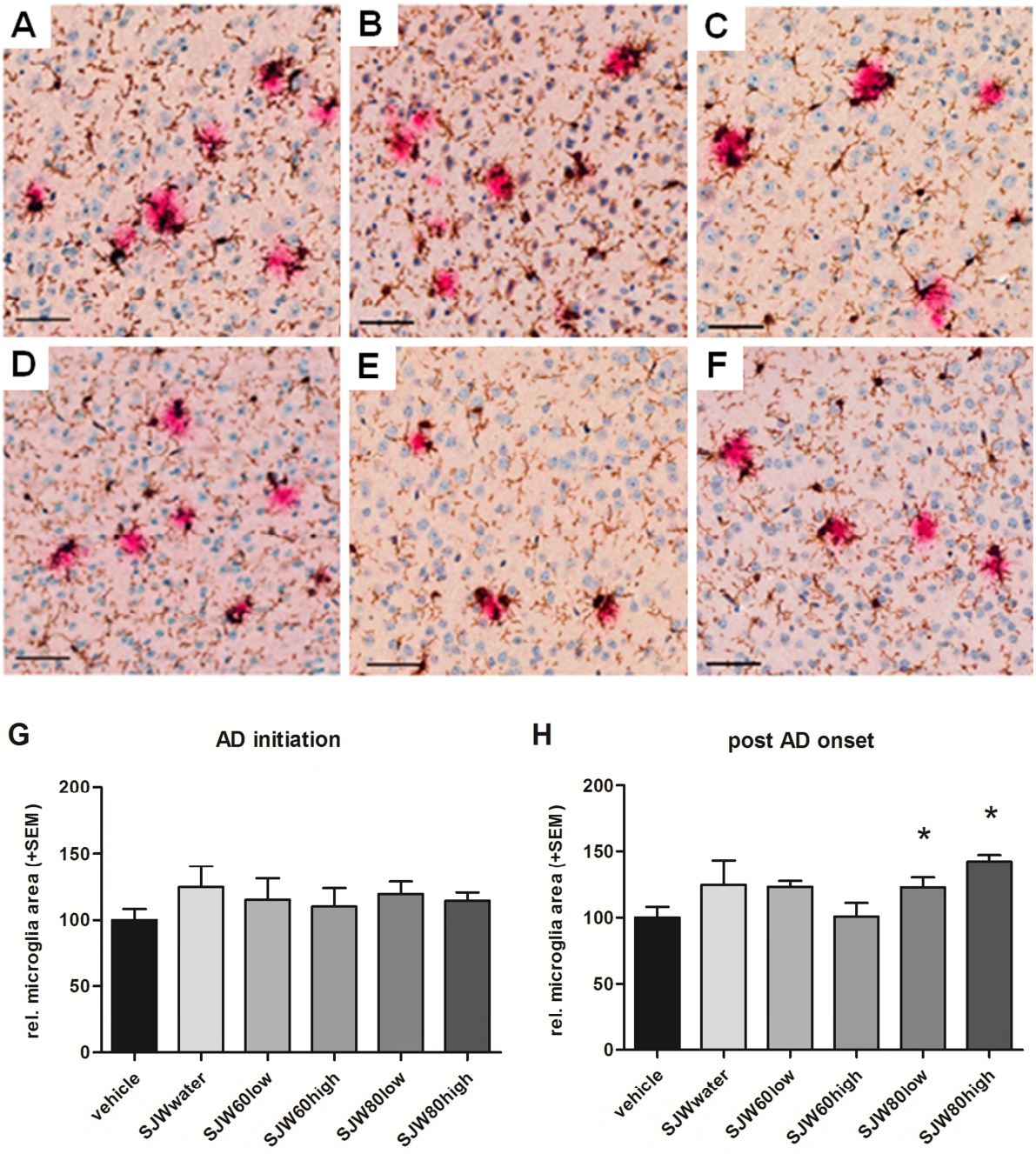

Figure 19. Activation of phagocytic microglia *in vivo*: (A-F) Microphotographs of representative cortical regions of post AD onset treated mice; for microglial classification immunohistochemically stained against microglia (brown) and Aβ (red): (A) vehicle, (B) SJWwater, (C) SJW60low, (D) SJW60high, (E) SJW80low, (F) SJW80high (scale bars: 50 µm). (G) Quantification of microglial area in the vicinity of Aβ-plaques in APP/PS1 mice after AD initiation treatment showed no changes in microglia activation. (H) Post AD onset treatment with SJW80 extracts resulted in a significant activation of microglia (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.2.6 *Hypericum perforatum* extracts' influence on Aβ generating secretases

To determine whether the diminishing effect of low-hyperforin SJW extracts on intracellular $A\beta_{42}$ is based on cleavage processes, Western blot analyses were performed to quantify the most important APP-cleavage enzymes. While the beta-secretase (BACE1) is involved in the process of generating toxic Aβ, an active alpha-secretase (ADAM10) generates non-toxic Aβ peptides. The protein expression of both secretases with regard to their influence on APP processing was observed. However, oral application of the different extracts did not alter the expression of both secretases, BACE1 and ADAM10, compared to the vehicle control group (Figure 20).

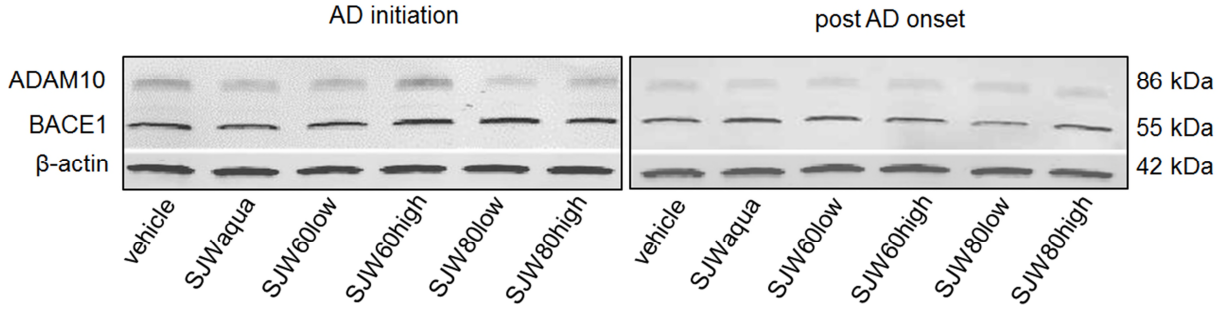

Figure 20. Ineffectiveness on secretase expression: SJW extracts independent of the hyperforin content do not affect expression levels of APP secretases BACE1 and ADAM10 shown by western blot quantification. Treating mice with neither the AD initiation nor the post onset paradigm lead to any changes in secretase expression in comparison to the vehicle treated control group.

4.2.7 A hyperforin low extract stimulates ABC transporter activity *in vitro*

Since the microglia activating effect was only achieved *in vivo* using the post AD onset treatment paradigm, there must be another route of action that accounts (i) for the effects seen with the AD initiation treatment and (ii) explains the greater effectiveness of the 80% EtOH extracts. Because SJW has been shown to affect ABC transporter expression, the protein abundance of ABCB1 and ABCC1 as the main contributors to amyloid clearance within the protein family was determined (Krohn et al., 2011). No change in protein expression for neither ABCB1 (Figure 21A) nor ABCC1 (Figure 21B) was found in mice treated with either of the extracts, chosen exemplarily for low hyperforin (SJW80low) and non-80% EtOH treatments with high hyperforin content (SJW60high), because of the similar results obtained within those extract groups. However, natural substances as well as synthetic drugs can interact with ABC transporters in several ways. Therefore, protein abundance is not necessarily a representative of transport activity. Accounting for this, we used an *in vitro* assay system to assess ABCB1 and ABCC1 transporter activity in presence and absence of a substrate and extracts. The assays revealed that both tested extracts contain substance(s) that enhance ABCB1 activity in presence of a known substrate by 12% and 19%, respectively (Figure 21C, D). Nevertheless, for both extracts the substance(s) are typical substrates per se,

shown by an activation of 20% in absence of the known substrate. Taken together the higher ABCB1 activity is consumed (at least) completely for the transport of this/these substance(s). In contrast, ABCC1 activity is elevated to 170% by the 80% EtOH extract only.

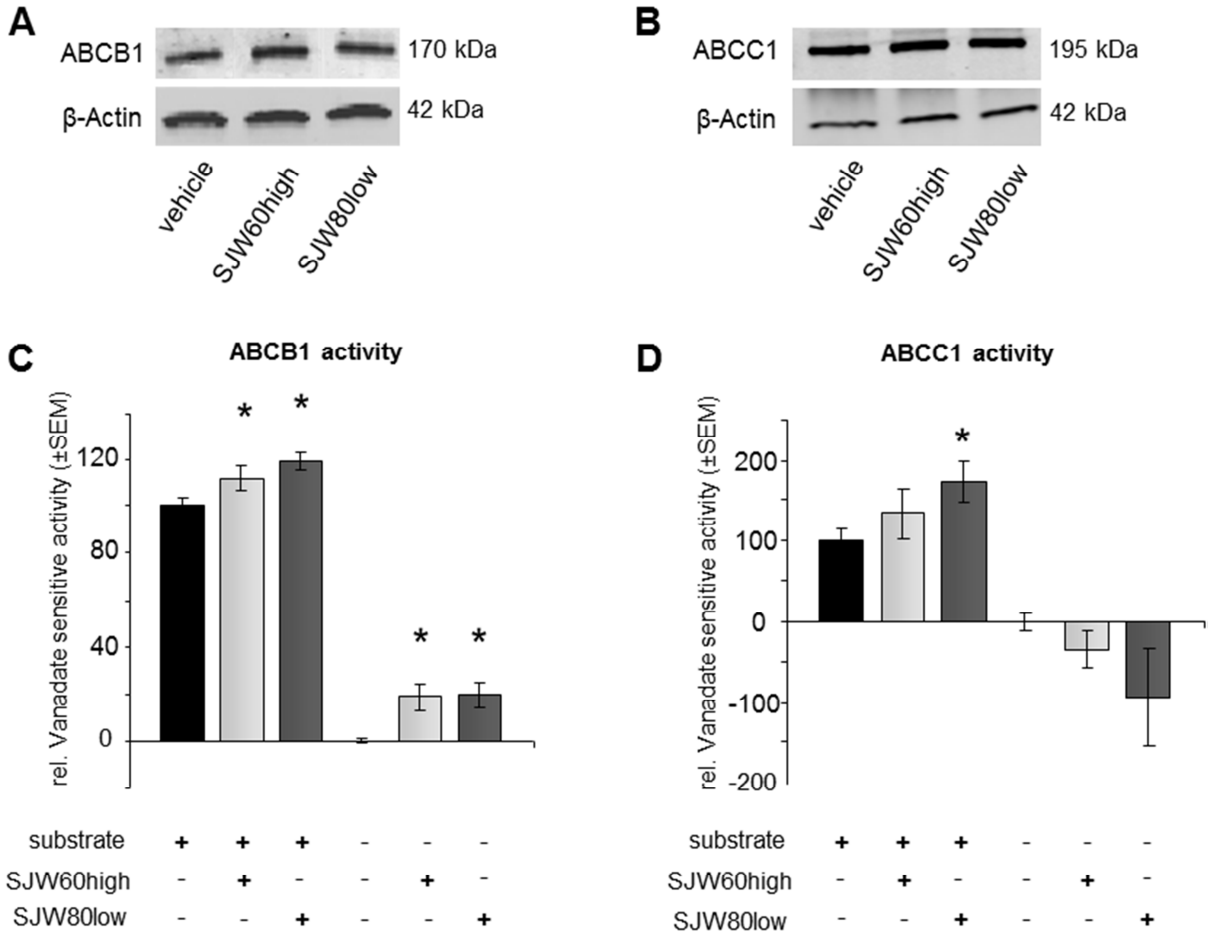

Figure 21. Activation of ABC transporter function in vitro: (A, B) Western blot analyses of brain homogenates of APP/PS1 mice revealed no changes in protein expression of ABCB1 or ABCC1 after hyperforin-high and hyperforin-low extract treatment. (C) Both extracts enhance transport activity in presence of an ABCB1 substrate (Verapamil), but the elevated activity is consumed by the active substance itself as indicated by activity elevation in absence of Verapamil. (D) Only the SJW80low extract significantly enhanced ABCC1 activity, whereas SJW60high did not (mean ± SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.3 The influence of herbal extracts of two *Sideritis* spp. species on the APP/PS1 mouse model

Due to the known vitalising effect of *Sideritis* spp. (Vasilopoulou et al., 2011), the question emerged whether extracts of this plant species are also able to act on AD pathology in a positive way. Therefore ethanolic extracts of two species of this genus were tested: (i) *Sideritis scardica* (S. scardica) and (ii) *Sideritis euboea* (S. euboea) as well as (iii) an extract combination of both (termed "combi"). Again, extracts were applied daily in an AD initiation as well as post AD onset paradigm until the age of 100 days.

4.3.1 *Sideritis* spp. decreases neurotoxic brain $A\beta_{42}$ levels

Because levels of soluble Aβ are closely associated with the degree of dementia, the intracerebral $A\beta_{42}$ concentration in APP/PS1 mice after treatment with extracts of *Sideritis* spp. was investigated. Buffer-soluble Aβ fractions of brain homogenates were measured via ELISA to quantify soluble Aβ peptides (monomers, oligomers and soluble fibrils) which are presumed to be the most toxic Aβ species (Naslund et al., 2000, Xia, 2010).

Significantly decreased $A\beta_{42}$ levels were detected in 100d old mice after AD initiation as well as after post onset treatment in all extract groups. AD initiation extract application reduced the intracerebral buffer-soluble $A\beta_{42}$ level significantly by 42.1% (S. scardica), 39.7% (S. euboea) and 40.1% (combi) (Figure 22A). Similarly, a treatment using the post onset paradigm led to a late, significant decrease of the $A\beta_{42}$ level by 57.8% (S. scardica), 59.8% (S. euboea) and 56.0% (combi) (Figure 22B).

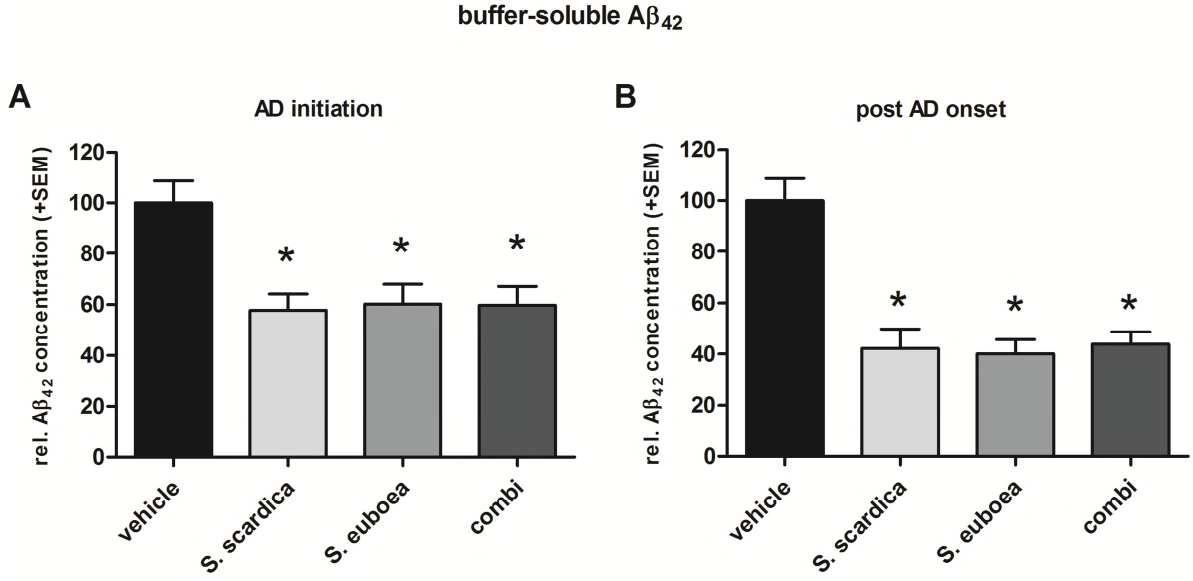

Figure 22. Decrease of intracerebral buffer-soluble $A\beta_{42}$: ELISA-measurements of buffer-soluble $A\beta_{42}$ in brain homogenates of SJW treated mice in comparison to vehicle treated littermates. (A) S. scardica, S. euboea and the extract combination reduce buffer-soluble $A\beta_{42}$ levels significantly when applied according to the AD initiation treatment strategy (B) and even more potently according to the post onset paradigm (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.3.2 Reduction of the cortical plaque burden by *Sideritis* spp. is dose and time dependent

Brain sections stained immunohistochemically for beta amyloid (6F3D; Figure 23A-D) were analysed to evaluate the effect of the various Sideritis extracts on Aβ depositions and plaque development. Comparison of changes in plaque morphology and overall amyloid pathology between control mice and the AD initiation treatment group revealed no significant changes regarding plaque numbers (Figure 23E). Still, this treatment paradigm led to a significant decrease of the Aβ burden in the extract combination group due to a mean plaque size reduced by 25.5% (Figure 23G). Contrary, daily post onset treatment with either extract or their combination led to a strong decrease in plaque number by 32.4% (*S. scardica*), 40.5% (*S. euboea*) and 42.2% (combination), respectively (Figure 23F). Additionally, the plaque size was also reduced in all groups by 31.2% (*S. scardica*), 35.7% (*S. euboea*) and 25.2% (combination) (Figure 23H).

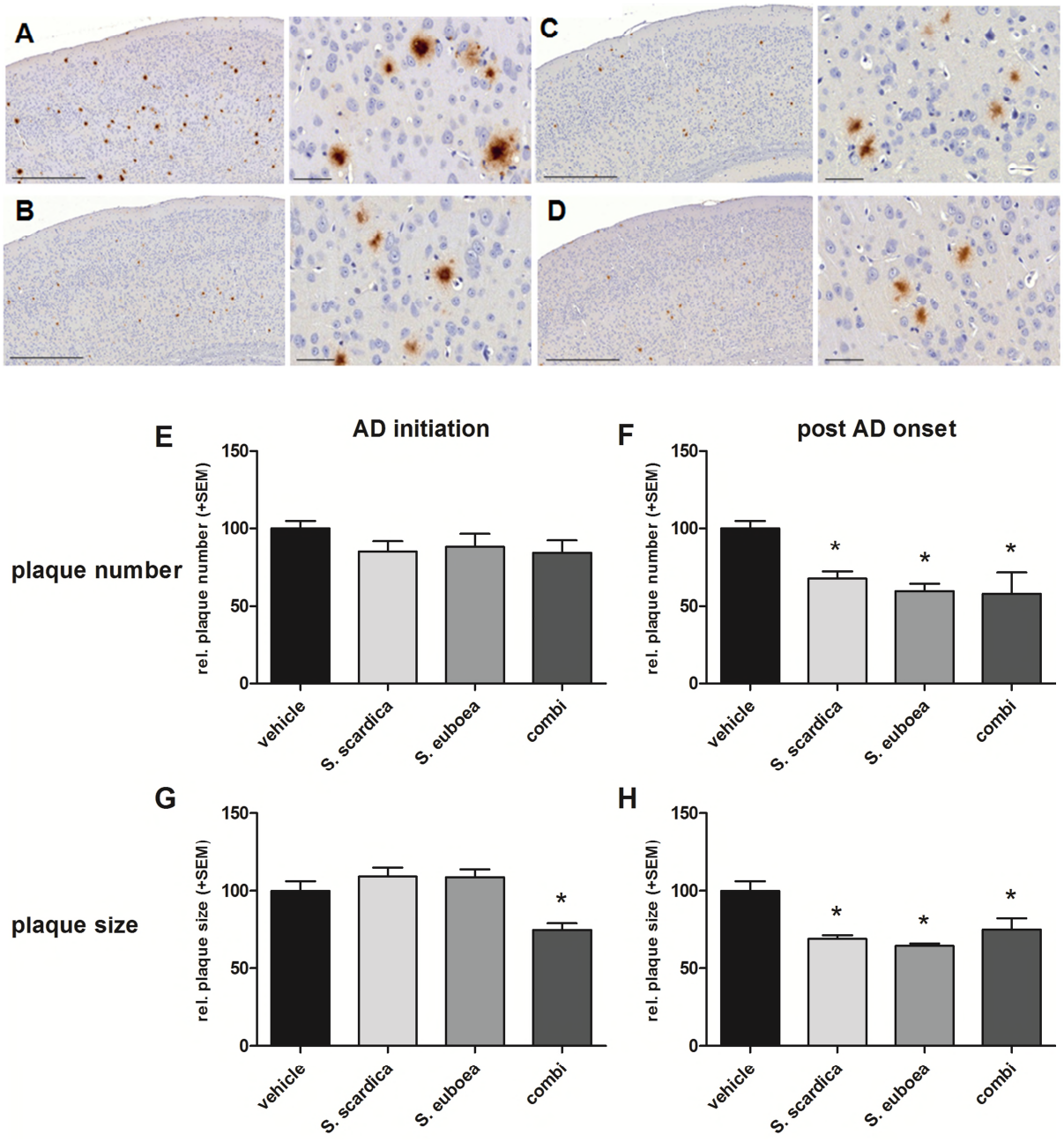

Figure 23. Lowering the neocortical plaque burden: Exemplary microphotographs of cortical plaques immunohistochemically stained with 6F3D after (A) vehicle, (B) S. scardica, (C) S. euboea, and (D) extract combination treatment (scale bars: 500 µm, 50 µm). (E, F) Sideritis extracts did only reduce plaque number when applied according to post AD onset treatment strategy. (G, H) Only the Sideritis extract combination reduced the plaque size significantly when applied during AD initiation. With post AD onset paradigm, plaque size was significantly reduced in all extract groups (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.3.3 An extract combination of two *Sideritis* spp. species improves the spatial memory performance in APP/PS1 mice

To evaluate if the two exemplars of the *Sideritis* genus might have improving effects on spatial memory in AD affected mice, Morris water maze tests were performed for four consecutive days. Results indicated strong memory enhancing effects after administration of Sideritis extracts. While both single extracts applied according to the AD initiation paradigm showed no improvement of spatial memory, the extract combination of both showed a strong memory enhancement with escape latencies reduced by 59.1% (day 3) and 45.8% (day 4) (Figure 24A). Moreover, the post AD onset treatment indicated a rapid memory enhancement. Using this treatment paradigm, mice already reached a significantly improved memory performance at the first test day (day 2) in comparison to the vehicle treated control group with escape latencies reduced by 55.4% (combi). The extract combination was able to maintain this significantly improved memory performance during the whole test period (day 3: -60.4%, day 4: -61%) (Figure 24B). A high dose treatment with both single extracts led to significantly escape latencies reduced by 43.4% (*S. scardica*) and 51.0% (*S. euboea*) at the second test day (day 3). In contrast to *S. scardica*, *S. euboea* also led to a significantly decreased escape latency by 40.0% at the last test day. There were no significant differences between Sideritis and vehicle treated mice regarding to the duration of stay within the target quadrant observed after AD initiation and post AD onset treatment (Figure 24A, B).

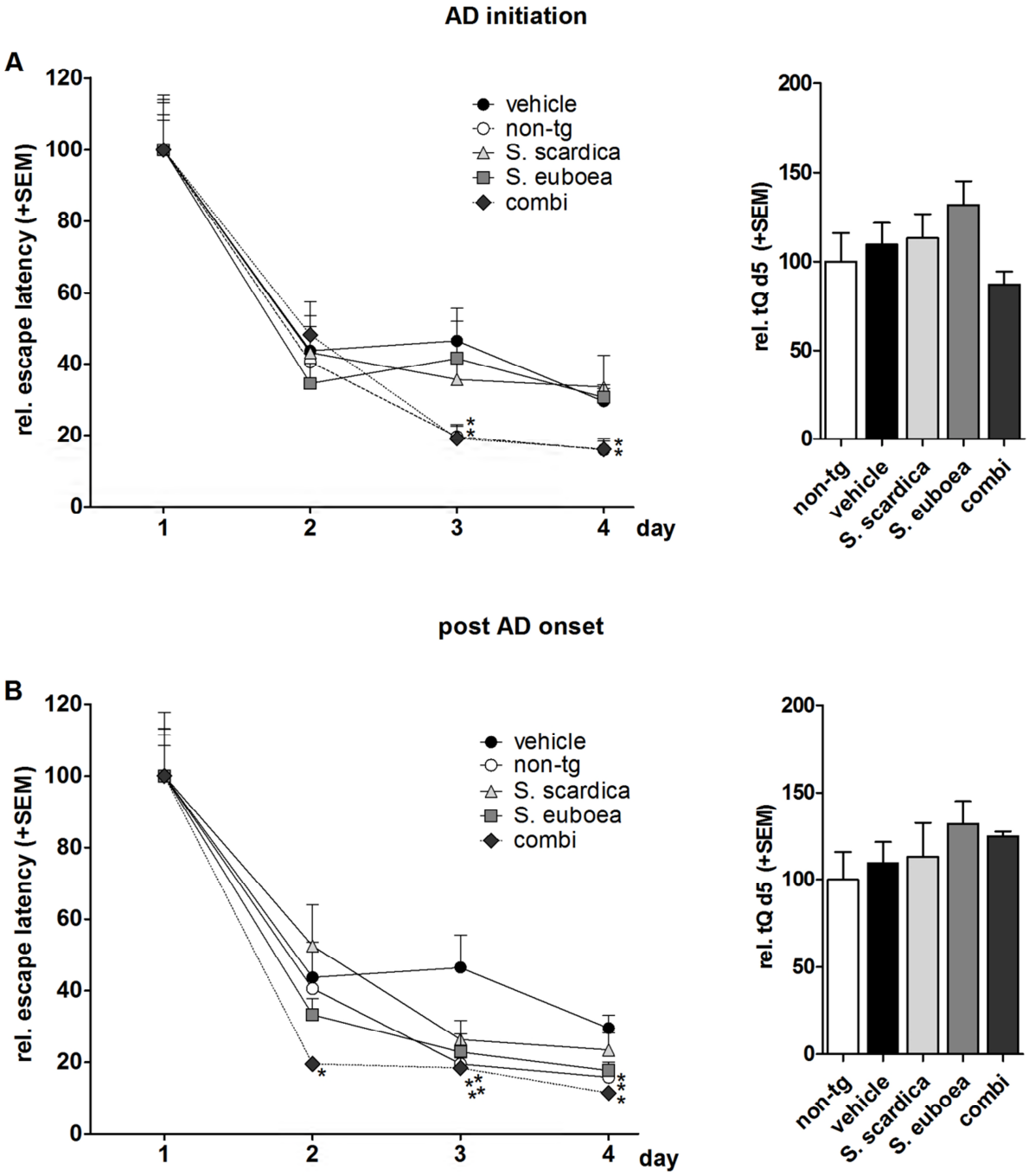

Figure 24. Improvement of spatial memory performance: Morris water maze test of APP/PS1 mice treated with herbal extracts in comparison to vehicle treated and non-transgenic littermates. Mean escape latencies were normalised to their day one performances. (A) Treatment with *Sideritis* extract combination during AD initiation improved retentiveness and learning aptitude shown by significantly reduced escape latencies, which are similar to vehicle treated non-transgenic littermates. (B) Decreased escape latency values of mice treated in post AD onset manner with *S. euboea* and the extract combination showed increased spatial memory capabilities in comparison to vehicle treated littermates (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.3.4 *Sideritis* spp. extract combination prevents neuronal loss

Based on results of behavioural assessments and $A\beta_{42}$ measurements, the influence of the extracts on neuronal cell loss was determined to indicate neuroprotective properties. Brain sections of 100d old mice were immunohistochemically stained for the neuronal marker NeuN after treatment with the most potent treatment paradigm, the post AD onset extract application (Figure 25A, B). Afterwards, NeuN positive areas within the cortex were quantified. Results unveiled a significant neuronal loss by 16.4% caused by AD in comparison to vehicle treated non demented mice (wild type mice). Intriguingly, AD initiation treatment with the extract combination as well as the single extract of *S. euboea* prevented Aβ-related neuronal cell loss, indicated by a significant increase in neuronal area compared to vehicle treated AD mice by 26.7% (combi) and 32.0% (*S. euboea*)(Figure 25C). Post onset treatment with the extract combination significantly increased neuronal area by 49,6% in comparison to vehicle treated littermates (Figure 25D).

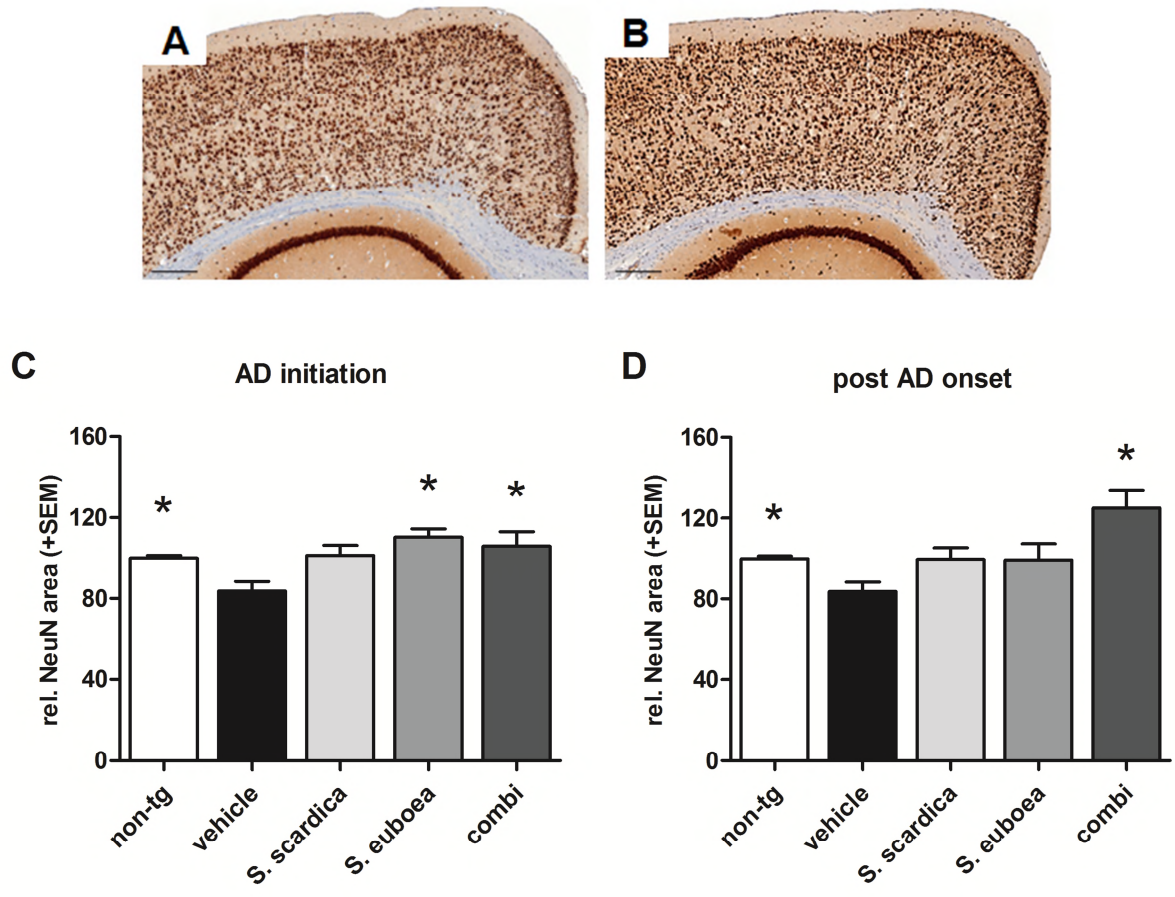

Figure 25. Protection from neuronal decline: (A, B) Exemplary histological brain sections of (A) APP/PS1 vehicle treated mice in comparison to (B) *Sideritis euboea* littermates after post AD onset therapy, stained against neuronal protein NeuN (Scale bar 50 µm). (C, D) Quantification of neuronal area is normalised to non-transgenic littermates. It indicated significant neuronal loss in APP/PS1 mice in contrast to not demented mice. A significantly increased neuronal area indicates neuroprotective effects for (C) the *S. euboea* and Sideritis extract combination when applied during AD initiation. (D) The post AD onset treatment strategy revealed significant effects for the combined extract (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.3.5 *Sideritis* spp. stimulates microglia activity *in vivo*

It has been reported that microglia are closely connected with soluble Aβ load and its depositions. To determine phagocytosis enhancing properties of Sideritis extracts on microglia *in vivo*, an immunohistochemical co-staining against both β-amyloid peptides (6F3D) and microglial cells (Iba-1) on paraffin embedded brain sections were performed (Figure 26A-D). After AD initiation extract application, computer-assisted analyses of the cortical microglia area in the surrounding of Aβ deposits showed a significantly enhanced microglia response shown by an increased microglia area in the surrounding of plaques by 107.0% (*S. scardica*), 111.3% (*S. euboea*) and 139.2% (combi) (Figure 26E). Similarly, with the post onset treatment a significantly enhanced microglia activity could be observed. The quantification of microglia area in relation to plaque area after post onset treatment showed an augmentation of the cortical microglia area by 150.4% (*S. scardica*), 137.9% (*S. euboea*) and 180.6% (combi) (Figure 26F). A comparison of the effect intensity of both treatment strategies did not reach significance.

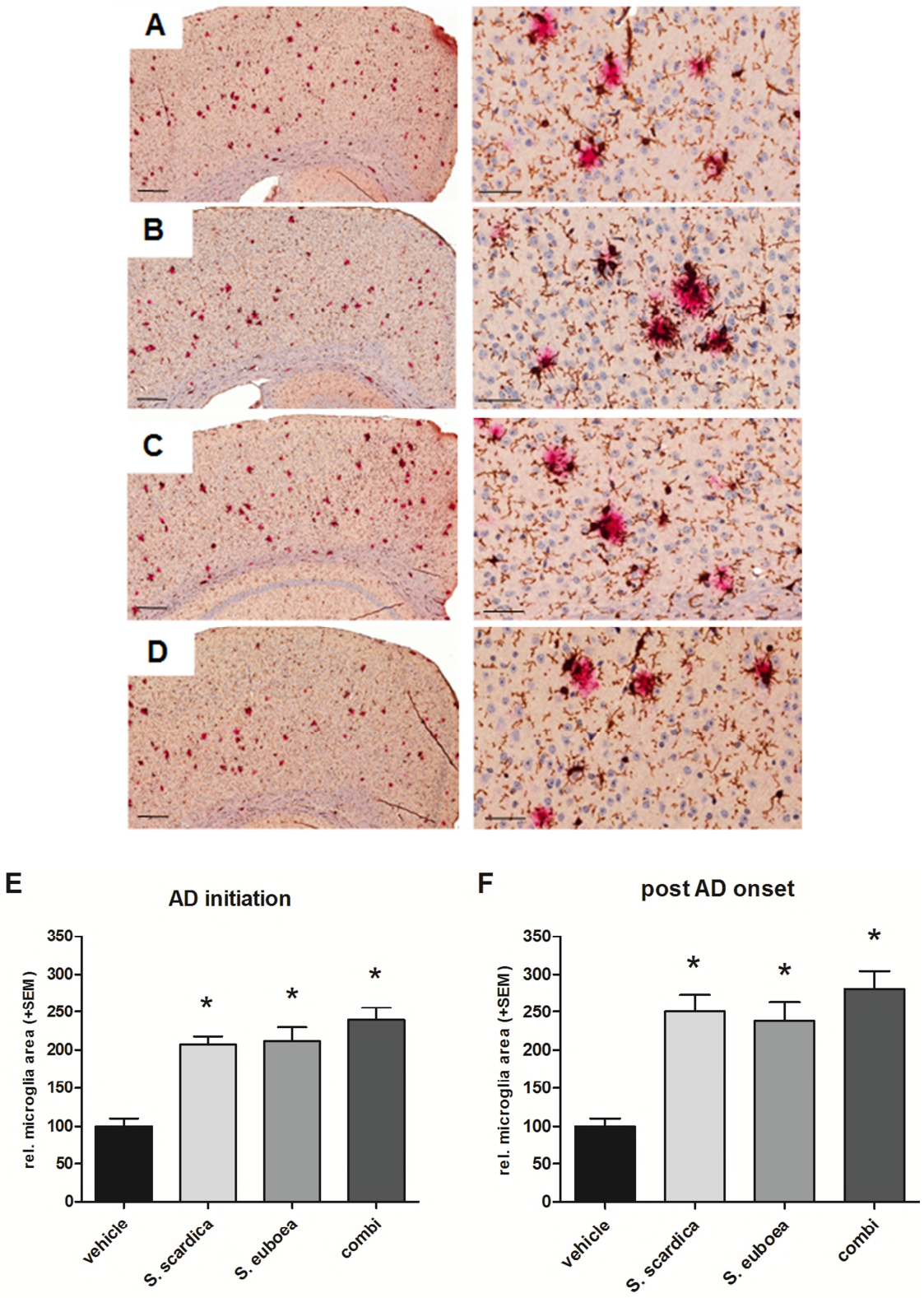

Figure 26. Activation of phagocytic microglia *in vivo*: (A-D) Microphotographs of representative cortical regions of post AD onset treated mice, co-stained for microglia (brown) and Aβ-depositions (red): (A) vehicle, (B) *Sideritis scardica*; (C) *Sideritis euboea*, and (D) the extract combination (scale bars: 500 µm left, 50 µm right). (E, F) For quantification of the microglia area, only microglia in the vicinity of plaques was included. It indicated strongly improved microglial responses. (E) Microglia area was significantly increased after AD initiation extract application and (F) post AD onset treatment (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

4.3.6 ADAM10 expression is increased by *Sideritis* spp.

To reveal whether Sideritis extracts are able to alter the APP processing machinery, the protein expression of the most important secretases, the alpha- (ADAM10) and beta-secretase (BACE1) was quantified by Western blot (Figure 27A, B). In conjunction with gamma-secretase, BACE1 is responsible for generating toxic Aβ species. In contrast, ADAM10 generates non-toxic Aβ peptides. Quantification analyses of protein expression levels revealed that oral administration of the two Sideritis species according to AD initiation and post AD onset paradigm significantly increased ADAM10 expression. Protein content exceeded that of the vehicle treated littermates by 104.2% (combi), 67.3% (*S. euboea*), and 90.4% (*S. scardica*) after AD initiation treatment (Figure 27C) and 248.5% (combi), 96.5% (*S. euboea*), and 73.5% (*S. scardica*) after post AD onset treatment (Figure 27D). The expression level of BACE1 proteins was not significantly changed, neither after AD initiation treatment nor post AD onset treatment (Figure 27E, F).

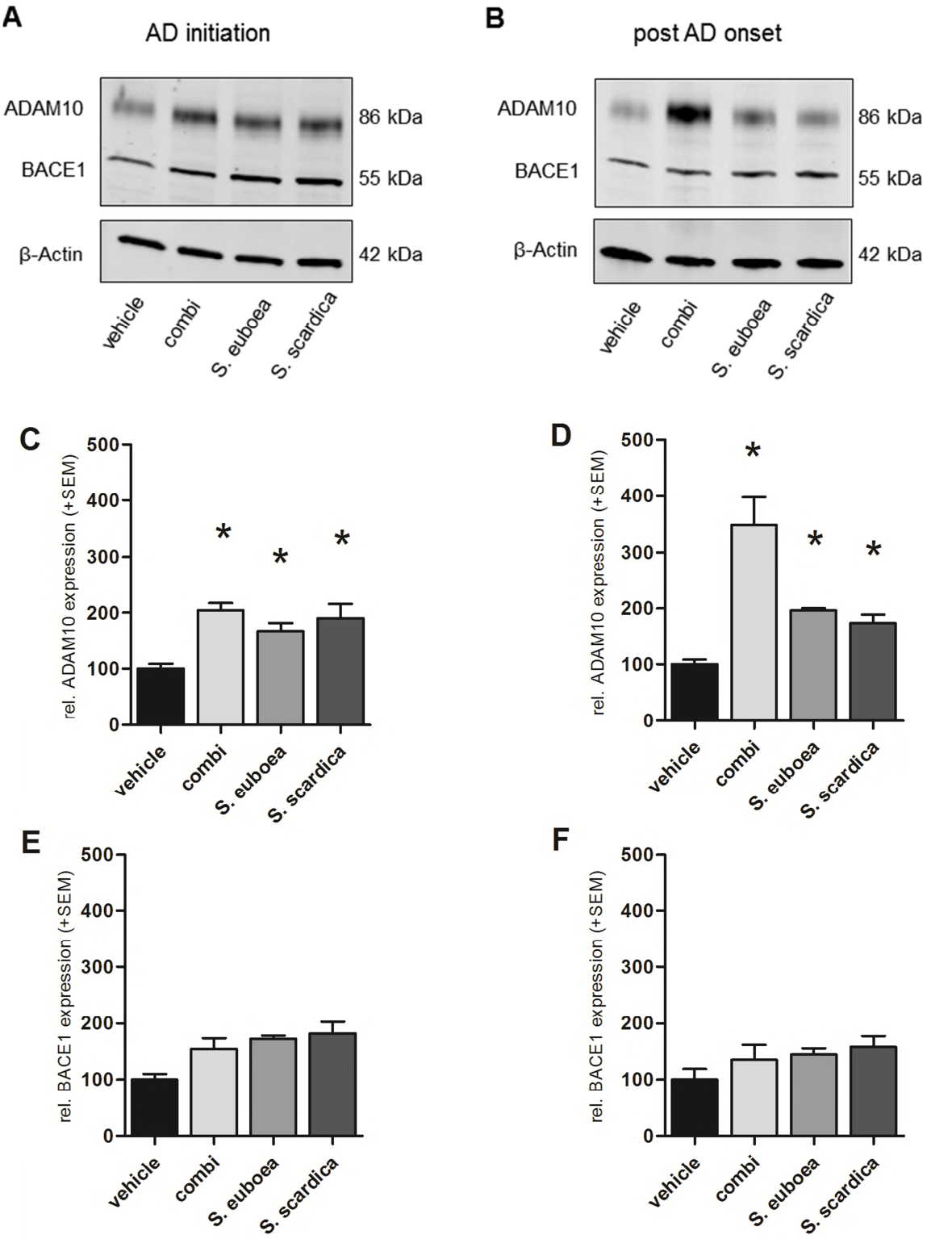

Figure 27. Activation of the α-secretase ADAM10: Sideritis extracts increased ADAM10 expression shown by western blot analyses of brain homogenates of APP/PS1 mice. (A, C) Mice treated with *Sideritis* spp. according to AD initiation paradigm showed an increased ADAM10 expression in all extract groups as well as (B, D) with post AD onset treatment in comparison to the vehicle treated littermates. (E, F) A change in the expression of BACE1 was not observed (mean + SEM, Mann-Whitney vs. vehicle, *p≤0.05).

5 Discussion

It is well known that $A\beta_{42}$ is a neurotoxic remnant of APP cleavage by β- and γ-secretases, and its deposits are one of the morphological hallmarks in AD pathogenesis. With increasing age and aetiopathology, $A\beta_{42}$ accumulates to oligomers, to fibrils and at last to diffuse or dense cored plaques (Larson and Lesne, 2012). The consequence of the formation of pathological amyloid deposits is the disruption in neuronal communication as well as reduced synaptic density, followed by neuronal inflammatory processes and neuronal cell death which manifests in reduced memory performance (Kuchibhotla et al., 2008, Xia, 2010, Paranjape et al., 2012).

AD is still incurable, and the only help for patients is a growing number of diverse drugs, which are at least able to mellow the symptoms but are not able to slow down the progression. There are for example cholinesterase inhibitors which can have a marginal beneficial effect on cognitive function in light to moderate AD (Howard et al., 2012). The aim of other therapy strategies is the activation of the α-secretase, which results in the amplification of the non-amylogenic pathway of APP processing (Citron, 2000). Additionally, non-steroidal anti-inflammatory drugs (NSAIDs) are discussed to be protective as well (Breitner et al., 1994, Shah et al., 2008). In summary, all of these therapy options share three shortcomings: (i) a marginal slowdown of the progression, (ii) significant side effects and influence on psychological as well as physiological processes, and (iii) an occasionally ephemeral effect.

An AD affected human brain underlies many morphological changes during aetiopathologies caused by Aβ generation. In contrast to humans, mice cannot develop AD pathology due to their APP's different amino acid sequence which makes it unable to form aggregates. Hence, a transgenic mouse model is required that actually develops morphological AD characteristics, as previously described by Radde et al. (Radde et al., 2006). Such a transgenic mouse line modelling the Aβ deposition aspect of AD showed to be ideal for a pharmacological study like the one presented here.

Whole plant extracts or rather specific components might be gentle treatment alternatives in all fields of medicine, especially in neurology. To date their typical applications comprise amongst other neurological disorders like depression, anxiety, and agitation as well as sleep disorders (Bonda et al., 2010). Amongst a variety of clinical drugs, there are some renowned ones originating from plants with a long history of usage that affect the central nervous system like ephedrine (*Ephedra sinica*), morphine (*Papaver somniferum*), galantamine (*Galanthus* spp.) and physostigmine (*Physostigma veneosum*) (Perry et al., 1998). Extracts tested here are in part traditionally used in the field of neurology, while others were still unknown.

5.1 Specific herbal extracts have beneficial effects on AD pathologies in APP/PS1 mice

As a first step to discover potent candidates that are beneficial in AD pathology, twelve extracts of eight species were tested in a prescreening study considering pathological parameters in APP/PS1 mice. Two of the traditional medical herbs tested here are widely spread in European regions: *Valeriana officinalis* and *Hypericum perforatum*. The former one is used especially in the field of sleeping disorders and insomnia. In 2010 a Chinese study showed that this sleep-inducing aid also reduces species of $Abeta_{1-40}$ peptides in an AD rat model (Tolvanen et al., 2010) although the study was based on an Asian species of the genus *Valeriana*, namely *Valeriana amurensis*. Effects on the toxically more relevant Aβ species $Aβ_{42}$ were not studied.

Herbal extracts of Hypericum perforatum and Sideritis spp. affect morphological changes caused by toxic levels of beta amyloid in APP/PS1 mice

The most widespread species of the genus Valeriana, *Valeriana officinalis* was screened regarding AD relevant parameters like the Aβ load of (i) toxic soluble $Aβ_{42}$ and (ii) deposited Aβ peptides in the form of amyloid plaques in an APP/PS1 mouse model. Results indicated no influence of this form of root extracts used here. Quantitative analyses of the soluble intracerebral $Aβ_{42}$ load and $Aβ_{42}$ plaques showed no changes regarding relevant Aβ parameters comparable with results of the vehicle treated control group. Since $Aβ_{42}$ is the toxically more significant cleavage product of APP during AD pathology (Lue et al., 1999, Radde et al., 2006, Larson and Lesne, 2012), the results shown here do not imply a potential medical usability of *Valeriana officinalis* against AD, not at least due to the lack of significant cognitive improvement after administration.

In contrast, *Hypericum perforatum* (SJW) and particularly its active constituent hyperforin already play an important role in modern medicine in the therapy of mental disorders, being used in the medication of mild to moderate depression for decades (Mennini and Gobbi, 2004, Linde et al., 2008). To date, SJW and its constituents are promising subjects of Alzheimer research. Hyperforin showed a few appreciable results in some basic aspects of AD, amongst others the avoidance of plaque formation, reduced Aβ-neurotoxicity, protection of memory and inhibited inflammatory processes according to a study of Dinamarca et al. (Dinamarca et al., 2006, Dinamarca et al., 2008, Griffith et al., 2010). Other neurobiological effects have also been described, including the inhibition of neurotransmitter re-uptake, an increase of intracellular calcium levels, N-methyl-D-aspartic acid (NMDA) receptor antagonism as well as antioxidant properties (Griffith et al., 2010). Notably, in the study of Dinamarca and colleagues, neurotoxic Aβ peptides as well as hyperforin were intracerebrally coinjected (Dinamarca et al., 2006). As part of the present study an orally administered 80%

ethanolic SJW extract (similar to Laif®900 Steigerwald Arzneimittelwerk GmbH), showed significantly reduced toxic Aβ levels in APP/PS1 mice down to 54%. In addition, the overall cortical plaque burden was diminished by around 50% resulting from significantly reduced plaque number and size by this 80% ethanolic extract, which is characterised by a low hyperforin content of 0.32%. Dinamarca et al. used a high hyperforin concentration of 6 µM, an amount which according to Biber and colleagues does not cross the blood-brain barrier after oral application (Biber et al., 1998). They showed that it insufficiently crosses the blood brain-barrier by finally 0.19 ± 0.1%. Hence, beneficial results regarding Aβ burden which Dinamarca reached with intracerebral injection of this hyperforin derivate (Dinamarca et al., 2006, Kraus et al., 2007) are not directly comparable with results achieved with oral application of crude plant extracts as presented here.

Different studies of extracts of *Humulus lupulus* (hops) suggest a sedative action and showed amongst others positive effects against anxiety and insomnia (Salter and Brownie, 2010). Studies regarding AD indicated hops' agent colupulone as responsible for the increase in expression of the blood brain barrier transporter P-glycoprotein (P-gp, ABCB1), an alleged Aβ transporter, by interaction with the pregnan X (PX) receptor (Teotico et al., 2008). Results of the prescreening of two hops extracts differing in active compounds (i) colupulone and (ii) xanthohumol indicated no changes in plaque load in comparison to vehicle treated littermates. This is in contrast to the study of Teotico, which pointed to colupulone as the ABCB1 expression increasing agent (Vogelgesang et al., 2011), which showed consequently decreasing Aβ levels. This study detected no effects at all for one hops extract and an increase in intracerebral $A\beta_{42}$ in APP/PS1 mice after post onset treatment strategy for the other.

Likewise, *Camellia sinensis* is a famous traditional plant especially in traditional Asian medicine. Its components are highly antioxidative with potent properties in the treatment of cancer chemoprevention and cardiovascular diseases (Mak, 2012, Trudel et al., 2012). Its secondary plant metabolites are notably investigated in the field of cognitive disorders (Gomez-Pinilla and Nguyen, 2012). The component theanine reduces intracerebral $A\beta_{42}$ levels, attenuates Aβ-induced neuronal cell death and cognitive dysfunction while acting antioxidatively (Kim et al., 2009). Caffeine is also an antioxidant agent and attenuates inflammatory processes, reducing the $A\beta_{42}$ levels as well as the number of plaques in the hippocampus in different AD mouse models. Another study of Chu and colleagues showed that caffeine acts neuroprotectively against Aβ-induced cell death and suppresses Aβ-induced caspase-3 activity (Chu et al., 2012). A recent study of Kim and others detected inhibitory effects on the acetylcholinesterase (Pohanka and Dobes, 2013). Various studies evaluated especially Epigallocatechin-3-gallate (EGCG) to interact with Aβ (Lopez del Amo et al., 2012) and to be able to enhance the activity of the AD relevant α-secretase ADAM10, which accounts for the non-amyloidogenic pathway and thereby averts the genesis of toxic Aβ

species like $A\beta_{42}$ (Obregon et al., 2006, Fernandez et al., 2010). In this study, two green tea extracts with varying compositions were tested. Camellia/ct had a catechin content of 60-70% and a caffeine content of 11% whereas camellia/tn exhibited only a theanine content of 6%. Quantification of soluble $A\beta_{42}$ by ELISA measurements exposed at least one of the two green tea extracts which contains a very high catechin content to be very promising. In comparison to vehicle treated controls, mice daily treated with camellia/ct extract showed significantly decreased intracerebral $A\beta_{42}$ levels which is in agreement with Obregon et al. who already indicated a strong influence of catechin on ADAM10 activity and hence the development of non-toxic Aβ species (Obregon et al., 2006). In this study, the catechin rich extraction form showed also strong plaque reducing effects resulting in a significantly lower plaque number and size, consequently resulting in significantly lower cortical plaque coverage.

Ginkgo biloba looks back on a long history as a traditional medical device with its origin in Asian regions. To date it also became very popular in the Western world, especially in the treatment of concentration problems and cognitive dysfunction. It became famous for memory enhancing properties and hence is in the focus of science especially in the research field of neurodegeneration and cognitive aging (Wu et al., 2006, Birks and Grimley Evans, 2009, Daffner, 2010). Here, two extracts of *Ginkgo biloba* were tested. The first (Ginkgo/T) was the standardised *Gingko biloba* product Tebonin® (W. SCHWABE GmbH & Co. KG), the second one (Ginkgo/TA) was an analogous extract to Tebonin®. Both extracts showed positive effects on pathological aspects in the APP/PS1 model. Complementary to other studies which indicated Ginkgo as an Aβ lowering agent either indirectly through the inhibition of Aβ production by lowering free cholesterol levels or through APP lowering activity (Yao et al., 2004, Augustin et al., 2009, Shi et al., 2012), this study proved a significant reduction of soluble toxic $A\beta_{42}$ levels. Correlating with reduced $A\beta_{42}$ levels, Ginkgo/TA also highly influenced the Aβ aggregation and deposition, lowering plaque number as well as plaque size, and in consequence the overall frontal cortex coverage by plaques as previously shown by Luo and colleagues (Luo et al., 2002).

Aloysia citrodora showed strong antioxidative properties *in vitro* which amongst others also plays an important role in AD (Guimaraes et al., 2011). Regarding AD pathology, this study first screened two different extracts, an aqueous and a 20% ethanolic extract. In comparison to vehicle treated littermates, both extract types showed no effect on Aβ burden concerning neither soluble peptide species nor fibril formations nor plaques. Results indicated that neither extraction forms had beneficial effects on pathological mechanisms of AD.

Rhodiola rosea showed beneficial effects in a wide range of diseases like depression and anxiety as well as in reducing fatigue (Chan, 2012). Studies of the influence of daily oral application of an aqueous extract in APP/PS1 mice showed no significant effects on

intracerebral $A\beta_{42}$ levels as well as on plaque deposition rate which was still comparable to vehicle treated littermates.

The beta amyloid related cognitive decline in APP/PS1 mice is lowered by herbal extracts of Hypericum perforatum and Sideritis spp.

Entirely unknown in the field of AD research are members of the Greek herb *Sideritis* spp. They have always been very popular in Mediterranean folk medicine. Besides its anti-inflammatory and antioxidative aptitudes and hence their potential as a treatment option in the field of neurodegenerative disorders, this study for the first time detected strong impacts on pathological hallmarks in APP/PS1 mice for two Sideritis species, namely *S. scardica* and *S. euboea*. Although the exact composition of active agents is not entirely known yet, the extraction method suggests that the extracts are mostly flavonoid enriched. Numerous *in vitro* as well as *in vivo* studies support a neuroprotective activity of flavones in neurodegeneration models. Further they act as (i) antioxidative agents, (ii) modulators of signalling cascades and gene expression, and finally they appear as (iii) highly anti-inflammatory agents, hence they are the main protective mechanism against uncontrolled and chronic inflammation, and oxidative damage. Besides metabolic and oxidative situation, the survival or death of neurons highly depends on the bioavailability of flavonoids, its concentration and exposure time (Dajas et al., 2013). All of this suggests flavonoids as a potential multi-targeted therapy option for protecting the brain.

Memory disruption is the first indication and one of the most important hallmarks in the aetiopathology of neurodegenerating processes like those in Alzheimer's disease. This cognitive decline also characterises the APP/PS1 mouse model for AD. Mice with human transgenes in both genes, APP and PS1, exhibit an early onset and severe progress of the disorder, further leading to decreased memory performance already at the age of 100 days. While there is no difference in escape latency at the first day of testing the extracts, already at the second and third day, transgenic mice showed a significantly lowered memory capacity by about 11% in comparison to their littermate wild-type control mice. Radde and colleagues used a food-rewarded four-arm spatial maze to detect a decrease in memory performance shown at the age of 8 month (Dajas et al., 2013). In contrast to the Morris water maze results shown here, the four arm spatial maze of the Radde group was obviously not sensitive enough to detect the respective changes at the age of 5 months. Conceivable reasons lie in the modality of motivation which distinguishes the two from each other. While the food-rewarded four-arm spatial maze based on the perception of hunger, the Morris water maze based on the rudimentary mortal fear which demonstrably is still the most potent motivation (Hodges, 1996). The difference and effectiveness of Morris water maze (MWM) in comparison to radial arm maze (RAM) are defined by the speed of learning, the strategy adapted and the necessity of accurate navigation. Hunger as motivation is subjective and

failure to find a food reward incurs no great penalty, whereas failure to find the platform may be life threatening. Both mazes are not measuring the same aspects and do not lead to comparable results as shown by Jarrad et al. with a hippocampal ischemia model (Hodges, 1996). Their findings indicate that behaviours in the MWM and RAM in the same animals are not necessarily closely correlated.

Corresponding to results of the morphological analyses of different Aβ species including soluble peptides as well as insoluble species and beta-amyloid depositions, behavioural tests using the Morris water maze, which aims at spatial memory, showed no increase in learning aptitude after daily oral treatment with *Valerina officinalis* in comparison to vehicle treated control mice. A comparison to other studies is not possible because studies concerning *Valeriana officinalis* and its potential on memory are still missing.

Contrary, a study of *Hypericum perforatum* regarding cognitive impairments indicated some beneficial effects on stress-induced memory impairments in a chronic restraint stress model in rats (Trofimiuk et al., 2005). In contrast, a study with healthy volunteers regarding the effects of hypericine rich SJW extracts on cognitive performance showed no effect on short-term memory according to psychometric tests and quantitative EEG (Siepmann et al., 2002). Yet, the Morris water maze, applied to the APP/PS1 mouse model, indicated enhancing effects on spatial memory by a specific SJW extract. The screening of a hyperforin and hypericin depleted extract by Morris water maze showed strong beneficial effects on the cognition of APP/PS1 transgenic mice. There was a significant improvement of spatial memory in comparison to vehicle treated littermates. Escape latencies were decreased by at least 40% (SJW80low), which is a performance comparable to the level of non-transgenic healthy mice. While this finding is consistent with results of lowered $A\beta_{42}$ levels, assuming a high toxicity for soluble $A\beta_{42}$ peptides and oligomers (Lesné et al., 2008, Lord et al., 2009), the results are still contrary to most studies, which indicate hyperforin and hypericin as the active components and mitigating substances against Aβ induced toxicity (Klusa et al., 2001, Dinamarca et al., 2006, Griffith et al., 2010).

In literature *Humulus lupulus* is only cited as a calming, relaxing and sedative agent (Salter and Brownie, 2010). For the first time, two hops extracts were screened regarding memory enhancing properties. Morris water maze analyses showed no differences between hops treated APP/PS1 mice and vehicle treated littermates, which correlates to unaffected Aβ levels corresponding to control mice. In addition, one *Rhodiola rosea* extract showed similar results concerning Aβ levels, plaque burden and spatial memory performance. In contrast, studies report cognition improving properties in stress induced fatigue because of antioxidative and neuroprotective effects (Fan et al., 2001, Fintelmann and Gruenwald, 2007, Qu et al., 2009).

Different studies of *Camellia sinensis* showed that components like caffeine prevent cognitive decline in young and middle aged rats after chronic consumption (Vila-Luna et al., 2012, Arab et al., 2013). These rats consumed 5 mg/kg/day for maximal 6 month. APP/PS1 mice of this study received 4 g/kg/day Camellia extract with a caffeine content of 11% administered for 50 consecutive days. Additionally, various studies showed that catechin like EGCG inhibits Aβ-induced cognitive dysfunction after catechin intake of 3 mg/kg or 35 mg/kg per day, respectively (Unno et al., 2007, Lee et al., 2009). In this study, the extract Camellia/ct is characterised by a catechin content of 60-70% and high levels of both active components; it showed $A\beta_{42}$ as well as plaque reducing properties, but the extract did not consistently affect the cognitive impairment probably due to the transgenesis triggered rapid cognitive decline. This agrees with Shukitt-Hale and colleagues, who showed that caffeine alone did not account for improved memory performance in aged rats whereas a complete coffee extract did (Shukitt-Hale et al., 2013). The second extract (Camellia/tn) was characterised by a theanine content of 6%. It did not have any effect on spatial memory in APP/PS1 mice administered with 4 g/kg powdered extract per day, which corresponds to a theanine concentration of 240 mg/kg, even though other studies reported neuroprotective and hence preventive effects on cognitive dysfunction even for Camellia extracts. The structural similarity between theanine and the neurotransmitter glutamate was discussed as a possible explanation for their effectiveness (Kakuda, 2011, Song et al., 2012). In elderly volunteers, who ingested theanine rich powdered green tea extract (45.5 mg/kg theanine), theanine lowered the cognitive decline compared with that of the placebo group (Kakuda, 2011).

Morris water maze tests of mice treated with an extract of *Sideritis scardica* in contrast detected a strong beneficial influence on cognition. Treatment with *S. scardica* significantly decreased the escape latency by 30%. Although the exact composition of active agents in this extract is unclear, the extraction method suggests that they are mostly of the flavonoid family. Several studies indicate that flavonoid consumption may be capable of inducing improvements in cognitive performance especially because of its anti-inflammatory properties (Commenges et al., 2000, Letenneur et al., 2007, Spencer, 2009). Recent evidence has indicated that this group of plant-derived compounds may exert particularly powerful actions on mammalian cognition and may reverse age-related declines in memory and learning (Spencer, 2010).

In contrast to the promising data of the Sideritis study, several randomised studies with Ginkgo extracts on AD patients never showed any significant differences between treatment groups and placebo groups (Weinmann et al., 2010). In 2008 a study with a cohort of 482 volunteers aged 75 years or older with mild cognitive impairments (MCI) were assessed every 6 months after twice-daily doses of *Ginkgo biloba* extract. The study detected no effect in reducing either the overall incidence rate of dementia or AD incidence in individuals with

MCI (DeKosky et al., 2008). However, the Ginkgo/T extract in this study reduced soluble Aβ species as well as especially plaque formations, which corresponds to former studies in which behavioural experiments regarding spatial memory indicated no improvement in cognitive abilities in 100 days old female mice (Stackman et al., 2003). Various *Ginkgo biloba* extracts also showed protective effects against Aβ mediated toxicity and neuronal loss in different animal models characterised by cognitive decline (Stackman et al., 2003, Wu et al., 2006). Furthermore, with LTP measurements as an indicator for memory storage, Vitolo demonstrated that a Ginkgo extract completely blocked the damaging effect of Aβ on LTP (Vitolo et al., 2009). Other studies showed decreased age-related cognitive deficits after chronic Ginkgo treatment with spatial memory tests (Stackman et al., 2003). In addition, the standardised *Ginkgo biloba* extract EGb 761 alleviated Aβ-induced pathological behaviours in a transgenic model of *Caenorhabditis elegans* (Wu et al., 2006). The mouse model studied herein showed no consistent improvement in spatial memory performance after daily treatment for 50 days with both Ginkgo preparations although soluble and insoluble $A\beta_{42}$ species including plaques were significantly reduced. One of those extracts even was the commercial Ginkgo concoction Tebonin® which is used against concentration problems und memory deficits. Similar to other investigation (Holmes et al., 2008), the present study also indicates that Aβ deposition rates do not correlate with the cognitive decline in AD affected individuals.

Besides the intracerebral Aβ load, oxidative stress plays an important role in AD and leads amongst others to neuronal decline and memory loss in consequence (Zhu et al., 2004). According to *in vitro* studies, *Aloysia citrodora* had very potent and promising antioxidative properties (Guimaraes et al., 2011). This study showed no differences in the Morris water maze performances between *Aloysia citrodora* treated mice and their vehicle treated littermates. Morphological analyses showed that these two extraction forms of *Aloysia citrodora* neither seemed to activate microglia, which would enhance Aβ phagocytosis, nor act neuroprotectively against neuronal destruction and declined memory performance, triggered by oxidative stress as a result of neuroinflammation due to neurotoxic Aβ species.

Summarising, the explorative screening of herbal extracts of various plant species detected numerous effects on AD pathologies in an APP/PS1 mouse model: (i) on the toxic intracerebral $A\beta_{42}$ level and (ii) on the plaque load as well as (iii) an influence on memory. Five descriptive morphological and behavioural parameters indicate the quality of the herbs' effects on the disease severity (see Table 22). Out of twelve extracts, two plant species complied the prerequisites by showing beneficial effects on at least four parameters, which was the basic condition for further analyses. *Hypericum perforatum* and *Sideritis* spp. applied daily also led to increased memory performance and to a highly decreased Aβ burden, beside a significantly decreased $A\beta_{42}$ level as well as plaque load in APP/PS1 mice.

5.2 The medicative effectiveness of SJW extracts on AD related pathology in APP/PS1 mice depends on its extraction form

The prescreening detected strong effects of one SJW extract on main pathological hallmarks in the transgenic APP/PS1 mouse model. Two principal components, hyperforin and hypericin make the herb SJW an interesting treatment option in the field of neurology. Hyperforin is one of the best known active compounds of SJW; it is the responsible component for the effectiveness in depression treatment (Butterweck, 2003, Mennini and Gobbi, 2004). Because of it, the medicinal herb has played an important role in the treatment of mild to moderate depression for several decades (Mennini and Gobbi, 2004, Linde et al., 2008) and it is also reputed to affect cognitive performance, which brought it into focus of AD research (Griffith et al., Dinamarca et al., 2006, Dinamarca et al., 2008). *In vitro* studies indicated disruptive effects of hyperforin on Aβ oligomerisation and positive impacts on neuronal survival, while *in vivo* studies only showed minor effects after intrathecal or intraperitoneal injection of either hyperforin sodium salt or hyperforin derivatives like tetrahydrohyperforin (Dinamarca et al., 2006, Carvajal and Inestrosa, 2011, Inestrosa et al., 2011) but *in vivo* studies of hyperforins' effectiveness on AD pathology are still challenging.

Because of studies reporting a very low bioavailability of hyperforin after oral administration (Biber et al., 1998), it was of fundamental interest to evaluate the importance of hyperforin for the effects on AD pathologies in the APP/PS1 mouse model, as demonstrated by the prescreening. Keller and Biber showed that after oral administration of a high hyperforin dosage (15 mg/kg), only minor fractions of the initially administered amounts of hyperforin (~0.19%) could be intracerebrally detected (Biber et al., 1998, Keller et al., 2003). The need for circumventing the low hyperforin availability, which is by magnitudes lower after oral administration, led to the approach of injecting hyperforin derivatives directly into the brain or at least intraperitoneally by different groups (Inestrosa et al., 2011). Obviously, such kind of highly invasive applications are not easily to be discussed comparing to the oral administration approach presented here, and are of course also not easily conceivable to be converted into clinical use. Therefore this study based on two intensions: (i) to enhance the availability of hyperforin and by this to ascertain the importance of hyperforins availability and (ii) to prove its potential on and stake in Aβ lowering effects. Hence, two treatment strategies were performed consisting of daily oral administrations of low and of high dosed extracts. Dosages were chosen according to literature where dose rates vary between 300 mg/kg and 600 mg/kg body weight (Klemow et al., 2011). High dosages of 2 g/kg as well as a maximum dose of 4 g/kg body weight were also reported (Barnes et al., 2001, Mueller et al., 2004, Klemow et al., 2011). Regardless of the low bioavailability of hyperforin, the goal of this study was to elucidate the effectiveness of different extraction forms of SJW to lower the AD pathology in order to possibly reveal a new and gentle treatment option and to elucidate the significance of hyperforin for the extract's functions. One treatment regime with a low dosage

(400 mg/kg body weight) starts at the age of 40 days during initial phase of Aβ deposition to restrict the onset of AD pathology because according to Radde *et al.* first deposits are detectable at the age of six weeks (Radde et al., 2006). At the age of 50 days, the second treatment paradigm starts with applications of the 10fold extract dosage (4 g/kg body weight) to maximize the extract effectiveness on existing diseased processes and first Aβ proteopathies.

Independently of hyperforin content, SJW extracts beneficially affect cerebral beta amyloid pathologies in APP/PS1 transgenic mice

Consequently the presented results indicate (i) a significant effect of SJW extracts on AD proteopathy, and (ii) that the hyperforin concentration does not correlate with clinical effects in APP/PS1 transgenic mice. Furthermore, the data also present strong evidence against a direct role of hyperforin in the clinical potential of SJW extracts, since two ethanolic extracts, both characterised by high hyperforin concentrations (SJW60low and SJW60high), showed no significant effects on morphological aspects of AD's pathological hallmarks like amyloid plaque level in the AD mouse model. There are several studies which documented a greater importance of soluble Aβ species, i.e. peptides and small oligomers than of insoluble aggregates and depositions (Lesné et al., 2006, Holmes et al., 2008, Lord et al., 2009). Separate analyses of both Aβ species showed a high effectiveness only for both 80% ethanolic extracts. It could also be detected that SJW80 extracts (hyperforin concentration: 0.32% in SJW80low, 2.88% in SJW80high) were consistently able to significantly reduce soluble $A\beta_{42}$ levels by at least 38% starting early in AD pathogenesis and 50% starting post AD onset. Using *in vivo* studies, Dinamarca and colleagues showed that the intracerebral injection of hyperforin prevented Aβ mediated neurotoxicity after coinjection of Aβ peptides (Dinamarca et al., 2006). In contrast, this study did neither observe morphological nor behaviour-modifying effects after oral application of the hyperforin rich extract (SJW60high, 6.08%), not even in the post-onset group with its tenfold dose application. These SJW60 extracts were not effective in reducing Aβ levels. However, treatments with SJW80, which has a similar hyperforin concentration, resulted in a significantly lower amyloid burden, a fact that clearly demonstrates that the beneficial effects of SJW extracts are independent of the hyperforin concentration. Several former studies indicated that hyperforin and hyperforin analogues are able to disaggregate plaque structure at least *in vitro* (Dinamarca et al., 2006, Dinamarca et al., 2008, Cerpa et al., 2010). Accordingly, the present study detected that low-hyperforin extracts, especially SJW80, significantly reduced the cortex coverage by senile plaques using AD initiation and post AD onset treatment strategy. Although hyperforin is hypothesised to be the main active ingredient, treatment with extracts containing high amounts of hyperforin did not lead to significant effects on Aβ aggregation.

Application of hyperforin low SJW extracts prevents neuronal degeneration and cognitive decline triggered by toxic Aβ levels

In addition, hyperforin was suggested to act against Aβ induced neurotoxicity. Quantitative analyses of the neuronal density indicated a significant decline of neurons caused by Aβ without SJW80 treatment or contrariwise a prevention of neuronal loss by hyperforin low extraction forms. Summarising, the results suggest that hyperforin is not the ingredient accounting for the treatment efficacies. Furthermore, since Lesné and Lord previously reported that especially oligomeric Aβ species are causative for memory dysfunction (Lesné et al., 2008, Lord et al., 2009), this study consequently observed that the amount of soluble Aβ species correlated more with the decline of spatial memory than quantities of insoluble fibril formations and Aβ plaques do. Interestingly, the effect of a more condensed neuronal area within the neocortex was even more pronounced in the post-onset paradigm. Only SJW80 extracts showed a significant attenuating effect on the neuronal decline. Concluding, also this effect is not due to hyperforin but rather due to yet unknown substances in the tested SJW extracts. Along the decline of neurons, a significant memory loss occurs as shown by comparative Morris water maze analyses of non-transgenic mice and its APP/PS1 transgenic littermates. With both treatment paradigms a strong cognitive improvement in the APP/PS1 mice could be achieved after application of both SJW80 extracts. The aqueous and even the hyperforin-containing SJW60 extracts did not improve cognitive performance at all. In contrast, mice of the SJW80 groups showed a performance comparable to the level of non-transgenic mice with significantly decreased escape latencies by 40% (SJW80low) to 50% (SJW80high). This finding is consistent with results of $A\beta_{42}$ ELISA measurements, assuming a higher toxicity for soluble $A\beta_{42}$ oligomers (Lesné et al., 2008, Lord et al., 2009). While Trofimiuk and colleagues observed the ability of SJW to enhance spatial working memory in stressed mice (Trofimiuk and Braszko, 2008, Trofimiuk et al., 2011), the present data show that SJW extracts are not effective *per se,* instead only certain 80% ethanolic extracts are.

Phagocytic activity of microglia is stimulated by 80% ethanolic extracts in a dose dependent manner

To discover the responsible mechanism behind the beneficial effects of SJW80 extracts, APP processing pathways, ABC transporter-mediated export, and microglial activation were explored. Since microglia have been recognised to clear Aβ (Kraus et al., 2007, Griffith et al., 2010), they came into focus of interest regarding the function of herbal extracts. *In vitro* experiments of Kraus and colleagues indicated that the treatment with complex SJW extracts may restore and improve microglial viability, and thereby attenuate Aβ-mediated toxicity (Kraus et al., 2007). In line with those findings, *in vitro* phagocytose experiments utilising a primary microglia cell culture and fluorescent labelled $A\beta_{42}$ peptides revealed a significant enhancement of microglia activation. The amount of phagocytosed $A\beta_{42}$ was significantly

increased after treatment with every SJW extract. In addition, this effect might be somehow dose-dependent since only animals of the post-onset group receiving a 10fold dosage showed significant effects. Comparative *in vivo* analyses of brain sections immunohistochemically double-stained for microglia and Aβ plaques in contrast indicated an increased microglial activity only in both SJW80 treated groups. These results showed that *in vitro* experiments using crude SJW extracts or pure hyperforin must be critically reviewed. Together with studies of other work groups, which indicated strong beneficial effects for hyperforin *in vitro*, these data suggest that factors accounting for beneficial effects do not enter the brain *in vivo* in sufficient amounts or only in insufficient dosages when given orally. In conclusion, since SJW60 extracts containing comparable or higher hyperforin concentrations performed worse than SJW80 extracts in a variety of *in vivo* experiments, this study showed that effects of SJW extracts on AD pathology are not hyperforin dependent.

Reduced Aβ levels are not caused by enhanced transport activity of ABCB1 facilitated by SJW extracts

The presented inverse correlation between soluble $A\beta_{42}$ species and enhanced cognitive performance as well as the previously proposed relevance of soluble Aβ species in the AD pathology by Xia and Lord (Lord et al., 2009, Xia, 2010) imply the importance of Aβ clearance mechanisms for treatment prospects in AD. Abuznait et al. showed an up-regulation of the blood-brain barrier transporter ABCB1 by hyperforin in *in vitro* experiments. They reported a reduction of intracellular Aβ accumulation in a human cell line, assumingly based on the sensitivity of the PX-receptor to hyperforin (Abuznait et al.). Beside human PXR, Ott and colleagues indicated that the ligand hyperforin also activated pig PXR but did not activate rodent PXR at the BBB. Hence, hyperforin did not induce mRNA and protein expression of ABCB1 (Ott et al., 2009). Furthermore they detected in *in vitro/ex vivo* investigations that hyperforin directly inhibited ABCB1 transport activity (Ott et al., 2010). In agreement, this study observed no effects of the hyperforin rich extract SJW60high on soluble $A\beta_{42}$ levels; neither increase nor decrease of these toxic species were observable. The analyses of the hyperforin influence on ABCB1 activity in a cell-free *in vitro* assay showed no impact. Contrary, Brenn and colleagues stated a novel AD treatment approach targeting Pgp activation. In experiments with APP/PS1 mice they showed that oral extract treatment with a hyperforin content of 5% enhanced the protein expression of Pgp and reduced intracerebral Aβ accumulation (Brenn et al., 2013). However, they neither showed any effect of hyperforin on the transport capacity, nor the effect of solely increased Pgp expression on quantities soluble toxic Aβ species, or even any evidence regarding hyperforin's effect on memory impairments, the main infirmity of AD patients. Since we know about the very low bioavailability of hyperforin in the CNS, the effect of hyperforin in the tested extract is disputable.

A hyperforin low extract of SJW in vitro stimulate ABCC1 transporter function

Additionally, the present study showed hyperforin independent effects on ABC transporter activity *in vitro*. These effects were also independent of the reported enhanced transporter expression because of the insensitivity of the murine PX-receptors to hyperforin (Ott et al., 2009). Furthermore, this study presents evidence for SJW extracts having an activating impact on ABCC1 transporters in a cell-free *in vitro* assay probably due to competitive substances within crude plant extracts. Krohn reported that ABCC1 has a strong influence on cerebral Aβ levels. Activation of this transporter by thiethylperazine leads to a significant reduction in cerebral Aβ concentration (Krohn et al., 2011). Interestingly, the ABC transporter activating function of a hyperforin-low extract (Hofrichter et al., 2013) appeared to be comparable to that reported for thiethylperazine (Krohn et al., 2011). In this study the hyperforin rich extract SJW60high was compared with the low hyperforin extract SJW80low in an ABCC1 transporter activity assay. Results revealed strong differences between the SJW80low and SJW60high extracts. While SJW60high showed no influence on ABCC1, a significant increase in activity could be detected for SJW80low. Furthermore the assay unveiled that both extracts do not contain typical transport substrates, but substance(s) that seem to enhance transport effectiveness, since they reduce the baseline activity of the transporter. It can be hypothesised that the prominent *in vivo* effects of SJW treatment, especially the strongly reduced Aβ burden in APP/PS1 mice, are likely the result of the activation of microglial phagocytosis as well as enhanced clearance of Aβ due to increased ABCC1 transport activity.

Extracts of SJW consist of a multitude of different organic compounds, while there is only knowledge about the concentration of the main active compounds hyperforin and hypericin. The detection of the composition of all other compounds is very defined and limited to the amount of classes of organic compounds, like flavonoids. The focus of intention needs to be shifted away from hyperforin as the "only" main active substance of SJW. The effective compounds still have to be determined in further bio-organic chemistry studies.

5.3 AD aetiopathology in APP/PS1 mice followed by cognitive impairments is prevented by *Sideritis* spp.

As outlined for SJW80low, the extract of *Sideritis scardica* also showed promising effects on AD pathology in the prescreening of APP/PS1 transgenic mice. Currently, *Sideritis* spp. is unknown in the field of dementia research. For the first time, extracts of two Sideritis species are now reported to have an effect on Aβ pathology as the main characteristic of AD by reducing the intracerebral concentration of the toxic protein itself. The exact composition of *Sideritis* spp. regarding its active agents is unclear, but the extraction method using ethanol as eluent suggests that they are probably of the flavonoid family as reported by the manufacturer. That this extraction method generates flavonoid rich extracts was also shown by various

studies (Hernandez-Perez et al., 2004). The group around Xue-Feng for example generated various flavonoid fractions by different ethanol concentrations: 20% ethanol (38.0% flavonoid content), 40% ethanol (48.6% flavonoid content), 60% ethanol (23.2% flavonoid content) and 80% ethanol (7.3% flavonoid content) from a bamboo species (Xue-Feng Guo, 2012). Beside terpenes and essential oils, *Sideritis* species are abound in a variety of flavonoids and are thereby famous for their action especially as antioxidants and anti-inflammatory agents (Tsaknis and Lalas, 2005, Gonzalez-Burgos et al., 2011, Zhao and Zhao, 2012). To enter the central nervous system and act biologically, agents basically have to cross the BBB. *In vitro* studies demonstrated that the permeation of polyphenols through the BBB is dependent on (i) the degree of lipophilicity with less polar polyphenols or metabolites capable of greater brain uptake than the more polar ones, (ii) on their interactions with blood brain barrier efflux transporters, like P-glycoprotein (P-gp), and (iii) their stereochemistry (Youdim et al., 2003, Youdim et al., 2004a). Otherwise, compounds would need to rely on some kind of active molecular transport systems to overcome the brain's insulation. Different studies confirmed that phenolic compounds are able to undergo permeation of the BBB (Kawabata et al., 2010, Cheng et al., 2013) and to exert direct neuroprotective effects (Youdim et al., 2003, Youdim et al., 2004b). Using an *in vitro* model of brain endothelial cells co-cultured with glioma cells of mouse and rat, Youdim reported that citrus flavonoids were significantly taken up by the *in vitro* BBB model.

Sideritis spp. extracts reduce cerebral $A\beta_{42}$ levels and lower plaque burden in a dose dependent manner in APP/PS1 transgenic mice

The effects detected in the prescreening, especially a significantly reduced soluble Aβ fraction, and the subsequent in profound analysis, results indicated the ability of Sideritis' compounds, presumably flavonoids to pass the BBB. This fact is immensely important because studies pointed out that flavonoids might delay the initiation of neurodegenerative disorders or slow down their progression (Williams and Spencer, 2012). Paris and colleagues indicated that the highly antioxidant flavonoid celastrol in root extracts of Tripterygium is able to decrease intracerebral Aβ e.g. by slowing down the Aβ production indirectly via interaction with the transcription factor NFκB, hence the inhibition of BACE1 transcription (Paris et al., 2010). In accordance with those reports, the present study points to flavonoid-rich extracts of two Sideritis species to be potent $A\beta_{42}$ level lowering agents. Both single extracts as well their combination consequently diminished the $A\beta_{42}$ level by at least 41% in both treatment strategy groups. This in turn led to a significant decrease of the plaque load. Moreover, Youdim reported that the uptake of flavonoids increased significantly in a time and concentration manner (Youdim et al., 2003). Although results imply a higher Aβ reductive potency of extracts given in higher dosages, this study detected no significant dose dependent effect on soluble Aβ decline. This is probably due to the two heterogeneous experimental strategies, starting at two different points within AD aetiopathology of APP/PS1 transgenic mice.

Interestingly, extract concentration differences in contrast resulted in cortical plaque burden differences. Only the high-dose treatment finally led to a decreased plaque load concerning plaque number and size. In conclusion, there is a concentration but not time dependent manner in which Sideritis extracts take effect, since the high-dose post AD onset treatment reduced the overall Aβ burden, whereas the ten days longer treatment duration of the AD initiation strategy did not. However, the mode of Aβ proteopathy diminishing action of Sideritis extracts remains to be elucidated while several explanations are already discussed. Some studies detected an inhibitory capacity of flavonoids on $A\beta_{25-35}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ fibrillation *in vitro* (Grosso et al., 2013) but also *in vivo* (Hamaguchi et al., 2009). A flavonoid effective against Aβ proteopathy *in vivo* is silibinin. It is derived from the herb milk thistle *Silybum marianum* and acts as an inhibitor of Aβ aggregation (Murata et al., 2010) in a dose dependent manner and is preventive against oxidative stress initiated by $A\beta_{1-42}$ in neurons derived *in vitro* from a SH-SY5Y cell line (Yin et al., 2011). Also polyphenols like morin from *Maclura pomifera* or the ubiquitous flavonoid quercetin are active on beta-amyloid aggregation and exhibit strong protecting effects from $A\beta_{25-35}$ as well as $A\beta_{1-40}$ and $A\beta_{1-42}$ toxicity as shown before by Kim and Rivière (Kim et al., 2005, Riviere et al., 2008). Mori and colleagues orally administered the flavonoid tannic acid to mice exhibiting a cerebral amyloidosis and showed that cerebral parenchymal Aβ deposits are mitigated in tannic acid-treated transgenic mice (Mori et al., 2012). In this study, plaque analyses after treatment with both Sideritis species, especially in combination, indicated strong effects on Aβ fibril formation and plaque deposition, resulting in an overall significantly decreased Aβ burden. But within occurring Aβ species, pre-fibrillar aggregates are primarily responsible for amyloid toxicity *in vitro* as well as *in vivo* (Walsh et al., 2002). Plaques and its insoluble fibril elements therefore are not directly toxic but still function as reservoirs for toxic species (Nicoll et al., 2006, Stefani, 2012) since it is believed that there is a delicate equilibrium where Aβ peptides switch between soluble oligomers and insoluble fibrils (Krohn et al., 2011). However, by different mechanisms even plaques lead to structural and functional disruption of neuronal networks and micro-lesions of the surrounding tissue (Grutzendler et al., 2007, Kuchibhotla et al., 2008).

Oral application of an extract combination of two Sideritis spp. species prevents Aβ triggered neuronal and cognitive decline

Amyloid plaques occur in a variety of conditions and they all are accompanied by dystrophic neurites and disrupted axonal transport. Studies of Grutzendler et al. demonstrated that plaques and their surrounding micro-environment are toxic to dendrites and contribute to a significant disruption of neuronal conjunctions (Grutzendler et al., 2007). These findings correlate with the results of the memory performance of treated APP/PS1 mice ascertained by Morris water maze. Like other studies, this study showed a close correlation between soluble Aβ and memory loss during AD pathogenesis (Cleary et al., 2005, Shankar et al., 2008, Lord et

al., 2009) and a prevention of flavonoids against Aβ-induced memory impairments and oxidative stress in mice (Lu et al., 2009). Lue and Shankar stated that the soluble Aβ concentration can be a predictor of synaptic change and cognitive decline (Lue et al., 1999). In fact, mice which received the extract combination with AD initiation therapy and showed improved water maze performances also showed an overall reduced $A\beta_{42}$ burden including plaques. Both single extracts where able to reduce soluble Aβ, but not its deposited forms. These mice also showed no changes in memory performance comparable to the vehicle treated littermates. Contrary, members of the post AD onset treatment group which received the tenfold extract dosage showed a reduction in Aβ peptides and oligomers as well as amyloid plaques.

As already reported, different studies indicated strong correlations between intracerebral Aβ burden in terms of soluble peptide species and cognitive decline (Cleary et al., 2005). Mice showing a decreased Aβ burden also showed an improved cognitive performance comparable to non-transgenic littermates. This effect is dose dependent since mice receiving the high-dose post AD onset treatment showed significantly reduced escape latencies in comparison to low-dose treated littermates in the AD initiation group. The extract combination of both species in contrast constantly showed a memory improving capacity. Although mice of the AD initiation group treated with both single extracts also showed highly reduced intracerebral Aβ concentrations, they did not reveal an enhanced memory performance in comparison to the extract combination or vehicle treated littermates. In contrast to both single extracts, the extract combination only showed a decreased plaque load in the low-dose AD initiation treatment strategy. While soluble Aβ peptides act highly neurotoxic, aggregated forms and protein depositions highly act on network communication. It was shown that pathogenic Aβ assemblies elicit aberrant excitatory activity in cortical-hippocampal networks and thereby disrupt the neuronal network communication (Harris et al., 2010). High concentrations of Aβ assemblies reduce glutamatergic transmission and hence increase synaptic long-term depression and in consequence network dysrhythmias like epileptic activities. Synaptic depression, aberrant network synchronization and abnormal patterns of neuronal activity interfere with activity-dependent synaptic regulation, which is critical for learning and memory and if abnormal may trigger neurodegeneration (Palop and Mucke, 2010). With *in vivo* functional magnetic resonance imaging (fMRI) of Aβ, Sperling and colleagues demonstrate that high levels of Aβ deposition are associated with impaired default network function (Sperling et al., 2009). In the present study, mice treated with the extract combination showed decreased soluble toxic Aβ and reduced plaque load with both treatment strategies. Only these showed reduced escape latencies as well, hence, indicate a strong relation between Aβ deposits and network function. Additionally, the consequence of an interaction of soluble oligomers with receptor proteins is as well a network dysfunction of dendrites and synapses, hence an imbalance of neurotransmitter uptake and release (Danysz

and Parsons, 2012). Flavonoids have been shown to act as acetylcholinesterase inhibitors (Uriarte-Pueyo and Calvo, 2011). Interestingly, Danysz et al. studied the effect of various ethanolic *S. scardica* extracts on serotonin, noradrenaline and dopamine uptake in rat brain synaptosomes and found that they are promising monoamine reuptake inhibitors (Knorle, 2012). Summarising, memory improvements by the treatment with Sideritis extracts is possibly a consequence of additive effects of the (i) reduction of soluble toxic Aβ species, (ii) restored communication between neurons by reduced plaque load, (iii) in consequence neuroprotective, possibly antioxidative properties and (iv) enhanced microglia activity assuming neurotrophic effects.

In vitro studies indicate protective effects of polyphenols against Aβ-induced cytotoxicity. They act highly anti-inflammatory and anti-oxidative (Ansari et al., 2009, Qin et al., 2012, Grosso et al., 2013). They have been shown to be very capable as multi-targeted therapeutic for protecting the brain against oxidative damage (Choi et al., 2012). Ishige et al. detected three protective mechanisms of flavonoids against oxidative stress in neurons: (i) increasing intracellular glutathione, (ii) directly lowered ROS levels, and (iii) prevention of Ca^{2+} influx despite high ROS levels (Ishige et al., 2001). Flavonoid concentrations and their respective exposure times have been shown to be correlated to survival or death of neurons, especially by antioxidative effects (Dajas et al., 2013). Summarising *in vitro* as well as *in vivo* studies indicated potential protective mechanisms of flavonoids in neuronal cell death (Zhao, 2009). Brain slices were analysed regarding beneficial effects. It is proposed that especially combinations of agents may produce additive neuroprotective effects in neurodegenerative diseases. There is also the assumption that a mixture of diverse antioxidants and anti-inflammatory agents may be more beneficial in the prevention of neurodegenerating processes (Wang et al., 2006b, Lin, 2011). Indeed, quantification of neuronal area revealed protective effects of *Sideritis* extracts, especially when administered in combination. AD initiation as well as post AD onset treatment of APP/PS1 mice with the extract combination led to a significant increase of neuronal area. Moreover, these mice had a neuronal density comparable to that of vehicle treated littermates. Beside the extract combination, a single high dosed *S. euboea* extract showed neuroprotective effects as well. However, the agents accounting for this effect still have to be identified just as the hypothesised underlying anti-inflammatory and anti-oxidative mechanisms (Wang et al., 2006b).

Sideritis spp. highly activates microglia activity without inducing inflammatory and neurodegenerating processes in APP/PS1 mice

After Sideritis extract treatment, the $A\beta_{42}$ load is significantly decreased which might be a consequence of enhanced microglia dependent plaque degradation. Microglia are associated with plaques, and studies indicate that soluble Aβ species and aggregated proteins are able to highly trigger the phagocytic activity of microglia (Sastre et al., 2006, Streit, 2006). Analyzing

brains of Sideritis treated APP/PS1 mice in this study indicated a link between the extract and microglia activity. The microglial response was highly increased after 60 days of treatment. The overall microglia area as well as the number of plaques which are covered by microglia by more than fifty percent was significantly increased. At the same time, the $A\beta_{42}$ burden in contrast to vehicle treated littermates was significantly reduced. Microglia also plays a relevant role in inducing and activating multiple cell signalling pathways that promote neuroinflammation and thereby promoting the development of AD. Inflammation is a pathological hallmark of AD and may actively contribute to disease progression and chronicity (Rojo et al., 2008, Azizi and Mirshafiey, 2012). Uncontrolled and chronic inflammatory processes may result in the production of neurotoxic factors that amplify underlying disease states, resulting in cognitive decline by various mechanisms (Rao et al., 2012) leading to neuronal death (Glass et al., 2010, Rao et al., 2012). Chronically activated microglia mortifies adjacent neurons by releasing proinflammatory and neurotoxic factors, amongst others TNFα, IL-1β, reactive oxygen intermediates, nitric oxide, and various proteolytic enzymes (Rojo et al., 2008, Rubio-Perez and Morillas-Ruiz, 2012). These mediators enhance APP production and the amyloidogenic processing of APP to $A\beta_{42}$ peptides, and in contrast inhibit the formation of the soluble APP fraction that has neuroprotective effects (Del Bo et al., 1995, Ringheim et al., 1998, Fassbender et al., 2000, Friedlander, 2003). It is notable that although there is an increased microglia response after Sideritis application, there is no evidence of pro-inflammatory mediators which induced oxidative stress and lead to enhanced APP, PS and BACE1 expression by various cytokines and in return to enhanced intracerebral $A\beta_{42}$ concentrations (Sastre et al., 2008). Affirming this, besides measured $A\beta_{42}$ levels,, western blot analyses showed no effect on BACE1 expression in APP/PS1 mice after treatment with both Sideritis extracts or the extract combination. Hence, there is no evidence for inflammatory processes that result in the production of neurotoxic mediators resulting in oxidative stress, cellular damage, neuronal dysfunction and cell death (Glass et al., 2010). Contrary, beside the reduction of Aβ by phagocytosis, acute inflammatory stimuli also induce beneficial effects like tissue repair processes (Frautschy et al., 1998). Microglia secrete a number of neurotrophic factors such as the glia-derived neurotrophic factor (GDNF), IL-6 or BDNF by enhancing mRNA expressions that are beneficial to the survival of neurons (Siegel and Chauhan, 2000, Liu and Hong, 2003). Flavonoid rich food like *Camellia sinensis*, *Theobroma cacao* and *Vaccinium* spp. demonstrated beneficial effects on memory and learning in both humans and animal models (Rendeiro et al., 2013). IL-6 is a multifunctional cytokine that has both direct and indirect neurotrophic effects on neurons. It promotes astrogliosis, activates microglia, and stimulates the production of acute phase proteins (Castell et al., 1989, Selmaj et al., 1990, Heyser et al., 1997, Benveniste, 1998). Oxidative stress, resulting in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is an attribute of neurodegeneration and leads to significant damage of cell structures (Huber et al., 2006, Wang et al., 2006b). Different studies showed that antioxidant therapies

have enjoyed general success in different ways: (i) trapping of free radicals, (ii) antioxidant activity in neurons per se, as well as (iii) a suppression of genes induced by pro-inflammatory cytokines and other mediators released by glial cells (Wang et al., 2006b). Extracts with antioxidants and anti-inflammatory agents may be very beneficial in the prevention of neurodegenerative disease like AD (Pandey and Rizvi, 2009). Especially secondary plant metabolites are able to increase the immune response against soluble Aβ species as well as protein aggregates, inflammatory processes and neuronal damage by oxidative stress (Sastre et al., 2006, Streit, 2006, Zhao, 2009). Sideritis extracts are able to activate microglia, to reduce $A\beta_{42,}$ and to improve cognitive performance significantly. Additionally, the extract composition, regardless of dosage, is highly able to significantly prevent neuronal loss shown by increased neuronal area in contrast to vehicle treated APP/PS1 mice as detected by NeuN staining. Although both singe extract groups revealed a tendency with regard to neuroprotection, the number of experimental animals did not suffice to reach significance. Nevertheless, proinflammatory and neurotrophic factors have to be reviewed.

Sideritis spp. enhances the non-amyloidogenic pathway of Aβ generation by improved expression of the metalloprotease ADAM10

Other strategies to avoid Aβ genesis are enhanced ADAM10 activity or decreased action of BACE1. Flavonoids like EGCG showed an effect on the expression of the α-secretase ADAM10 as well as indirectly on β-secretase BACE1 transcription (Obregon et al., 2006, Fernandez et al., 2010, Paris et al., 2011), and in consequence on the processing of APP to non-toxic Aβ-peptides and in preventing the formation of toxic Aβ species, respectively. This was shown before for *Camellia sinensis* containing the active component epigallo-catechingallate (EGCG) (Obregon et al., 2006). Flavonoid rich extracts of both Sideritis extracts, especially in combination, highly enhanced ADAM10 expression according to the Western blotting results, indicating a lowered $A\beta_{42}$ genesis. Contrary, BACE1 expression remains unaffected after treatment.

In conclusion, specific SJW extracts and the extract combination of two species of *Sideritis* spp., namely *S. scardica* and *S. euboea,* showed highly beneficial potential in counteracting Aβ pathology followed by highly increased memory performance as well as enhancing microglia activity without the initiation of uncontrolled and chronic inflammation cascades. It is effective at low doses at as well as in high dosages after disease onset, with no adverse side effects known so far at least for Sideritis. The study evidences that specific extraction forms of SJW, independently of hyperforin and both *Sideritis* spp. species, especially the extract mixture of both bears a promising cognition improving potential for memory impaired patients, here with regard to Alzheimer's disease.

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Curriculum vitae

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List of publications

PUBLICATIONS

Hofrichter J, Krohn M, Schumacher T, Lange C, Feistel B, Walbroel B, Heinze HJ, Crockett S, Sharbel TF, Pahnke J (2013) Reduced Alzheimer's disease pathology by St. John's Wort treatment is independent of hyperforin and facilitated by ABCC1 and microglia activation in mice. Curr Alzheimer Res 10:1057-1069.

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Baltic Winter School (2008) Oral presentation: Function of ABC Transporters for the Pathogenesis of idiopathic Alzheimer's Disease. Binz/Rügen, Germany

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Hofrichter J, Kraft K, (2011), Einfluss von Pflanzenextrakten aus Hypericum perforatum auf die Amyloidbelastung und Kognition von Mäusen eines APP/PS1-B6 Mausmodells. FORUN Program of the University of Rostock

Declaration

Ich gebe folgende Erklärung ab:

1. Die Gelegenheit zum vorliegenden Promotionsvorhaben ist mir nicht kommerziell vermittelt worden. Insbesondere habe ich keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen/Betreuer für die Anfertigung von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

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Rostock, den 28.03.2014