Natural products against neurodegenerative diseases: effects in the model organism *Caenorhabditis elegans*

DISSERTATION

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany

for the degree of
Doctor of Natural Sciences

presented by
Dipl.-Biol. Pille Link
born in Tallinn, Estonia

DISSERTATION

submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany

for the degree of
Doctor of Natural Sciences

presented by
Dipl.-Biol. Pille Link
born in Tallinn, Estonia

Oral examination: 24/02/2016

Natural products against neurodegenerative diseases: effects in the model organism *Caenorhabditis elegans*

Referees: Prof. Dr. Michael Wink
Prof. Dr. Gert Fricker

Acknowledgements

First, I would like to thank Prof. Dr. Michael Wink for giving me the opportunity to work in his group at IPMB, Heidelberg University.

Special thanks are due to Prof. Dr. Gert Fricker for serving as second referee for this work.

I would also like to acknowledge Prof. Dr. Yujie Fu (Key Laboratory of Forest Plant Ecology, China), Dr. Quijun Lu (Wangjing Science and Technology Park, China), Prof. Dr. Thomas Efferth (University of Mainz, Germany), and Dr. Egon Koch (Dr. Willmar Schwabe GmbH & Co. KG, Germany) for providing the plant material and pure substances used in this work.

I would like to thank Dr. Christopher Link (University of Colorado at Boulder, USA) for providing me the *C. elegans* strains CL2355 and PD8120, as well as for his kind help whenever I was at a loss about my worms. Furthermore, I also thank the former and current members of the *C. elegans* group at IPMB, especially Dr. Sami Abbas, Dr. Leila Rezaizadehnajafi, Dr. Chen Wei, Steffen Breinlinger, and Felix Heiner, for many useful tips and insightful discussions about our worms.

I owe my thanks to Dr. Dorothea Kaufmann, who gave me a first introduction to Alzheimer's disease and always brightened the day with her colourful personality. She and Dr. Florian Herrmann also provided me with the first TCM extracts and helpful tips to get started with my project.

Special thanks go to Kevin Roth and Mariam Baalbaki, who contributed to this work during their laboratory practicals. You did a nice job.

The analytical work presented here was supported by Dr. Bernhard Wetterauer, who taught me more about mass spectrometers than I wanted to know, and Frank Sporer, who contributed the GLC-MS data. I would also like to acknowledge Eva Arnold and Dr. Ikhwan Sudji for their help with HPLC analysis and Dr. Ahmad Tahrani for initial LC-MS data. Thank you for a fun time in the analytics lab.

I am grateful for the support of Hedwig Sauer-Gürth and Dr. Thomas Tietze with DNA sequencing and data analysis.

Many thanks are also due to Heidi Staudter and Astrid Backhaus for practical tips and troubleshooting, to Dr. Holger Schäfer for asking nasty questions and for wide-ranging scientific discussions, to Petra Fellhauer for helping me through the German bureaucracy, and to the whole AG Wink for a friendly working atmosphere.

Microscopic data evaluation was mostly conducted in the Nikon Imaging Centre at Heidelberg University. I am grateful to Dr. Ulrike Engel, Dr. Christian Ackermann, and Peter Bankhead for providing the equipment and their knowledge to support my work.

Last but not least I thank my mother Sirje Link, who has supported me throughout my studies.

Publications based on this work

Journal articles

Link P, Wetterauer B, Fu Y, Wink M. Extracts of *Glycyrrhiza uralensis* and the pure compound isoliquiritigenin counteract amyloid-beta toxicity in *C. elegans*. *Planta Medica* **81**: 357-362

Conference abstracts

Link P, Roth K, Wink M (2014) *Carlina acaulis* has antioxidant effects and ameliorates beta-amyloid toxicity in a *C. elegans* model. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association* **10**: P465.

Link P, Wink M. *C. elegans as a model for Alzheimer's disease: screening medicinal plants for potential activity against Alzheimer.* Berlin *C. elegans* Meeting. 2014
Berlin *C. elegans* Meeting book of abstracts #P82.

Link P, Wink M. *Effects of liquorice on beta-amyloid aggregation and toxicity in Caenorhabditis elegans.* 27th International Conference of Alzheimer's Disease International. 2012.
ADI Conference book of abstracts #OC001.

Link P, Abbas S, Wink M. *Traditional Chinese medicinal drugs inhibiting beta-amyloid aggregation in C. elegans.* Jahrestagung der Deutschen Pharmazeutischen Gesellschaft. 2010.
DPhG Conference book of abstracts #P205.

Articles in preparation

Link P, Roth K, Sporer F, Wink M. *In vivo* antioxidant activity and amelioration of Aβ toxicity by *Carlina acaulis* and its active compound Carlina oxide.

Link P, Wink M. Antioxidant activity of isoliquiritigenin in *C. elegans* via activating the transcription factor DAF-16/FOXO.

Table of contents

Acknowledgements iii

Publications iv

Abbreviations ix

Summary xi

Zusammenfassung xii

1 Introduction 1
- 1.1 Alzheimer's disease . . . 1
  - 1.1.1 β-Amyloid . . . 2
  - 1.1.2 Tau . . . 6
  - 1.1.3 Oxidative stress, mitochondrial dysfunction, and inflammation . . . 8
  - 1.1.4 Risk factors and biomarkers . . . 10
  - 1.1.5 Treatment . . . 11
- 1.2 Medicinal plants . . . 13
  - 1.2.1 Phytotherapy in Traditional Chinese Medicine . . . 14
  - 1.2.2 *Glycyrrhiza uralensis* . . . 21
  - 1.2.3 *Carlina acaulis* . . . 23
- 1.3 *Caenorhabditis elegans* . . . 24
  - 1.3.1 Insulin-like signalling pathway . . . 26
  - 1.3.2 Nervous system . . . 29
  - 1.3.3 *Caenorhabditis elegans* as a model for AD . . . 30
- 1.4 Objectives . . . 32

2 Material and methods 35
- 2.1 Material . . . 35
  - 2.1.1 Instruments . . . 35
  - 2.1.2 Laboratory material . . . 36
  - 2.1.3 Chemicals . . . 36
  - 2.1.4 Buffers, solutions, media . . . 39
  - 2.1.5 Plants . . . 44
  - 2.1.6 *Caenorhabditis elegans* strains . . . 45
  - 2.1.7 Software . . . 46
- 2.2 Methods . . . 47
  - 2.2.1 Preparation of extracts . . . 47
  - 2.2.2 Isolation of Carlina oxide . . . 47
  - 2.2.3 HPLC analysis . . . 47
  - 2.2.4 LC-MS/MS analysis . . . 47
  - 2.2.5 GLC-MS analysis . . . 48
  - 2.2.6 Identification of *Glycyrrhiza uralensis* via DNA barcoding . . . 48

2.2.7 DPPH• assay . . . 49
2.2.8 SDS-PAGE and Western blot analysis of Aβ . . . 49
2.2.9 *Caenorhabditis elegans* culture conditions . . . 51
2.2.10 Quantification of Aβ aggregates in CL2006 . . . 51
2.2.11 Paralysis assay in CL4176 . . . 52
2.2.12 Chemotaxis assay in CL2355 . . . 53
2.2.13 Serotonin sensitivity assay in CL2355 . . . 53
2.2.14 Heat shock protein expression . . . 53
2.2.15 Survival assay . . . 54
2.2.16 DAF-16 delocalisation . . . 54
2.2.17 Lifespan assay . . . 54
2.2.18 Statistical analysis . . . 55

3 Results **57**
3.1 Screening the TCM extracts against Aβ aggregation . . . 57
3.2 *Areca catechu* . . . 61
3.2.1 Dose-dependence . . . 61
3.2.2 Paralysis assay . . . 61
3.3 *Alpinia oxyphylla* . . . 61
3.3.1 Paralysis assay . . . 61
3.4 *Glycyrrhiza uralensis* . . . 63
3.4.1 Identification of the species . . . 63
3.4.2 Phytochemical analysis of the extracts . . . 63
3.4.3 Dose-dependence . . . 77
3.4.4 Western blot analysis . . . 79
3.4.5 Paralysis assay . . . 79
3.4.6 Chemotaxis assay . . . 81
3.4.7 Serotonin sensitivity assay . . . 81
3.4.8 Antioxidant properties . . . 83
3.4.9 DAF-16 delocalisation . . . 84
3.4.10 Lifespan assay . . . 86
3.5 *Carlina acaulis* . . . 88
3.5.1 GLC-MS analysis of the extract . . . 88
3.5.2 Paralysis assay . . . 90
3.5.3 Chemotaxis assay . . . 91
3.5.4 Serotonin sensitivity assay . . . 91
3.5.5 Antioxidant properties . . . 92
3.5.6 DAF-16 delocalisation . . . 93

4 Discussion **95**
4.1 Screening of TCM drug extracts . . . 95
4.2 *Areca catechu* . . . 98
4.3 *Alpinia oxyphylla* . . . 99
4.4 *Glycyrrhiza uralensis* . . . 100
4.4.1 Determination of the used *Glycyrrhiza* species . . . 100
4.4.2 *Glycyrrhiza uralensis* and its major compounds decrease Aβ aggregation 100
4.4.3 *Glycyrrhiza uralensis* and isoliquiritigenin counteract Aβ toxicity . . . 101
4.4.4 Isoliquiritigenin has antioxidant activity via activating DAF-16 . . . 104
4.4.5 Toxicity of long-term treatment with *Glycyrrhiza uralensis* . . . 106
4.4.6 Is isoliquiritin a viable drug candidate? . . . 107

4.5 *Carlina acaulis* 108
4.5.1 Isolation of Carlina oxide 108
4.5.2 Dichlormethane extract of *Carlina acaulis* but not Carlina oxide has an effect against Aβ toxicity 108
4.5.3 Carlina oxide exhibits *in vivo* antioxidant activity 109
4.5.4 Toxicity of *Carlina acaulis* and Carlina oxide 110
4.6 Conclusion 111

References **113**

Abbreviations

AAP-1	AGE-1 adaptor protein
Aβ	beta-amyloid
ABAD	Aβ-binding alcohol dehydrogenase
ACh	acetylcholine
AChE	acetylcholine esterase
AChEI	acetylcholine esterase inhibitor
ACN	acetonitrile
AD	Alzheimer's disease
ADAM	a disintegrin and metalloproteinase
AGE-1	ageing alteration 1
AICD	APP intracellular C-terminal domain
AKT	protein kinase B
ANOVA	analysis of variance
APOE	apolipoprotein E
APL-1	APP-like 1
APP	amyloid precursor protein
APS	ammonium persulfate
ATP	adenosine triphosphate
BACE1	β-site APP-cleaving enzyme 1
BSA	bovine serum albumin
°C	degrees Celsius
Car0	residual extract of *Carlina acaulis* after isolation of Carlina oxide
CarOx	Carlina oxide fraction isolated from the dichlormethane extract of *Carlina acaulis*
CGC	Caenorhabditis Genetics Center
CI	chemotaxis index
DAF	abnormal dauer formation
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	mean effective concentration
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia*
EGCG	(−)-epigallocatechin gallate
eV	electron volt
FOXO	forkhead box O
FTT-1	14-3-3 family protein 1
FYN	FYN proto-oncogene
g	gram
GA	glycyrrhizic acid
GAA	glycyrrhizic acid monoammonium
GLC	gas-liquid chromatography
GFP	green fluorescent protein
GRA	glycyrrhetinic acid
h	hour
HPLC	high pressure liquid chromatography
HSP	heat shock protein
HSF-1	heat shock factor 1
HRP	horse radish peroxidase
IGF-1	insulin-like growth factor 1
ILG	isoliquiritigenin
ILS	insulin-like signalling
INS	insulin related peptide
IRS	insulin receptor substrate, protein in *H. sapiens*
IST-1	insulin receptor substrate, protein in *C. elegans*
ITS	internal transcribed spacer
JNK-1	c-Jun N-terminal kinase 1
kDa	kilodalton
l	litre
LG	liquiritigenin
LRP1	low density lipoprotein receptor-related protein 1
µg	microgram
µM	micromolar
µl	microlitre
M	molar
$[M-H^+]^-$	negatively charged molecular ion
mA	milliampere
MAP	microtubule-associated protein
mAU	arbitrary unit
mg	milligram
min	minute
mM	millimolar
ml	millilitre
mRNA	messenger ribonucleic acid

MS	mass spectrometer
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NGM	nematode growth medium
nm	nanometre
NMD	non-sense mediated mRNA decay
NMDAR	N-methyl-D-aspartate receptor
NRF2	nuclear factor erythroid 2-like 2
p	probability
PAGE	polyacrylamide gel electrophoresis
PFA	paraformaldehyde
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing 0.05 % Tween® 20
PCR	polymerase chain reaction
PDK-1	3-phosphoinositide-dependent kinase-1
PHA-4	defective pharynx development 4
pH	potential of hydrogen
PI3K	phosphoinositide 3-kinase
PIP_3	phosphatidylinositol-3,4,5-trisphosphat
PLSD	protected least significant difference
ppm	parts per million
PrP	prion protein
PSEN	presenilin
PT_{50}	mean time of paralysis
PTEN	phosphatase and tensin homologue
RAGE	advanced glycosylation end product-specific receptor
rbcL	ribulose bisphosphate carboxylase large chain
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT	retention time
s	second
sAPPα	soluble APP ectodomain released by α-secretase
sAPPβ	soluble APP ectodomain released by β-secretase
SDS	sodium dodecyl sulphate
SIR-2.1	yeast silent information regulator related 2.1
SKN-1	skinhead 1
SMG-1	suppressor with morphogenetic effect on genitalia
SOD	superoxide dismutase
SGK-1	serum- and glucocorticoid-inducible kinase 1
spp.	species
ssp.	subspecies
TCM	Traditional Chinese Medicine
TE	Tris-EDTA
TEMED	tetramethylethylenediamine
ThT	thioflavin T
ThS	thioflavin S
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)-aminomethane
V	volt

Summary

Neurodegenerative diseases are a growing burden in the modern ageing societies. Especially Alzheimer's disease (AD)—the most common form of dementia—has gained lot of attention lately. Although several drugs are available to enhance the life-quality of people with AD, none of them can stop the progression or cure this disease. Therefore new medications for treatment and prevention are needed. Medicinal plants are a rich source for drug leads and active compounds. Furthermore, plant extracts are potential multitarget drugs that can be particularly useful for diseases with complex pathology like AD. Therefore, in the present work plants from Traditional Chinese Medicine (TCM) were tested for their efficacy against two prominent pathological markers in AD: beta-amyloid (Aβ) aggregates and oxidative damage.

For the present study the model organism *Caenorhabditis elegans* was deployed. In a screening of 55 TCM plant extracts on a *C. elegans* strain expressing human Aβ peptide in muscles, several extracts that could reduce Aβ aggregation were identified. From those the three most active ones were chosen for further evaluation. The methanol extract of *Glycyrrhiza uralensis* proved to have the best characteristics for therapeutic use. Additionally to the reduction of Aβ aggregates by 30 %, this extract could also counteract Aβ toxicity in a paralysis assay by increasing the mean time of paralysis (PT_{50}) by 1.8 h and showed antioxidant activity in the heat shock protein (HSP) expression assay.

The major compounds in the *G. uralensis* extract were identified via LC-MS/MS. Four substances—glycyrrhizic acid (GA), glycyrrhetinic acid (GRA), liquiritigenin (LG), and isoliquiritigenin (ILG)—were chosen as possible active compounds. From those ILG showed the strongest activity by reducing Aβ aggregation by 26 % and counteracting Aβ toxicity in paralysis assay (1.2 h delay in PT_{50}). It also affected serotonergic neurotransmission in *C. elegans* with neuronal Aβ expression. Furthermore, significant antioxidant activity was shown in the HSP expression assay, and the survival of worms under oxidative stress was increased by 82 % after treatment with ILG. This compound could induce nuclear translocation of the transcription factor DAF-16 that is responsible for stress resistance and longevity in *C. elegans*. The mechanism of action of ILG in counteracting Aβ toxicity could therefore involve hormesis and modulation of serotonergic neurotransmission.

The present work also reports for the first time the effect of *Carlina acaulis* against Aβ toxicity and its antioxidant activity *in vivo*. The dichloromethane extract of this plant delayed the Aβ-induced paralysis by 1.6 h. GLC-MS analysis identified Carlina oxide as the main compound in this extract. Carlina oxide alone was not as active as the extract in paralysis assay, but it was responsible for the antioxidant activity. Both the extract and Carlina oxide were active in the HSP expression assay and could induce DAF-16 delocalisation. The mechanism of action for *C. acaulis* against Aβ toxicity still needs further study, although hormetic effects and the antioxidant activity may contribute to this effect.

The plants and compounds identified in this study should be considered for further investigation in vertebrate models. Especially their bioavailability and drug safety need broader attention. The initial results reported here suggest *G. uralensis*, *C. acaulis*, and ILG as possible candidates for prevention or treatment of AD. Their positive effects counteracting protein aggregation and oxidative stress might also be useful against other neurodegenerative diseases and for healthy ageing in general.

Zusammenfassung

Neurodegenerative Erkrankungen werden immer mehr zu einer größeren Last in der zeitgenössischen, alternden Gesellschaft. Besonders die Alzheimer Erkrankung (AD) – die am häufigsten vorkommende Demenzart – wurde diesbezüglich in den letzten Jahren in den Fokus gerückt. Obwohl mehrere Medikamente zur Verbesserung der Lebensqualität der Menschen mit AD zur Verfügung stehen, kann keines dieser den Krankheitsverlauf anhalten oder die Patienten heilen. Daher besteht Bedarf an neuen Medikamenten zur Behandlung und Prävention. Arzneipflanzen stellen eine reichhaltige Quelle für Leitsubstanzen und Wirkstoffe dar. Darüber hinaus sind Pflanzenextrakte in der Lage mit mehreren Targets zu interagieren, was bei einer komplexen Pathologie wie die der AD von Vorteil sein kann. Daher wurden in der vorliegenden Arbeit Pflanzen aus der Traditionellen Chinesischen Medizin (TCM) auf ihre Wirksamkeit an zwei prominenten pathologischen Markern von AD – Beta-Amyloid (Aβ)-Aggregate und oxidativer Schaden – getestet.

Für die vorliegende Arbeit wurde der Modellorganismus *Caenorhabditis elegans* benutzt. In einem Screening von 55 Extrakten aus TCM Pflanzen an einer *C. elegans* Linie, die das menschliche Aβ-Peptid in den Muskeln exprimiert, wurden mehrere Extrakte gefunden, die die Aβ-Aggregation hemmten. Von diesen wurden die drei Effektivsten für weitere Untersuchungen ausgewählt. Der Methanolextrakt aus *Glycyrrhiza uralensis* erwies sich als der am besten Geeigneteste für eine Behandlung. Zusätzlich zu der Hemmung der Aβ-Aggregation um 30 % konnte dieser Extrakt auch die Toxizität von Aβ in einem Paralyse-Test herabsetzen, wobei die mittlere Zeit zur Paralyse (PT_{50}) um 1,8 h verlängert wurde, und zeigte antioxidative Wirkung in dem Hitzeschock-Protein (HSP)-Expressions-Test.

Die Hauptbestandteile des *G. uralensis* Extrakts wurden mittels LC-MS/MS identifiziert. Vier Substanzen – Glycyrrhizinsäure (GA) , Glycyrrhetinsäure (GRA) , Liquiritigenin (LG) und Isoliquiritigenin (ILG) – wurden als mögliche Wirkstoffe ausgewählt. Von diesen zeigte ILG die höchste Aktivität, indem es die Aβ-Aggregation um 26 % hemmte und der Aβ-Toxizität in dem Paralyse-Test entgegenwirkte (1,2 h Verzögerung in PT_{50}). ILG beeinflusste auch die serotonerge Signalübertragung in *C. elegans* mit neuronaler Aβ-Expression. Darüber hinaus konnte eine signifikante Aktivität in dem HSP-Expressions-Test gezeigt sowie die Überlebensrate von Würmern unter oxidativem Stress durch die Behandlung mit ILG um 82 % erhöht werden. Diese Substanz konnte die Translokation des Transkriptionsfaktors DAF-16, der für Stressresistenz und Langlebigkeit in *C. elegans* zuständig ist, in den Zellkern induzieren. Der Wirkmechanismus von ILG gegen Aβ-Toxizität könnte daher eine Hormesis und die Modulation der serotonergen Signalübertragung mit einschließen.

In der vorliegenden Arbeit wurde auch zum ersten mal eine Wirkung von *Carlina acaulis* gegen die Aβ-Toxizität und seine antioxidative Wirkung *in vivo* aufgezeigt. Der Dichlormethanextrakt von dieser Pflanze verzögerte die Aβ-induzierte Paralyse um 1,6 h. Eine GLC-MS Analyse identifizierte Carlinaoxid als den Hauptbestandteil dieses Extraktes. Carlinaoxid als Reinsubstanz zeigte in dem Paralyse-Test eine geringere Wirksamkeit als der Extrakt, aber war für die antioxidative Wirkung verantwortlich. Der Extrakt und das Carlinaoxid waren beide wirksam in dem HSP-Expressions-Test und induzierten die DAF-16 Delokalisation. Der Wirkmechanismus von *C. acaulis* gegen Aβ-Toxizität bedarf weiterer Untersuchungen, obwohl hormetische Effekte und die antioxidative Wirkung dazu beitragen können.

Die Pflanzen und Reinstoffe, die sich in dieser Arbeit als wirksam gezeigt haben, sollten für weitere Untersuchungen an Vertebraten in Betracht gezogen werden. Besonders die Bioverfügbarkeit und Arzneimittelsicherheit benötigen umfassende Aufmerksamkeit. Die Ergebnisse dieser Arbeit machen *G. uralensis*, *C. acaulis* und ILG zu möglichen Kandidaten für Prävention und Behandlung von AD. Ihre positive Wirkungen auf Proteinaggregation und oxidativen Stress könnten auch gegen andere neurodegenerative Erkrankungen sowie für ein gesundes Altern allgemein hilfreich sein.

1 Introduction

1.1 Alzheimer's disease

Alzheimer's disease (AD) was first described by Alois Alzheimer as a presenile dementia he observed in one of his patients, Auguste D. The 51 year old woman suffered from loss of memory, confusion, auditory hallucinations, and anxiety (Alzheimer, 1907). After the death of his patient, Alzheimer examined her brain. He found an uniform atrophy of the brain accompanied by fibrillar inclusions in the neurons—neurofibrillary tangles—and another kind of extracellular inclusions, that became known as senile plaques. The plaques are mostly found in the isocortex, whereas the neurofibrillary tangels first appear in the entorhinal cortex, then in limbic areas, and only in late stages of the disease affect also the isocortex (Braak & Braak, 1991). Memory dysfunction is the most common early symptom in AD. It is usually accompanied by a complex mixture of several other cognitive deficits and neuropsychiatric comorbidities that are partly related to the brain areas affected by the pathology (Lyketsos *et al.*, 2011; Peña-Casanova *et al.*, 2012).

The senile plaques can be divided, based on their morphology, into diffuse plaques and classical cored plaques. All of them contain a protein known as beta-amyloid (Aβ) and variable other constituents (reviewed in Armstrong, 2009). The cored plaques are characterized by dense cores consisting of fibrillar Aβ and are often accompanied by dystrophic neurites (then also called neuritic plaques). Due to the insolubility of Aβ fibrils in most common solvents it was possible to isolate and purify them (Selkoe *et al.*, 1986) and the protein was first sequenced by Masters *et al.* (1985). Comparison of the sequences of the protein forming the plaque cores and an amyloid from cerebral blood vessels from AD patients (Glenner & Wong, 1984b) showed that also the latter inclusions contain Aβ. The neurofibrillary tangles proved to be more difficult to purify, but by immunochemical methods they were shown to contain the protein tau (Grundke-Iqbal *et al.*, 1986a).

In the beginning of 20th century only a few presenile cases of AD caught the attention of physicians like A. Alzheimer, but as the disease became better known, it was also detected in many old patients. Today age is considered the most important risk factor for AD. In only about 4 % of the cases the symptoms occur before the age of 65 (Alzheimer's Association, 2015). A small portion of this early onset AD is caused by genetic mutations in genes related to Aβ (see section 1.1.1) and has an autosomal dominant heredity. In these cases the people affected can be as young as 30 years. In the much more common late onset AD no single gene is responsible for the condition (see section 1.1.4).

AD comprises 60–80 % of all dementia cases (Alzheimer's Association, 2015). About 5 % of people over the age of 60 worldwide suffer from AD, and since the population of many countries is ageing, the number of people with AD is predicted to triple until 2050 (Prince & Jackson, 2009). Although AD is the most common form of dementia and has been known and studied for over a century, it is still not clear what causes this syndrome. Figure 1.1 shows some of the proteins and cellular processes found to contribute to the pathology. These factors and their interplay as well as approaches for diagnosis and treatment are described in following sections.

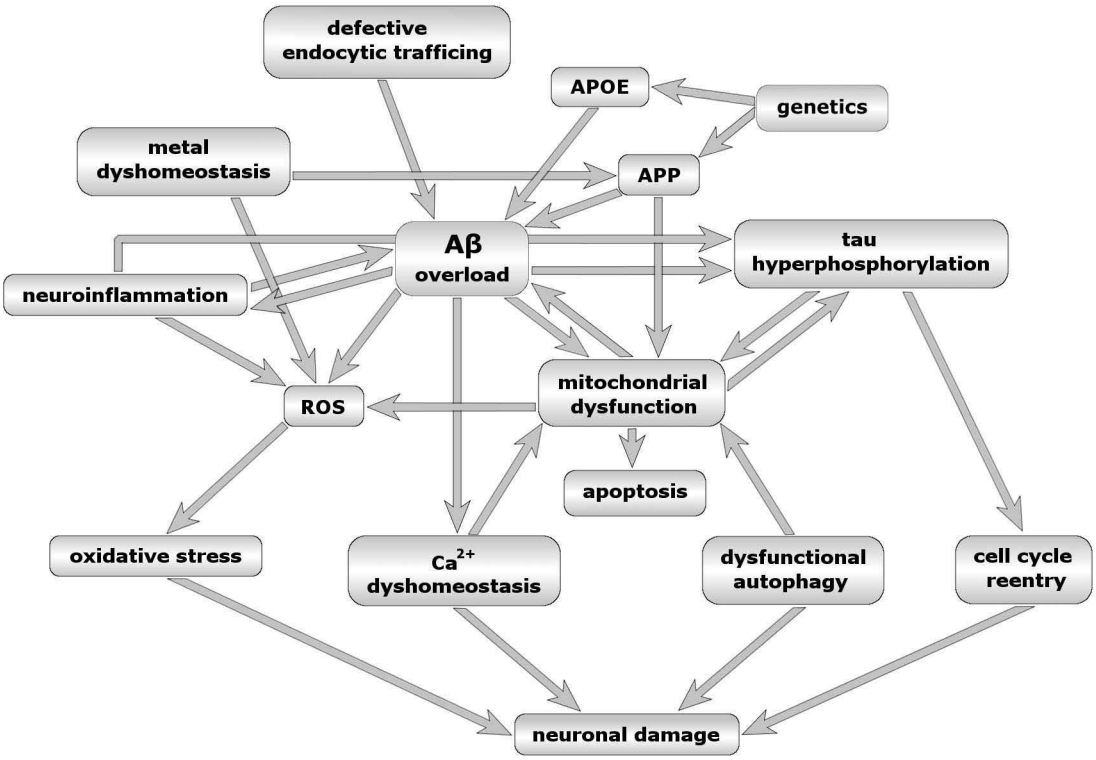

Figure 1.1: Overview of some cellular defects in AD. Next to Aβ overload and tau hyperphosphorylation also mitochondrial dysfunction, oxidative stress, neuroinflammation, and other abnormalities are found in AD brains, ultimately leading to neuronal damage and cell death. The arrows point to possible causative connections between them. APOE: apolipoprotein E; APP: amyloid precursor protein; Aβ: beta-amyloid; ROS: reactive oxygen species.

1.1.1 β-Amyloid

Extracellular depositions of Aβ are one pathological hallmark of AD. Soon after the sequencing of this peptide, the gene coding for it was discovered and located on chromosome 21 (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Robakis *et al.*, 1987). Aβ is a cleavage product of a type I transmembrane protein called amyloid precursor protein (APP) (Koo & Squazzo, 1994). APP is a glycosylated protein with a length of 695–770 amino acids that is expressed in several cell types with the highest expression rate in neurons (Beyreuther *et al.*, 1993; Dyrks *et al.*, 1988; Weidemann *et al.*, 1989). There predominantly the splicing form with 695 amino acids is found, and it is concentrated in synapses (Kang & Müller-Hill, 1990; Schubert *et al.*, 1991). Although the exact function of APP is still not clear, the different cleavage products of it have both trophic and toxic effects and are essential to the development of an organism (reviewed in Nhan *et al.*, 2015).

APP can be cleaved by different secretases (Fig. 1.2). α-Secretase has its cleavage site within the Aβ peptide between Lys-16 and Leu-17 (Esch *et al.*, 1990). Different zinc binding metalloproteinases from a disintegrin and metalloproteinase (ADAM) family have α-secretase activity, especially ADAM10 (reviewed in Lichtenthaler, 2011). This cleavage results in a soluble APP ectodomain (sAPPα) and a C-terminal membrane bound peptide of 83 amino acids (C83). The latter can be further cleaved to p3 peptide and APP intracellular C-terminal domain (AICD) by γ-secretase, a complex of presenilin (PSEN), nicastrin, anterior pharynx defective 1 (APH1), and presenilin enhancer (PEN2) (Edbauer *et al.*, 2003; Kim-

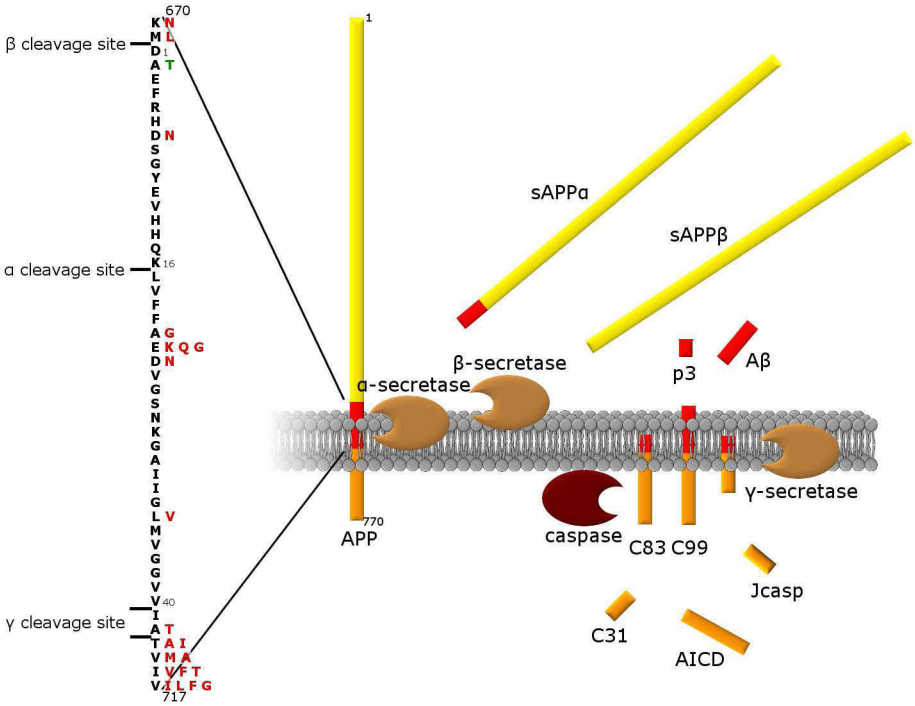

Figure 1.2: APP processing and mutations. The Aβ fragment is marked in red and its sequence is shown on the right together with possible mutations in or adjacent to this peptide (red: pathogenic; green: protective).

berly *et al.*, 2003). Alternatively the full length APP can be cleaved by β-site APP-cleaving enzyme 1 (BACE1), which leads to the production of sAPPβ and C99 (Hussain *et al.*, 1999; Lin *et al.*, 2000; Sinha *et al.*, 1999; Vassar *et al.*, 1999; Yan *et al.*, 1999). Subsequent cleavage of C99 by γ-secretase leads to the release of Aβ peptide and AICD. The C83 or C99 fragments can also be cleaved by caspases, resulting in C31 (the last 31 amino acids of the C-terminus of APP) and by subsequent γ-secretase cleavage in Jcasp (Gervais *et al.*, 1999). For a more detailed review on APP processing see Zhang *et al.* (2011).

The secretases and APP are transmembrane proteins. They are produced in endoplasmic reticulum, transported to Golgi for maturation, and from there sent to plasma membrane. Regulation of this trafficking has an impact on Aβ generation, depending on the colocalisation of APP with the different secretases. The α-secretase is active at the plasma membrane, where mostly sAPPα is produced (Parvathy *et al.*, 1999). BACE1 can cleave APP in the endoplasmic reticulum, in trans-Golgi network, at the plasma membrane where it competes with α-secretase, and in endosomes (Chyung & Selkoe, 2003; Cook *et al.*, 1997; Kinoshita *et al.*, 2003; Vassar *et al.*, 1999). Since BACE1 has its pH optimum at 4–5.5, the intracellular acidic compartments account for most of its activity (Knops *et al.*, 1995; Lin *et al.*, 2000; Sinha *et al.*, 1999; Vassar *et al.*, 1999). γ-Secretase complex needs complete assembly and maturation, it is first active at the plasma membrane and also has an acidic pH optimum (Kaether *et al.*, 2006; Pasternak *et al.*, 2003). Therefore, Aβ peptides are mostly generated in the endosome, where APP and its C-terminal fragments are sorted after internalisation to be recycled to the plasma membrane or degraded, and in lysosomes (Haass *et al.*, 1992; Koo & Squazzo, 1994; Pasternak *et al.*, 2003; Yamazaki *et al.*, 1996). In damaged neurons also the autophagic vacuoles have been shown to be a site of Aβ generation (Yu *et al.*, 2005). APP, BACE1, and γ-secretase localize to lipid rafts—membrane microdomains that are rich in cholesterol and sphingolipids (Riddell *et al.*, 2001; Vetrivel *et al.*, 2004). The interactions of secretases and APP with membrane lipids and cholesterol can modulate secretion of Aβ,

but also its aggregation and clearance as reviewed by Walter & van Echten-Deckert (2013).

The Aβ peptide has a length of 37–43 amino acids due to the multiple cleavage sites of γ-secretase (Qi-Takahara *et al.*, 2005). The most abundant form under normal conditions is $A\beta_{1–40}$, followed by a smaller amount of $A\beta_{1–42}$. Also N-terminal truncated forms ($A\beta_{x–40/42}$) and other C-termini can be found in small amounts (Miller *et al.*, 1993; Vigo-Pelfrey *et al.*, 1993). These peptides can aggregate into oligomers, protofibrils, and finally fibrils that deposit into plaques. Diffuse and cored plaques contain mostly $A\beta_{1/x–42}$, plaques in the walls of leptomeningeal and intracortical blood vessels (a sign of cerebral amyloid angiopathy often found in AD brains) contain both $A\beta_{1/x–40}$ and $A\beta_{1/x–42}$ (Gowing *et al.*, 1994; Gravina *et al.*, 1995; Iwatsubo *et al.*, 1994; Miller *et al.*, 1993; Roher *et al.*, 1993a,b). Monomeric Aβ is naturally unfolded in aqueous solutions. In solid plaque cores, fibrils of Aβ with a cross-β structure are found. The formation of fibrils is dependent on pH, solvent, and concentration of the peptide. $A\beta_{1–42}$ has a higher tendency to aggregate into fibrils because of a more stable β-sheet formation, and it is the first aggregate forming species in the course of AD (Barrow *et al.*, 1992; Burdick *et al.*, 1992; Iwatsubo *et al.*, 1994). This process probably starts with an intraneuronal aggregation (Capetillo-Zarate *et al.*, 2012; Gouras *et al.*, 2000; Ling *et al.*, 2014). The N-truncated forms and the p3 peptide ($A\beta_{17–x}$) are found in diffuse plaques and tend to aggregate more rapidly than the full length Aβ (Gowing *et al.*, 1994; Kumar-Singh *et al.*, 2000; Pike *et al.*, 1995). These species, Aβ with pyroglutamate at positions 3 or 11, and phosphorylated peptides are found in later stages of AD (Rijal Upadhaya *et al.*, 2014).

Several missense mutations have been found in the APP gene in families with heritable AD or cerebral haemorrhage. Most of the pathogenic mutations are located near the secretase cleavage sites (Fig. 1.2). The Swedish mutation (Mullan *et al.*, 1992) is a double mutation affecting two amino acid residues near the β-cleavage site (K670N/M671L, positions given for APP_{770} transcript) that increases the production of both $A\beta_{1–40}$ and $A\beta_{1–42}$ (Citron *et al.*, 1992; Scheuner *et al.*, 1996). Recently a mutation at codon 673 has been shown to be protective against AD by inhibiting the β-cleavage (Jonsson *et al.*, 2012). Many of the other mutations are located near the γ-secretase cleavage sites (Ancolio *et al.*, 1999; Chartier-Harlin *et al.*, 1991; De Jonghe *et al.*, 2001; Eckman *et al.*, 1997; Goate *et al.*, 1991; Guardia-Laguarta *et al.*, 2010; Kumar-Singh *et al.*, 2000; Murrell *et al.*, 1991, 2000; Pasalar *et al.*, 2002; Terreni *et al.*, 2002) and increase the $A\beta_{1–42}/A\beta_{1–40}$ ratio (De Jonghe *et al.*, 2001; Eckman *et al.*, 1997; Guardia-Laguarta *et al.*, 2010; Scheuner *et al.*, 1996). Two mutations lying at the cytoplasmic transmembrane junction at the residues 723 and 724 of APP_{770} have a similar effect (Kwok *et al.*, 2000; Theuns *et al.*, 2006). Consistent with the notion that γ-secretase cleavage plays an important role in familial AD, there are 207 pathogenic mutations in *PSEN1* gene and 13 in *PSEN2* (AD & FTD Mutation Database (http://www.molgen.vib-ua.be/ADMutations, accessed on 20/11/2015), Cruts *et al.*, 2012). PSENs also play a role in cellular processes independent of Aβ such as calcium homoeostasis, endocytosis, and autophagy that are found to be impaired in both familial and sporadic AD (reviewed in Smolarkiewicz *et al.*, 2013). Mutations lying inside the Aβ sequence are associated with cerebral amyloid angiopathy and cerebral haemorrhage, but in some cases also AD symptoms were accompanied with the vascular ones (Giaccone *et al.*, 2002; Grabowski *et al.*, 2001; Hendriks *et al.*, 1992; Kamino *et al.*, 1992; Levy *et al.*, 1990; Nilsberth *et al.*, 2001; Obici *et al.*, 2005; Rossi *et al.*, 2004). Some of these mutations lead to differences in APP processing, others affect the aggregation kinetics of the Aβ peptide (Haass *et al.*, 1994; Nilsberth *et al.*, 2001; Watson *et al.*, 1999). Additionally to these mutations, duplication of the APP gene can lead to AD symptoms as seen in people with Down's syndrome, a condition arising from trisomy of chromosome 21 (Glenner & Wong, 1984a).

Aβ-fibrils were first thought to be the toxic species, but due to the lack of correlation between plaques and cognitive decline (Gómez-Isla *et al.*, 1997; Lue *et al.*, 1999; Snowdon, 2003) and evidence that oligomers of Aβ are far more toxic than the fibrils (Lambert *et al.*, 1998; Walsh *et al.*, 2002), most of the recent research has concentrated on low molecular weight aggregates. The oligomers are heterogeneous and sensitive to solution conditions, which complicates the study of these aggregates (Benilova *et al.*, 2012). The exact size or structure of the toxic Aβ species is not yet known, as an example globular or annular oligomers that interact with membranes are shown to be toxic (Sebollela *et al.*, 2014; Tsigelny *et al.*, 2014). It is also not clear how these oligomers exert their toxicity. Since Ca^{2+} levels are raised in affected neurons and Aβ is known to interact with membranes, it has been suggested that the oligomers can increase membrane permeability unspecifically (Kayed *et al.*, 2004) or by forming pores that are selective for Ca^{2+} (Arispe, 2004; Durell *et al.*, 1994; Lin *et al.*, 2001a). This mechanism would lead to unspecific toxicity in all cells. Another hypothesis states that there is a 'toxin receptor' that allows the oligomers to target a specific population of cells at the presynaptic sites. Many such receptors for Aβ oligomers have been proposed (reviewed in Dinamarca *et al.*, 2012). One of them is the prion protein (PrP) that can activate FYN proto-oncogene (FYN), a cytoplasmic tyrosin kinase (Chin *et al.*, 2004; Lacor *et al.*, 2004; Laurén *et al.*, 2009; Um *et al.*, 2012). Binding to PrP or other possible receptors leads to exitotoxicity, aberrant morphology with loss of dendritic spines, and decreases in long term potentiation (Lacor *et al.*, 2007; Laurén *et al.*, 2009; Um *et al.*, 2012). Some of the possible mechanisms are discussed in a recent review by Viola & Klein (2015). For effects of Aβ on oxidative stress, mitochondrial dysfunction, and inflammation in AD see section 1.1.3.

The Aβ peptides can be cleared from brain by several routes. The observation of degreased Aβ clearance in patients with late onset AD (Mawuenyega *et al.*, 2010) has pointed out a possible defect in one of these pathways as a cause for this most common form of AD. One important way of clearance is the active transport of Aβ over the blood brain barrier. Transport from the brain into blood is mediated by P-glycoprotein (Cirrito *et al.*, 2005), low density lipoprotein receptor-related protein 1 (LRP1) (Shibata *et al.*, 2000), and low density lipoprotein receptor (Castellano *et al.*, 2012). The latter two are dependent on apolipoprotein E (APOE) (Castellano *et al.*, 2011), a major risk factor for late onset AD (see section 1.1.4). Aβ can also be transported in the other direction, from blood to brain, dependent on advanced glycosylation end product-specific receptor (RAGE) (Deane *et al.*, 2003). This raises the possibility that part of the Aβ burden in brain actually originates in systemic circulation. Changed levels and localization of LRP1 and RAGE in AD brains probably contribute to the pathogenesis (Donahue *et al.*, 2006). Another minor pathway for Aβ clearance is the perivascular drainage of interstitial fluid. This can also explain the arterial deposition of Aβ in cerebral amyloid angiopathy (Weller *et al.*, 1998). Along this pathway Aβ is removed by perivascular macrophages, whereas microglia and peripheral macrophages have only a minor role in phagocytotic Aβ clearance (Hawkes & McLaurin, 2009; Mildner *et al.*, 2011). Aβ can be taken up by cells and transported to lysosomes, where $Aβ_{1–40}$ is rapidly degraded. $Aβ_{1–42}$ is more resistant to degradation and tends to accumulate (Burdick *et al.*, 1997). This can lead to lysosomal dysfunction and release of the contents of lysosomes into cytosol (Yang *et al.*, 1998). Microglia, neurons, astrocytes, and cells of the blood vessels synthesise a variety of enzymes that are able to degrade Aβ (reviewed in Miners *et al.*, 2011). The most important ones among them are insulin degrading enzyme (Qiu *et al.*, 1998) and neprilysin, a membrane bound protein that can be released into extracellular space and accounts for the most Aβ degrading activity *in vivo* (Iwata *et al.*, 2000). The activity of these enzymes is up-regulated in ageing brain and in AD, probably as a physiological response to

increased Aβ level. However, since these enzymes also have a variety of other substrates, the up-regulation can lead to detrimental effects like vasoconstriction and reduced cerebral blood flow (Miners *et al.*, 2014). Others have also reported decreased levels of neprilysin (reviewed in Grimm *et al.*, 2013) and the ability of insulin degrading enzyme to process Aβ may be compromised due to brain insulin resistance (see section 1.1.4).

The mutations found in *APP*, causing autosomal dominant AD, and the observation of diffuse plaques before any other abnormal lesions or neuronal damage led to the formulation of amyloid cascade hypothesis (Hardy & Allsop, 1991; Hardy & Higgins, 1992). According to its original version the aggregation of Aβ is the first event in the pathogenesis of AD. The plaques then exert their toxic effects on neurons and lead to tau aggregation and neuronal death, eventually causing dementia. During the next decades new findings essentially supported this hypothesis with the replacement of Aβ fibrils by oligomers as the toxic species and addition of synaptic dysfunction as an early event in the pathology (Ferreira & Klein, 2011; Hardy & Selkoe, 2002; Selkoe, 2002). Opponents of this hypothesis have argued that there is no correlation between plaque load and cognitive decline; the cell culture and mouse models, used to produce most of the evidence supporting Aβ as the cause for AD, have little resemblance with *in vivo* disease conditions; and there are evidence that Aβ is in fact protective, produced as a response to cellular stress (Herrup, 2015; Lee *et al.*, 2004; Perry *et al.*, 2000). More controversy arose when the first drugs based on lowering the amount of Aβ failed to improve the cognitive function of patients in clinical trials. Alternatives and modifications to the amyloid cascade hypothesis have been proposed (Armstrong, 2014; Ethell, 2010; Pimplikar, 2009; Small & Duff, 2008). Others argue that the damage made by Aβ may be irreversible. Therefore, possible treatments should be tested earlier in course of the disease, before the patients develop severe pathology and overt symptoms (Karran *et al.*, 2011; Musiek & Holtzman, 2015; Tam & Pasternak, 2012). To test this theory, biomarkers to detect early stages of the disease are needed (see section 1.1.4). Nevertheless, the amyloid cascade hypothesis is still the predominant explanation for the aetiology of AD.

1.1.2 Tau

Tau tangles are the other prominent aggregates in AD. Tau is a microtubule-associated protein (MAP) that is necessary for tubulin assembly and stabilisation of microtubules (Bré & Karsenti, 1990; Fellous *et al.*, 1977; Panda *et al.*, 1995; Weingarten *et al.*, 1975). Microtubules are part of the cytoskeleton that give differentiated cells their typical shape. They are also involved in movement, outgrowth of axons in developing neurons, and polarized transport of vesicles. There are several MAPs that regulate the dynamic equilibrium of microtubules with monomeric tubulin and they are specific for different cells and compartments. Tau is found specifically in axons of neurons where microtubules are important for both stability and axonal transport (Binder *et al.*, 1985; Dotti *et al.*, 1987).

The gene encoding tau is located on chromosome 17 (Neve *et al.*, 1986) and gives rise to 7 isoforms through alternative splicing. In peripheral nervous system a high molecular weight isoform is found that contains an additional exon 4A (Couchie *et al.*, 1992). The other 6 isoforms are expressed in central nervous system and differ in splicing of exons 2, 3, and 10 (Goedert *et al.*, 1989a,b). Exon 10 encodes one of the four possible tandem repeats at the C-terminal portion of tau that, together with their flanking regions, act as tubulin binding sites (Gustke *et al.*, 1994; Himmler *et al.*, 1989; Maccioni *et al.*, 1989). Therefore, isoforms lacking this exon have only three binding repeats, and their binding to microtubuli is weaker. In early developmental stages only the shortest isoform lacking exons 2, 3, and 10 is expressed (Goedert *et al.*, 1989a,b). Mutations in tau gene are not associated with AD,

but they can lead to frontotemporal dementia (reviewed in Ghetti *et al.*, 2015).

Tau protein can be posttranscriptionally modified. A number of these modifications affect its interaction with microtubules and aggregation to neurofibrillary tangels (reviewed in Fontaine *et al.*, 2015). The best studied modification is phosphorylation at the tandem repeats or residues flanking this region by different kinases (Lovestone & Reynolds, 1997; Wang *et al.*, 2007). This occurs in a healthy brain, varying at different ages, to regulate the binding affinity of tau to microtubules (Lindwall & Cole, 1984a,b; Trinczek *et al.*, 1995). In AD brain tau is hyperphosphorylated and loses its ability to bind to microtubules (Grundke-Iqbal *et al.*, 1986b). In this form tau can aggregate into paired helical filaments and further into the tangles found in AD brains (Bancher *et al.*, 1989; Ihara *et al.*, 1986; Kidd, 1963; Luna-Muñoz *et al.*, 2007). All tau isoforms found in adult brain are involved in formation of paired helical filaments (Greenberg *et al.*, 1992). The aggregation of tau can be prevented by molecular chaperones that alter its binding to microtubules and prevent toxicity (Abisambra *et al.*, 2010; Dou *et al.*, 2003; Patterson *et al.*, 2011b; Voss *et al.*, 2012).

By aggregating tau loses its function as stabilizer of microtubules, leading to dysfunctions in cytoskeleton and axonal transport. Additionally to this loss of function effect, the soluble tau oligomers or hyperphosphorylated monomers might gain a toxic function, similar to the Aβ oligomers (Ding & Johnson, 2008). As for Aβ, it is also not clear for tau what kind of oligomers mediate the toxicity (Cowan *et al.*, 2012). However, they have been shown to accumulate in AD brain early in the disease progression and correlate with memory loss and synapse dysfunction in tauopathy models (Berger *et al.*, 2007; Lasagna-Reeves *et al.*, 2011; Patterson *et al.*, 2011a; Sydow *et al.*, 2011). Several mechanisms have been proposed to explain the toxicity of tau. A phosphatase-activating domain at the N-terminus, that becomes accessible through conformation change in pathogenic tau, can inhibit kinesin dependent axonal transport (Kanaan *et al.*, 2011). Defects in mitochondrial distribution have been observed probably as a consequence of dysfunctional axonal transport (Kopeikina *et al.*, 2011), and tau fragments contribute to mitochondrial dysfunction (see section 1.1.3). Tau can also mediate some aspects of Aβ toxicity (Roberson *et al.*, 2007). Aβ oligomers lead to activation of kinases that phosphorylate tau, missorting of tau and other axonal proteins into dendrites, elevated Ca_2^+ levels, and destabilization of microtubules (Yu *et al.*, 2012; Zempel & Mandelkow, 2012; Zempel *et al.*, 2010). Tau is necessary for postsynaptic targeting of FYN that can phosphorylate a subunit of N-methyl-D-aspartate receptor (NMDAR) leading to its anchoring at the postsynaptic sites. This causes exitotoxicity and seizures in AD models (Ittner *et al.*, 2010). Tau itself can also be phosphorylated by FYN that is activated by Aβ via PrP (Larson *et al.*, 2012). Phosphorylation by this and other kinases leads to aberrant cell cycle re-entry that leads to cell death (Seward *et al.*, 2013). It is not clear which of these mechanisms is most relevant for AD.

It is widely accepted that tau pathology occurs downstream of Aβ in accordance with the amyloid cascade hypothesis. What remains unclear are the mechanisms by which Aβ leads to changes in tau. Aβ seems to activate kinases that lead to tau hyperphosphorylation like discussed above and reviewed by Lloret *et al.* (2015). On the other hand, there are reports that human tau can increase Aβ levels (Bright *et al.*, 2015), suggesting a more complex interaction between these proteins. While most of the treatment strategies today aim at Aβ as the main culprit of the disease, some researchers argue that tau should be the target (Crespo-Biel *et al.*, 2012). Indeed, tau pathology correlates better with cognitive decline than Aβ (Arriagada *et al.*, 1992; Gómez-Isla *et al.*, 1997), and tau knockout in APP expressing mouse models rescues the memory deficits (Ittner *et al.*, 2010; Roberson *et al.*, 2007). Still, neuronal and synapse loss are better correlates for cognitive decline than either Aβ or tau tangles, suggesting the possibility of other pathogenic mechanisms. Oxidative stress and

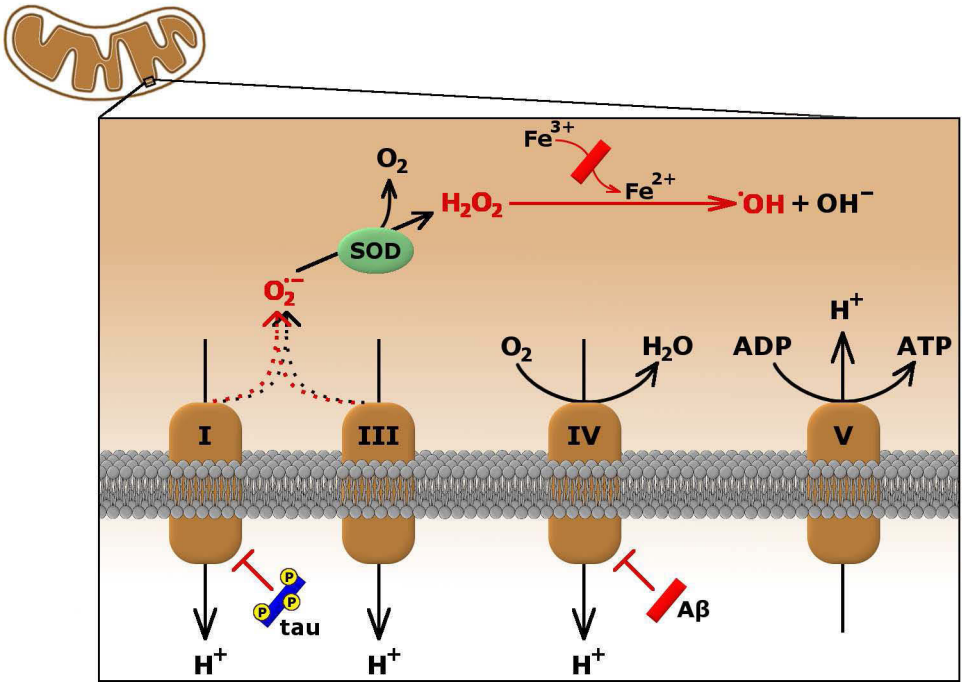

Figure 1.3: ROS production in mitochondria. The respiratory chain (complexes I–V; complex II not shown) establish a pH gradient and use it to produce ATP. Aβ and tau can inhibit complex IV and I, respectively, leading to excess superoxide production. Superoxide can react further to form other ROS. Here shown the Fenton reaction with Fe^{2+} as catalyst. Aβ can facilitate the reduction of Fe^{3+} to Fe^{2+}, thereby indirectly affecting the formation of $OH^{\bullet}$ (Smith *et al.*, 2007).

mitochondrial dysfunction play an important role at the junction between Aβ and tau and have an important role in disease progression as described in the next section.

1.1.3 Oxidative stress, mitochondrial dysfunction, and inflammation

Aerobic cells are constantly exposed to reactive oxygen species (ROS) generated by metabolic processes. Low levels of ROS have a physiological signalling function and can protect cells from serious damage (Ristow & Schmeisser, 2011; Yan, 2014). But when the level of oxidative stress exceeds the antioxidant capacity of the cells, ROS become toxic. They can react with different biomolecules like phospholipids, proteins, or nucleic acids, leading to ageing and various diseases including AD (reviewed in Phaniendra *et al.*, 2015). An excess of oxidised proteins, lipids, and ribonucleic acid (RNA) is seen at early stages in AD brains (Abe *et al.*, 2002; Lovell *et al.*, 1995; Markesbery & Lovell, 1998; Nunomura *et al.*, 1999; Smith *et al.*, 1991; Williams *et al.*, 2006). Deoxyribonucleic acid (DNA) oxidation, especially in mitochondrial DNA, has also been found (Mecocci *et al.*, 1994; Prasad Gabbita *et al.*, 1998). Taken together, this indicates prominent oxidative damage in AD brain that may have an important role in the pathogenesis.

The major source of ROS in an eukaryotic organism are mitochondria. Mitochondria produce adenosine triphosphate (ATP)—the main energy carrier in the body—by oxidative phosphorylation. Thereby electrons are carried through four protein complexes of the electron transport chain that use the energy to establish a proton gradient (Fig. 1.3). At complex IV molecular oxygen (O_2) is reduced to water (H_2O). Subsequently complex V uses the proton gradient to produce ATP. If the electron transfer between complexes I–IV is inhibited, O_2 can serve as an alternative electron receptor and a superoxide radical ($O_2^{\bullet -}$) is formed. $O_2^{\bullet -}$ is detoxified by superoxide dismutase (SOD) which produces O_2 and hydrogen

peroxide (H_2O_2), another ROS that is further reduced to H_2O by catalase or glutathione peroxidase. If these ROS escape the cells defensive mechanism, they can either oxidise biomolecules themselves or form the more reactive hydroxyl radical ($OH^\bullet$). This can happen via two reaction mechanisms, both of which depend on metal ions (Me^{n+}) like copper or iron (reviewed in Halliwell & Gutteridge, 1984):

$$\text{Fenton reaction: } Me^{n+} + H_2O_2 \longrightarrow Me^{(n+1)+} + OH^\bullet + OH^-$$

$$\text{Haber-Weiss reaction: } O_2^{\bullet -} + H_2O_2 \xrightarrow{Me^{n+}} OH^\bullet + OH^- + O_2$$

Imbalances in copper, iron, and zinc ions have been reported in AD brains, and these metals are found in senile plaques (Deibel *et al.*, 1996; Lovell *et al.*, 1998). Copper can bind to Aβ, especially under acidic conditions, and facilitate the formation of H_2O_2, possibly followed by Fenton reaction (Atwood *et al.*, 2000; Huang *et al.*, 1999; Opazo *et al.*, 2002). The three histidine residues of Aβ are thereby involved in complexing the metal ion, whereas Met_{35} and Tyr_{10} are important for the redox chemistry (Smith *et al.*, 2007; Varadarajan *et al.*, 1999; Yatin *et al.*, 1999). In nanomolar concentrations Aβ binding to copper exhibits neurotrophic and antioxidant effects, although higher concentrations lead to pro-oxidant activity (reviewed in Atwood *et al.*, 2003). Binding of zinc to Aβ leads to aggregation of the peptide and reduces its toxicity (Bush *et al.*, 1994; Cuajungco *et al.*, 2000). Zink can also bind to tubulin, and dyshomeostasis of this metal can lead to depolymerisation of microtubules (Craddock *et al.*, 2012). The expression of APP is regulated by iron (Rogers *et al.*, 2002), and copper binding to APP increases Aβ production (Noda *et al.*, 2013), further emphasising the important role of metal ions in AD pathogenesis.

Mitochondrial dysfunction is seen in different neurodegenerative disorders, such as AD, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease (reviewed in Lezi & Swerdlow, 2012). In AD interactions of Aβ with mitochondrial proteins can lead to inhibition of the electron transport chain (Fig. 1.3), increased ROS levels, and apoptosis (Cha *et al.*, 2012). The activity of complex IV is decreased in AD (Maurer *et al.*, 2000; Mutisya *et al.*, 1994), possibly because of decreased expression and deletions in mitochondrial DNA (Krishnan *et al.*, 2011; Rhein *et al.*, 2009) or blockage of the translocases—proteins needed for proper import of mitochondrial proteins encoded by nuclear DNA—by APP (Devi *et al.*, 2006). Furthermore, Aβ is taken up into mitochondria and is found in the inner membrane, making a direct interaction with the complexes of the respiratory chain or other mitochondrial proteins like Aβ-binding alcohol dehydrogenase (ABAD) possible (Hansson Petersen *et al.*, 2008; Lustbader *et al.*, 2004). Deficiency of complex IV is dependent on Aβ, whereas tau oligomers decrease complex I levels. Both these effects are synergistic in impairing mitochondrial membrane potential and ATP production (Amadoro *et al.*, 2012; Lasagna-Reeves *et al.*, 2011; Quintanilla *et al.*, 2014; Rhein *et al.*, 2009). Exposure to Aβ leads to higher ROS levels due to its interaction with mitochondrial proteins (Hernandez-Zimbron *et al.*, 2012; Lustbader *et al.*, 2004). Additionally, increased fission and decreased fusion of mitochondria have been observed in AD, resulting in changed morphology (Baloyannis, 2011). Aβ can influence the expression of proteins involved in mitochondrial dynamics and this leads to altered transport of mitochondria in neurons (see also section 1.1.2) that correlates with loss of dendritic spines (Calkins & Reddy, 2011; Wang *et al.*, 2009). Aβ can also support the expression of permeability transition pore, whereas tau oligomers activate caspase 9, implicating both peptides in induction of apoptosis (Lasagna-Reeves *et al.*, 2011; Moreira *et al.*, 2002). In normal cells dysfunctional mitochondria are degraded via autophagy, but under pathological conditions this process is impaired and leads to further damage of the cells (reviewed in Nixon & Yang, 2011; Schiavi & Ventura, 2014).

Decreased ATP production due to mitochondrial dysfunction can lead to higher Aβ levels (Scheffler *et al.*, 2012; Velliquette *et al.*, 2005), and phosphorylated tau has been detected after inhibition of complex IV (Szabados *et al.*, 2004). The evidence of defect mitochondria in AD have led to the formulation of a 'mitochondrial cascade hypothesis' (Swerdlow *et al.*, 2014; Swerdlow & Khan, 2004). It states that mitochondrial dysfunction is the driving force of sporadic late onset AD, leading to Aβ aggregation due to changes in electron transport chain as well as tau phosphorylation and aggregation due to cell cycle re-entry. This hypothesis includes the free radical theory of ageing (Harman, 1956), using it to explain the late onset of sporadic AD. The 'two hit' hypothesis also states that oxidative stress has an important role in AD pathogenesis, but according to this theory a second factor like mitotic stress is needed to initiate the disease progression (Zhu *et al.*, 2001, 2007, 2004).

Another possible source of ROS are activated immune cells, that use these reactive molecules for fighting intruders like bacteria. Indeed, fibrillar Aβ can induce $O_2^{\bullet -}$ production in macrophages (Colton *et al.*, 2000). Already Alzheimer observed microglia, the brain resident immune cells, surrounding the senile plaques (Alzheimer, 1911). Aβ aggregates activate microglia that can bind and phagocytose Aβ via different membrane receptors (reviewed in Yu & Ye, 2015) and recruit peripheral monocytes that also contribute to Aβ clearance in brain parenchyma (Krabbe *et al.*, 2013; Simard *et al.*, 2006). In the course of the disease, however, microglia exposed to Aβ lose this protective function and change to a more pro-inflammatory form, releasing cytokines that further inhibit clearance of Aβ (Hickman *et al.*, 2008; Krabbe *et al.*, 2013). Additionally, uptake of Aβ from the periphery by RAGE causes neurovascular stress and leads to further expression of cytokines (Deane *et al.*, 2003). On the other hand, chronic systemic inflammation has been shown to induce AD-like pathology in mice, suggesting that inflammatory conditions can trigger excess Aβ production and initiate AD (Krstic *et al.*, 2012). The ROS and pro-inflammatory cytokines produced by microglia can activate different signalling cascades in neurons and lead to damage of the cells. For example the activation of kinases can lead to tau hyperphosphorylation (Kitazawa *et al.*, 2011). The role of neuroinflammation in the aetiology of AD is still under debate, but its association with the disease is supported also by genetic studies discussed in the next section.

1.1.4 Risk factors and biomarkers

Since curing AD has proven to be a difficult task, more and more attention is paid to prevention and early detection of the people at risk. The most important risk factor is high age, followed by cerebrovascular disease, traumatic brain injury, cardiovascular disease, hypertension, and metabolic disorders like type II diabetes or obesity. Mediterranean diet and physical and mental activity, on the other hand, decrease the risk (reviewed in Mayeux & Stern, 2012; Reitz & Mayeux, 2014). Metabolic disorders and AD could be linked together via insulin resistance as proposed already decades ago (Hoyer, 1988; Hoyer *et al.*, 1991). Insulin has an important role in energy control and also in synaptic plasticity in central nervous system (reviewed in Ma *et al.*, 2015a). Signalling trough insulin and insulin-like growth factor 1 (IGF-1) receptors promotes clearance of Aβ oligomers (Zhao *et al.*, 2009) and is linked to other stress signals involved in AD by different pathways (reviewed in Lourenco *et al.*, 2015). One of the hubs where several of these pathways converge is the transcription factor forkhead box O (FOXO), that regulates the response of a cell to both oxidative stress and glucose deprivation, but can lead to cell death and several processes implicated in AD by prolonged activity (Manolopoulos *et al.*, 2010). The evidence for the involvement of insulin signalling in AD pathology has led to the notion that this disease can be seen as a form of brain diabetes (Lourenco *et al.*, 2015; Morgen & Frölich, 2015). According to a

recent study, however, Aβ oligomers can induce peripheral glucose intolerance by a mechanism involving inflammation and endoplasmic reticulum stress in hypothalamus (Clarke *et al.*, 2015). Therefore, the role of insulin resistance and diabetes in AD pathology remains a subject of further study.

The genetic background of familial AD is well known (see section 1.1.1), but the genetic component of the more prevalent sporadic AD seems to be more complex. Several genome wide association studies have been conducted to find risk genes, but most of the genes found have only a weak association with the disease. Among those are genes coding for proteins involved in lipid transport (APOE; clusterin), immune system (ATP-binding cassette sub-family A member 7; complement component receptor 1; CD33; membrane-spanning 4-domains, subfamily A, members 6A and 4E; CD-2 associated protein), and endocytosis (bridging integrator 1; phosphatidylinositol binding clathrin assembly protein; sortilin-related receptor 1) (AlzGene database (http://www.alzgene.org/, accessed on 20/11/2015), Bertram *et al.*, 2007; Reitz & Mayeux, 2014). The strongest association, found in almost all of these studies, is for *APOE*. There are three common alleles of the *APOE* gene: ε2, ε3, and ε4. The ε4 allele is associated with higher risk for late onset AD (Corder *et al.*, 1993; Strittmatter *et al.*, 1993), whereas ε2 is protective (Corder *et al.*, 1994; Hardy *et al.*, 1993). APOE is responsible for cholesterol transport and homoeostasis in brain. Lower cholesterol levels in microglia have been shown to enhance intracellular degradation of Aβ by facilitating endocytic trafficking (Lee *et al.*, 2012a). Also the degradation by insulin degrading enzyme is enhanced by lipidated APOE (Jiang *et al.*, 2008). Additionally, APOE can directly interact with Aβ and alter its aggregation properties (Arold *et al.*, 2012; Garai *et al.*, 2014; LaDu *et al.*, 2011).

The pathological diagnosis of AD relies on the extent of Aβ and tau pathology in the brain of the patient (reviewed in Serrano-Pozo *et al.*, 2011). In order to diagnose AD in living patients, biomarkers and imaging techniques are needed together with cognitive tests to separate AD from other dementias. Available biomarkers can be divided into two groups: biochemical markers found in cerebrospinal fluid and imaging biomarkers. In cerebrospinal fluid concentrations of Aβ and tau can be measured. $A\beta_{1–42}$ is lower in patients with AD whereas total and phosphorylated tau levels are higher than in controls. Especially the ratios $A\beta_{1–42}/A\beta_{1–40}$ and $A\beta_{1–42}$/phospho-tau have shown high predictive value for early AD (reviewed in Lewczuk *et al.*, 2014). Different imaging techniques can be applied for the diagnosis (reviewed in Ahmed *et al.*, 2014). Positron emission tomography using 18-F-flourodeoxyglucose typically shows bilateral hypometabolism and hypoperfusion in the AD brains. Specific tracers like Pittsburgh Compound B used with positron emission tomography can visualize amyloid plaques in living patients. Additionally, structural magnetic resonance imaging can detect brain atrophy. Using combinations of these biomarkers, it is possible to strengthen the diagnosis made by cognitive testing and differentiate between different types of dementias. Still, there are many problems including availability and cost of suitable instrumentation and insufficient standardisation of the acquisition methods, therefore these techniques are not everywhere used as a standard procedure for diagnosis. New methods and markers, that would be easier to handle and have better sensitivity and selectivity, are in development (Ahmed *et al.*, 2014).

1.1.5 Treatment

The first prescription drug for AD was tacrine (Cognex®), an acetylcholine esterase inhibitor (AChEI) (Summers, 2006). The neurotransmitter acetylcholine (ACh) has an important role in forming the concious awareness, and impairment of the cholinergic system

underlies symptoms of different dementias (Perry *et al.*, 1999). In AD there is a lack of ACh due to selective loss of cholinergic neurons (Davies & Maloney, 1976; Francis *et al.*, 1999). By inhibiting the enzyme that degrades ACh—acetylcholine esterase (AChE)—the amount of this neurotransmitter in the synaptic cleft can be increased. Hence, the treatment slows down the cognitive decline and helps to maintain activities of daily living. The usage of tacrine was discontinued due to hepatotoxicity (e.g. Blackard *et al.*, 1998), but newer AChEI donepezil (Aricept®), rivastigmine (Exelon®), and galantamine (Reminyl®) are still used in mild to moderate AD (Birks, 2006). For moderate to severe disease stages another drug called memantine (Namenda®) is approved (Matsunaga *et al.*, 2015). Memantine is a non-competitive NMDAR antagonist, that counteracts the impaired glutamate signalling and can protect neurons from excitotoxicity. Combinations of both drug types have shown greater benefits in mild to moderate AD than one drug alone (Parsons *et al.*, 2013). Still, these treatments only counteract the symptoms and cannot halt the course of the disease. Therefore, a lot of effort has been made to find new, more efficient drugs.

Most of the research in this area has concentrated on Aβ: substances that can inhibit Aβ oligomerization (reviewed in Doig & Derreumaux, 2015), reduce its production, or facilitate its clearance. An example of a small molecule that can reduce brain amyloid load, probably due to modulation of BACE1, and is currently tested in clinical trials is methylene blue (Mori *et al.*, 2014). BACE1 is the rate limiting enzyme for Aβ production and therefore a logical drug target. Several small molecules that can inhibit BACE1 or modulate its activity have been found and some of them have reached phase III clinical trials (reviewed in Evin & Hince, 2013; Vassar *et al.*, 2014). However some adverse effects have been noticed due to other targets of this enzyme and cross reactivity with other aspartyl proteases of the same family like BACE2 or cathepsin D. Similar problems occurred with γ-secretase inhibitors, where an increased risk of skin cancer and worsening of cognitive abilities have been observed (reviewed in Mikulca *et al.*, 2014). There the focus has shifted from inhibiting the enzyme to modulating its activity in favour of the shorter Aβ species. These modulators do not inhibit other functions of γ-secretase and therefore should have less side-effects (D'Avanzo *et al.*, 2015). For increasing the clearance of Aβ, immunotherapy is utilized (reviewed in Spencer & Masliah, 2014). Although the first clinical trials in this field failed due to severe side effects and no change in cognitive abilities of the patients was observed, new approaches for both active and passive immunization are hoped to lead to safer and more effective treatment.

Failure of the first anti-Aβ drugs has triggered a search for new targets and treatment possibilities. Immunotherapy against tau is considered along with antioxidant and anti-inflammatory approaches, and a lot of other targets are explored (Geldenhuys & Darvesh, 2014). Since AD is an multi-factorial disease with many possible drug targets, a trend of developing multi-target-directed-ligands has emerged. Also, prevention and early pharmacological intervention are gaining more and more attention (Sindi *et al.*, 2015), although there is still a lack of reliable biomarkers to identify those at risk in presymptomatic stages (see section 1.1.4). In the search for new multitarget treatments and lead substances for drugs traditional medicine, especially Traditional Chinese Medicine (TCM) (reviewed in Su *et al.*, 2014; Zeng *et al.*, 2015), and natural products have received considerable attention. Several natural products have been found to be effective: the AChEI galantamine was first isolated from snowdrop (*Galanthus* spp.) (Heinrich & Teoh, 2004); huperzin A, an AChEI from *Huperzia serrata*, is in phase II clinical trials (Yang *et al.*, 2013b); (–)-epigallocatechin gallate (EGCG) (*Camellia sinensis*), curcumin (*Curcuma longa*), resveratrol, scyllo-inositol (*Cocos nucifera*), and several flavonoids can modulate Aβ aggregation (reviewed in Bu *et al.*, 2015); the standardized *Ginkgo biloba* extract EGb761® has shown beneficial effects (Can-

evelli *et al.*, 2014; Gauthier & Schlaefke, 2014). Despite all these efforts, more research is still needed to find an effective treatment for AD.

1.2 Medicinal plants

Phytotherapy is one of the oldest forms of medicine, known to mankind already for thousands of years. The traditional practices differ by culture and region. Today we have access to a lot of ethnobotanical information from around the world. Some of the plants used traditionally are scientifically proved to be effective and are still used in modern phytotherapy, some others are used in alternative medicine or as food supplements. The basis of effectiveness of medicinal plants are almost exclusively secondary metabolites—small molecules produced by plants to protect themselves. These molecules can either directly interact with a pharmaceutical target in human body or they can be used as leads for synthesis of active compounds. Therefore, medicinal plants are interesting for both traditional medicine and modern pharmaceutical research.

Plants produce secondary metabolites as a means to interact with their environment. In order to reproduce, plants need the help of insects and animals for pollination and distribution of their seeds. Therefore, they have developed molecules that attract these helpers. They also have to protect their territory and resources from other plants, so they have developed phytotoxins. Plants do not have the ability to move away from their enemies. Hence, they depend on their chemical defence system to protect themselves from phytopathogens and herbivores. Upon an attack they produce phytoalexins—molecules that can ward off the danger and send a warning to systemic tissues. As a reaction to this signal protective secondary metabolites and proteins are produced for enhanced protection in whole plant, leading to a systemic acquired resistance (Ahuja *et al.*, 2012; Spoel & Dong, 2012). Next to this induced expression of phytoalexins, many protective secondary metabolites are produced constitutively, stored, and released upon an attack. These molecules, that have antimicrobial, antifungal, antioxidant, and several other properties, are most interesting for pharmaceutical research.

Secondary metabolites probably developed early in the evolution of plants and have been refined through natural selection (Wink, 2003), giving them an advantage over *de novo* synthesis of medical compounds. The chemical structures of these molecules are diverse, as are their mechanisms of action. Some mimic molecules in animals and can interact with specific receptors or enzymes (e.g. some alkaloids are similar to neurotransmitters and can activate or inhibit neuroreceptors or re-uptake transporters), but most (e.g. polyphenols, terpenes) have rather unspecific interactions with proteins, biomembranes, or nucleic acids (reviewed in Wink, 2008). One plant usually produces various compounds to have the best protection against as many different threats as possible. Hence, plant extracts are multitarget drugs that can modulate several cellular processes and possibly even act on targets that have not been identified yet.

Natural selection has not only helped the plants to perfect their toxins, the herbivores have gone through an evolutionary adaptation process as well. Thereby, mutations to the target proteins have led to resistance in some specialised herbivores. Other insects and animals have developed several mechanisms to either store the toxins and use them for their own protection or detoxify these phytochemicals. The latter option is mostly used by vertebrates. Proteins in these organisms can be protected by chaperones like heat shock proteins (HSPs); oxidative damage can be prevented by SOD, catalase, or other enzymes. Additionally, the toxic compounds are actively modified in liver and excreted, or their intake is inhibited by ATP-binding cassette transporters in epithelia (Murugaiyah & Mattson,

2015). Expression of protective proteins is controlled by transcription factors like nuclear factor erythroid 2-like 2 (NRF2), heat shock factor 1 (HSF-1), or FOXO (Murugaiyah & Mattson, 2015; Son *et al.*, 2008). Small amounts of toxic compounds, that manage to cross the epithelia, activate these transcription factors and trigger the transcription of the stress resistance proteins that can protect the organism from the toxin that was ingested, but also give it a wider resistance to many types of stress. This is the underlying mechanism of hormesis—the phenomenon that small amounts of a compound can be beneficial, even if a larger amount of the same substance is toxic (Calabrese, 2015; Mattson, 2008). Hormesis is proposed as the mechanism of action for several phytochemicals including polyphenols from green tea like EGCG (Murakami, 2014; Son *et al.*, 2008). Also other types of stress like exercise or caloric restriction lead to hormetic responses. Most interestingly, hormesis is essential for healthy ageing and the health of nervous system (Murugaiyah & Mattson, 2015).

1.2.1 Phytotherapy in Traditional Chinese Medicine

TCM is one of the oldest medicine systems dating back more than 5000 years. Since at that time there was no technology available to look inside a living body, TCM uses superficial observations—ranging from voice quality to pulse feeling—to diagnose a condition. A disease is seen as an imbalance in yin and yang, the two basic forces in Taoism. Without the modern knowledge about anatomy and physiology, the ancient healers used this philosophy to describe the function of human body. In TCM the body is divided into Organs, whereby 'Organ' does not refer to an anatomical entity but is rather a concept of body parts and functions that react together to certain changes in the body (for better distinction these Organs are written with a capital letter). Each Organ is governed by one of the five elements—wood, fire, earth, metal, water—and has either a yin- or yang-character depending on its function. The normal functioning of the Organs is ensured by the flow of qi, often translated as 'life energy'. Qi flows through meridians—'energy channels' that belong to the Organs—thereby nourishing all tissues. If the flow of qi is obstructed, the balance between yin and yang will be disturbed and the person feels sick.

TCM uses different forms of therapy like acupuncture, tuina massage, qigong exercises, and also phytotherapy. Using medicinal plants is common in different cultures and can be rationalised by the physiological activity of secondary metabolites. TCM, however, has its specific way of using plants. Each plant is described by its flavour—sour, bitter, sweet, acrid, salty. These flavours correspond to the five elements and thereby indicate the effect of the plant on different Organs. Some plants are processed, e.g. by roasting, to obtain the desired effect. A phytotherapeutical drug always consists of at least four plants: emperor—the plant with the main effect; minister—supports the effect of emperor; ambassador—guides the effect to the right Organ; assistant—helps to treat secondary symptoms, harmonizes the other plants, and reduces toxicity (Bürkland, 2014). This concept of using many plants together in one drug is in contrast to western medicine, where one plant or, better still, one pure compound is used. Extracting several plants together can, however, change the composition of the extract, possibly leading to less toxicity and synergistic effects (Wang *et al.*, 2013b; Yang *et al.*, 2009b).

One of the TCM herbs that has become popular also in western countries is *Ginkgo biloba* L. (Ginkgoaceae) (Fig. 1.4). This dioecious tree can grow up to 35 m high, it has unique fan-shaped leaves, and the female plants produce fruit-like seeds that smell like rancid butter due to the high content of butyric acid (Van Wyk *et al.*, 2015). In TCM originally the seeds are used, only later the leaves have been also listed as a medicinal drug. The fleshy

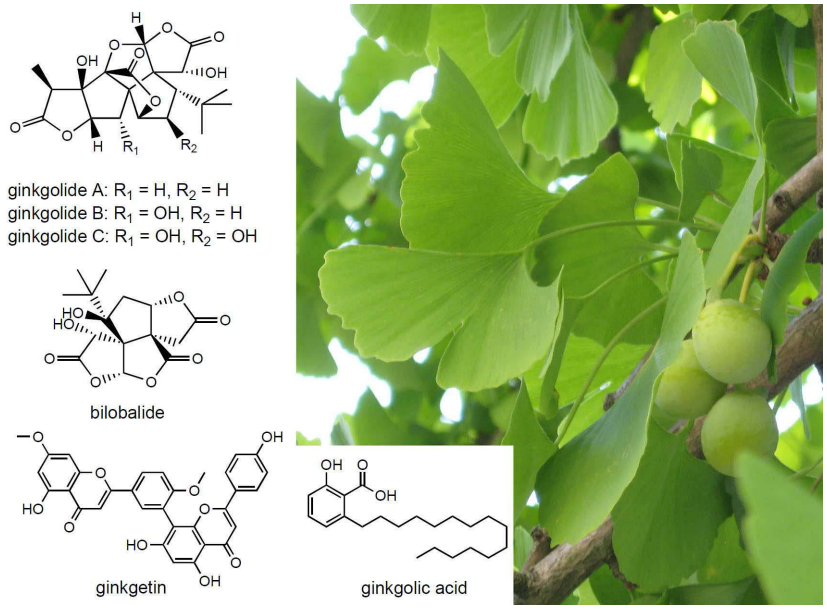

Figure 1.4: *Ginkgo biloba* leaves with unripe seeds (picture taken in the botanical garden of Heidelberg University, July 2015) and some of its compounds.

sarcotesta is removed and the gametophytes, also called 'nuts', are briefly steamed or boiled before baking them dry. This drug, bái guǒ, affects the Lung qi. It is used against cough and asthma, and it also reduces urination and leukorrhea (Qian, 2007). In western countries *Ginkgo* leaves extract has become a popular food supplement because of its assumed memory enhancing properties. A standardized extract called EGb761® with controlled content of flavonoid glycosides (25 %), terpene lactones (6 %), and ginkgolic acid (less than 5 ppm) has been developed by Beaufor-Ipsen Pharma (Paris, France) and Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). Many studies have demonstrated the antioxidant, neuroprotective, anticancer, and other beneficial effects of this extract in different models (reviewed in Mahadevan & Park, 2008; Smith & Luo, 2004), but the clinical value of it in humans with cognitive disorders like AD is still under debate (Schneider, 2012).

Many other TCM herbs have been studied for their neuroprotective and anti-dementia activities. Since in the TCM theory brain is considered as bone marrow and belongs to Kidney, many Kidney nourishing preparations have been tested, although the overall results have been inconclusive (Zeng *et al.*, 2015). Examples for plants with an effect on Kidney would be *Alpinia oxyphylla* (yì zhì), *Cnidium monnieri* (shé chuáng zǐ), *Polygonum multiflorum* (hé shǒu wū), *Epimedium* spp. (yín yáng huò), or *Phellodendron* spp. (huáng bǎi) (Qian, 2007). *A. oxyphylla* Miq. is a perennial herb from the family Zingiberaceae that is native to Asia and Australia. Its capsular elliptic fruits, that are the medicinally used part, contain terpenes, diarylheptanes, flavonoids, protocatechuic acid, and sterols (Fig. 1.5) (Li *et al.*, 2013b; Qing *et al.*, 2012). Antioxidant, anti-inflammatory, and neuroprotective activities have been reported for this drug and its compounds (Qing *et al.*, 2012; Shi *et al.*, 2006; Shui *et al.*, 2006; Yu *et al.*, 2003). It has also shown beneficial effects in models for AD and Parkinson's disease (Shi *et al.*, 2014; Zhang *et al.*, 2015). Other herbs like *Coptis chinensis* (huáng lián), *Evodia rutaecarpa* (wú zhū yú), *Magnolia officinalis* (hòu pò), *Paeonia lactiflora* (chì sháo), *Panax ginseng* (rén shēn), or *Verbena officinalis* (mǎ biān cǎo) have also been studied regarding their possible benefits against AD in different model systems and several secondary metabolites from different groups (alkaloids, polyphenols, saponins, and others) have been identified as their active ingredients (reviewed in Su *et al.*, 2014). Some studies involving effects on Aβ are summarised in Table 1.1.

Table 1.1: Activity against Aβ aggregation and toxicity of selected TCM plants.

Plant	Drug	Model	Effect	Reference
Alpinia galanga	ethanol extract of rhizome	mice treated with $A\beta_{25–35}$	memory enhancing, antioxidant	Hanish Singh *et al.* (2011)
Alpinia oxyphylla	ethanol extract of fruits	mice treated with $A\beta_{1–42}$	memory enhancing, decreased $A\beta_{1–42}$ levels, inhibits AChE, antioxidant	Shi *et al.* (2014)
	5-hydroxy-methylfurfural	mice treated with $A\beta_{1–42}$	memory enhancing, decreased $A\beta_{1–42}$ levels, inhibits BACE1, antioxidant	Liu *et al.* (2014)
Camellia sinensis	EGCG	α-synuclein, $A\beta_{1–42}$, cell culture	binds unfolded proteins, inhibits fibrillisation, leads to formation of non-toxic oligomers	Ehrnhoefer *et al.* (2008)
	green tea catechins	SAMP8 mice[1]	memory enhancing, reduced levels of $A\beta_{1–42}$ oligomers	Li *et al.* (2009)
	EGCG	*C. elegans*	reduced Aβ deposits and oligomerisation, decreased lipofuscin formation, antioxidant	Abbas & Wink (2010)
	EGCG	α-synuclein, $A\beta_{1–42}$, cell culture	binds to amyloid fibrils, remodels their structure	Bieschke *et al.* (2010)
	digested water extract of green tea	cell culture	antioxidant, protective against $A\beta_{1–42}$ toxicity	Okello *et al.* (2011)
	EGCG	metal-free and metal-associated $A\beta_{1–40}$, cell culture	higher antiamyloidogenic activity in metal-associated system, reduced Aβ toxicity, produces ternary complexes with metal-associated Aβ	Hyung *et al.* (2013)
Centella asiatica	extract	PSAPP mice[2]	decreased $A\beta_{1–40}$ and $A\beta_{1–42}$ in hippocampus, antioxidant	Dhanasekaran *et al.* (2009)
	water extract	Tg2576 mice[3], cell culture	memory enhancing, neuroprotective against Aβ toxicity, no change in Aβ levels	Soumyanath *et al.* (2012)
	water extract, caffeoylquinic acids	cell culture	antioxidant, normalizes calcium homoeostasis, attenuates mitochondrial dysfunction	Gray *et al.* (2015)

Continued on next page

[1]Naturally occurring mouse line with accelerated senescence and several features similar to AD.
[2]Doubly transgenic mice expressing APP with the Swedish mutation and PSEN1 with M146L mutation.
[3]Transgenic mice expressing APP with the Swedish mutation.

Table 1.1 – *Continued from previous page*

Plant	Drug	Model	Effect	Reference
Cnidium monnieri	osthole	APP/PS1 mice[4]	memory enhancing, enhanced neurogenesis in dentate gyrus, neurotrophic	Liu *et al.* (2015b)
Coptis chinensis	berberine	TgCRND8 mice[5], cell culture	memory enhancing, decreased Aβ levels, anti-inflammatory, decreased phosphorylation of APP and tau	Durairajan *et al.* (2012)
Eleutherococcus senticosus	water and methanol extracts of rhizome	rat cortical primary neuron cultures treated with $A\beta_{25–35}$	protective against axonal and dendritic atrophy, against cell death, and against synapse loss	Tohda *et al.* (2008)
	methanol extract of rhizome, isolated compounds	rat cortical primary neuron cultures treated with $A\beta_{25–35}$	protective against axonal and dendritic atrophy	Bai *et al.* (2011)
Epimedium brevicornum	icariin	APP/PS1-21 mice[6]	rescues nesting behaviour, reduced Aβ deposition, anti-inflammatory	Zhang *et al.* (2014)
	icariin	Tg2576	memory enhancing, reduced insoluble Aβ and APP levels, increased neurogenesis	Li *et al.* (2015)
Evodia rutaecarpa	evodiamine	APP/PS1 mice, SAMP8 mice	memory enhancing, increased glucose uptake in the brain, anti-inflammatory	Yuan *et al.* (2011)
Ginkgo biloba	EGb761®, flavonoids, bilobalide, ginkgolide B	rat hippocampal primary cell cultures treated with $A\beta_{25–35}$, $A\beta_{1–40}$, or $A\beta_{1–42}$	protective against Aβ toxicity, antioxidant (flavonoids)	Bastianetto *et al.* (2000)
	EGb761®, individual compounds	cell culture, *C. elegans*	antioxidant (especially kaempferol and quercetin)	Smith & Luo (2003)
	EGb761®, ginkgolides, bilobalide, flavonoids	*C. elegans*	counteracts Aβ toxicity, inhibits Aβ oligomerization, antioxidant	Wu *et al.* (2006)
	ginkgolides A, B, C, and bilobalide	$A\beta_{25–35}$	no specific interaction that would modulate Aβ aggregation	He *et al.* (2008)

Continued on next page

[4]Doubly transgenic mice expressing APP with the Swedish mutation and PSEN1 with exon 9 deletion.
[5]Transgenic mice expressing APP with the Swedish mutation and V717F mutation.
[6]Doubly transgenic mice expressing APP with the Swedish mutation and PSEN1 with L166P mutation.

Table 1.1 – *Continued from previous page*

Plant	Drug	Model	Effect	Reference
Ginkgo biloba	EGb761®	TgCRND8 mice, cell culture	memory enhancing, anti-inflammatory, induces autophagy, reduces Aβ deposits	Liu *et al.* (2015d)
Glycyrrhiza uralensis	water extract of the root	mice treated with $A\beta_{25–35}$	memory enhancing, antioxidant, reduces AChE activity	Ahn *et al.* (2006)
	liquiritigenin	rat hippocampal primary neuron cultures treated with $A\beta_{25–35}$	protects for apoptotic cell death and Aβ-induced toxicity, reduces $A\beta_{1–40}$ secretion	Liu *et al.* (2009)
	ethanol extract of the root, isoliquiritigenin	rat cortical primary neuron cultures treated with $A\beta_{25–35}$	protective against apoptotic cell death, interfere with increases in calcium and ROS concentration	Lee *et al.* (2012b)
	isoliquiritigenin and its synthetic derivatives	$A\beta_{1–42}$, 5-lipoxygenase	inhibit Aβ aggregation by binding to the peptide, inhibit 5-lipoxygenase	Chen *et al.* (2013)
Magnolia officinalis	magnolol, honkiol	cell culture	protective against apoptotic cell death, interfere with increases in calcium and ROS concentration	Hoi *et al.* (2010)
	ethanol extract of bark, 4-O-methylhonkiol	mice treated with $A\beta_{1–42}$, cell culture, $A\beta_{1–42}$, BACE1	memory enhancing, protective against neuronal cell death, antioxidant, inhibited Aβ aggregation, BACE1 activity, and BACE1 expression	Lee *et al.* (2010a)
	ethanol extract of bark	Tg2576	memory enhancing, decreases $A\beta_{1–42}$ levels, reduces expression of APP and BACE1	Lee *et al.* (2012c)
Paeonia lactiflora	paeoniflorin	rats treated with $A\beta_{1–42}$	memory enhancing, antioxidant, protective against apoptotic cell death, interferes with increase in calcium	Zhong *et al.* (2009)
	paeoniflorin	microglial cell culture treated with $A\beta_{1–42}$	reduced secretion of inflammatory mediators and chemokines, suppressed chemotaxis to $A\beta_{1–42}$	Liu *et al.* (2015a)
	isolated lignans	$A\beta_{1–42}$	inhibit $A\beta_{1–42}$ aggregation	Liu *et al.* (2015e)
Panax ginseng	extract from HerbPharm, pure compounds	cell culture, Tg2576 mice	reduce Aβ levels, ginsenosides Re, Rg1, and Rg3 selectively reduce $A\beta_{1–42}$ levels	Chen *et al.* (2006)

Continued on next page

Table 1.1 – *Continued from previous page*

Plant	Drug	Model	Effect	Reference
Panax ginseng	ginsenoside Rg3	cell culture	reduces Aβ levels, enhances neprilysin gene expression	Yang *et al.* (2009a)
	fermented water extract of root	cell culture, scopolamin-induced amnesic mice, APP/PS1 mice	memory enhancing, reduces $A\beta_{1–42}$ level	Kim *et al.* (2013)
	ginsenoside compound K	mouse primary astrocyte culture	promotes Aβ clearance through enhancing autophagy	Guo *et al.* (2014a)
Panax notiginseng	panaxynol, panaxydol	rat cortical primary neuron cultures treated with $A\beta_{25–35}$	protective against apoptotic cell death, interfere with increases in calcium and ROS concentration	Nie *et al.* (2008)
	quercetin 3-O-β-D-xylo-pyranosyl-β-D-galacto-pyranoside	cell culture, $A\beta_{1–40}$, scopolamin-induced amnesic mice	oestrogenic activity, antioxidant, inhibits Aβ aggregation, protective against apoptotic cell death, interferes with increase in calcium, memory enhancing	Choi *et al.* (2010)
	notoginsenoside R1	cell culture	protective against apoptotic cell death and mitochondrial damage, antioxidant	Ma *et al.* (2014)
	notoginsenoside R1	mouse hippocampal primary neuron cultures, acute brain slices, APP/PS1 mice	memory enhancing, increases intrinsic excitability of hippocampal neurons, rescues Aβ-induced decline in long term potentiation	Yan *et al.* (2014)
Phellodendron chinense / *amurense*	ethanol extract of cortex	cell culture	protective against apoptotic cell death, *P. amurense* is more potent than *P. chinense*	Xian *et al.* (2013)
Polygonum multiflorum	water extract of roots	mice treated with $A\beta_{25–35}$	memory enhancing, inhibits AChE, antioxidant	Um *et al.* (2006)
	ethanol extract of roots, 2,3,5,4'-tetrahydroxy-stilbene-2-O-β-D-glucoside	*Drosophila melanogaster* expressing $A\beta_{1–42}$, cell culture	ameliorated neurological phenotypes, protective against cell death, antioxidant	Liu *et al.* (2015c)
Punica granatum	pomegranate juice	Tg2576 mice	improved learning, reduced Aβ levels	Hartman *et al.* (2006)
Rhodiola rosea	salidroside	cell culture	protective against apoptotic cell death, antioxidant	Zhang *et al.* (2010)

Continued on next page

Table 1.1 – *Continued from previous page*

Plant	Drug	Model	Effect	Reference
Rhodiola rosea	salidroside	rats treated with $A\beta_{1–40}$	memory enhancing, antioxidant, anti-inflammatory, inhibits AChE	Zhang *et al.* (2013a)
Rosa laevigata	methanol extract	cell culture, mice treated with $A\beta_{1–42}$	antioxidant, protective against cell death, memory enhancing	Choi *et al.* (2006)
	ethanol extract, 1,2-benzenedicarboxylic acid dinonyl ester	cell culture, mice treated with $A\beta_{1–42}$	antioxidant; 1,2-benzenedicarboxylic acid dinonyl ester: memory enhancing, inhibits AChE	Choi *et al.* (2009)
Sanguisorba officinalis	gallic acid	rat cortical primary neuron cultures treated with $A\beta_{25–35}$	in low concentrations protective against apoptotic cell death, interfere with increases in calcium and ROS concentration and glutamate release	Ban *et al.* (2008)
Scutellaria baicalensis	baicalein, baicalin	cell culture	antioxidant, protective against cell death	Heo *et al.* (2004)
	oroxylin A	mice treated with $A\beta_{25–35}$	memory enhancing, anti-inflammatory, antioxidant	Kim *et al.* (2008a)
	stem-leaf total flavonoid	rats treated with $A\beta_{25–35}$	protective against apoptotic cell death	Wang *et al.* (2013a)
	baicalein	cell culture, mouse primary neuron culture, Tg2576 mice	reduces Aβ production, increases α-secretase activity, memory enhancing	Zhang *et al.* (2013b)
Verbena officinalis	water extract of aerial parts	rat cortical primary neuron cultures treated with $A\beta_{25–35}$ or $A\beta_{1–42}$	protective against apoptotic cell death and neurite destruction	Lai *et al.* (2006)

Plants with an effect on the acetylcholinergic system could also be beneficial in treatment of AD. One such plant from TCM is *Areca catechu* L. (Arecaceae). This palm tree is native to south and south-east Asia, it can grow 10–25 m high, and bears hard orange fruits with fibrous mesocarp (Van Wyk *et al.*, 2015). Medicinally used part are the seeds, that can be used raw (bīn láng), baked, or charred. Bīn láng is indicated for the treatment of parasitic infections, malaria, abdominal pain, and gastrointestinal disorders (Qian, 2007). Beyond the traditional usage, extracts of *A. catechu* seeds have been shown to exert several pharmacological activities such as antioxidant, anti-inflammatory, analgesic, antidepressive, regulating blood glucose and lipids, and others (reviewed in Peng *et al.*, 2015). The effects are in part mediated by a cholinomimetic activity due to stimulation of muscarinic ACh receptors and inhibition of AChE (Ghayur *et al.*, 2011; Gilani *et al.*, 2004). The main compounds in *A. catechu* are alkaloids, mainly arecoline, and polyphenols (Fig. 1.5). Arecoline, while being one of the main active compounds, has also been shown to have toxic effects including enhancement of oxidative stress and inducing apoptosis (Peng *et al.*, 2015; Shih *et al.*, 2010).

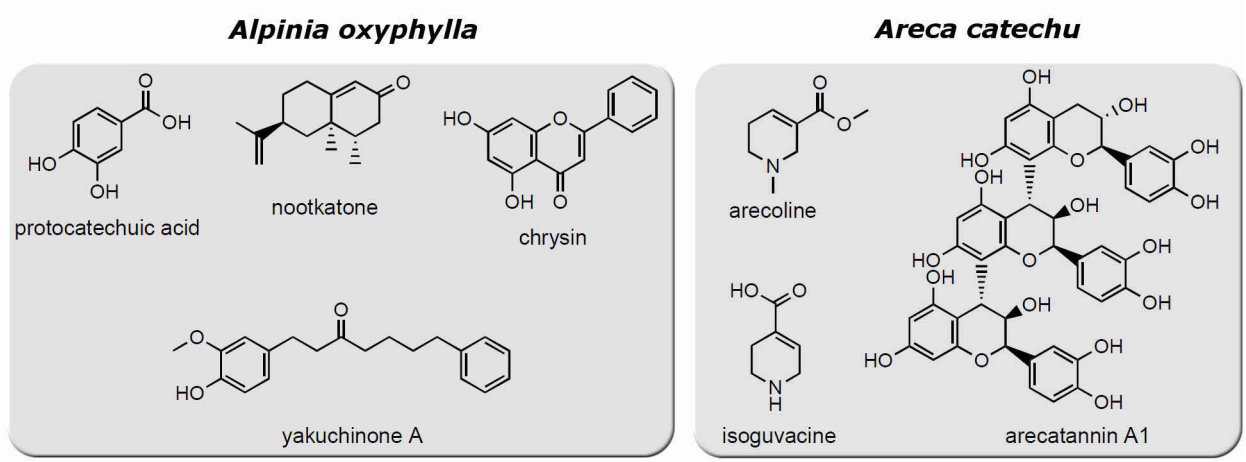

Figure 1.5: Selected compounds from *Alpinia oxyphylla* and *Areca catechu*.

1.2.2 *Glycyrrhiza uralensis*

Glycyrrhiza uralensis Fisch. (Fabaceae) is a perennial herb, that can grow around 1 m tall (Fig. 1.6). It has imparipinnate leaves with 5–17 leaflets, racemose inflorescence with violet papilionaceous flowers, and a branched root system. *G. uralensis* naturally grows in Asia, therefore it is traditionally used in TCM. The same can be said about *Glycyrrhiza inflata* Bat., whereas the third medicinally used species of the genus *Glycyrrhiza*, *Glycyrrhiza glabra* L., is known both in China and in Europe, as it also grows in Mediterranean areas (Van Wyk *et al.*, 2015). The medicinally used part of these plants are the dried roots and stolons. Besides their use in medicine, the roots can be also used to produce liquorice confectionery because of their sweet taste that has given the genus its name (Greek 'glukurrhiza' means 'sweet root').

In both western and Chinese medicine *Glycyrrhiza* has been traditionally used against cough, palpitations, and gastric pain. Other uses in Europe include other conditions of respiratory, gastrointestinal, cardiovascular, and genital-urinary systems, and skin (Fiore *et al.*, 2005; Hosseinzadeh & Nassiri-Asl, 2015; Nassiri-Asl & Hosseinzadeh, 2008). Today it is still used against cough and in treatment of gastric and duodenal ulcers. In China gān cǎo (the drug consisting of dried roots of either three *Glycyrrhiza* species) is one of

Figure 1.6: *Glycyrrhiza glabra* (picture taken in the botanical garden of University of Würzburg, June 2008) and the drug gān cǎo, dried root from *Glycyrrhiza uralensis*.

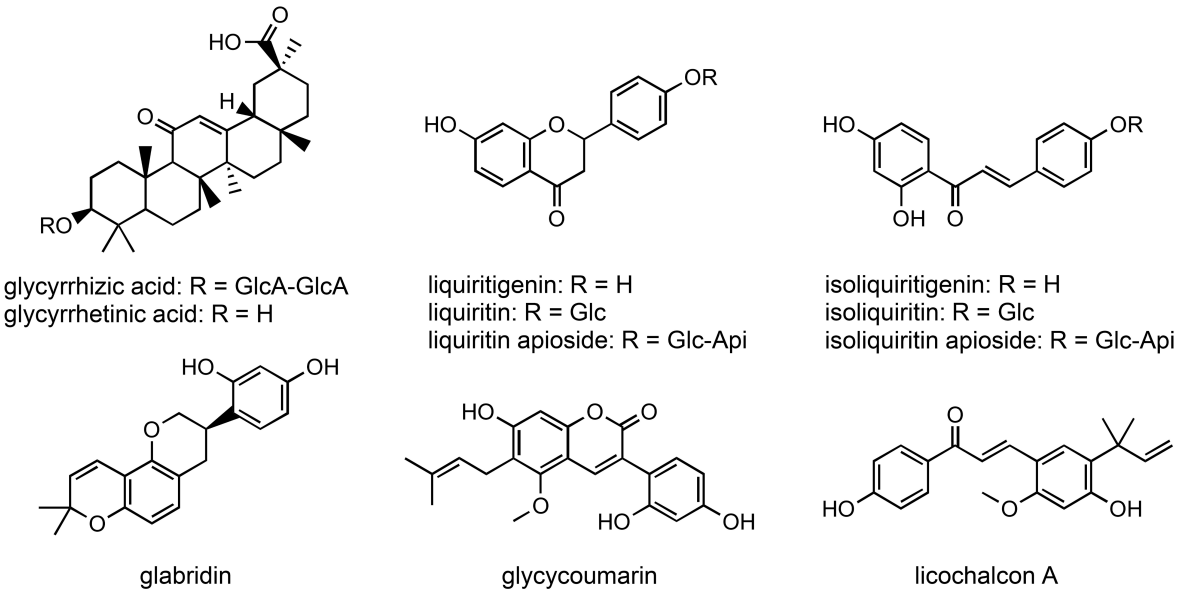

Figure 1.7: Some compounds found in *Glycyrrhiza* spp. Glc: glucose; GlcA: glucuronic acid; Api: apiose.

the most used herbal drug (Guo *et al.*, 2014b). According to TCM philosophy it can harmonize and strengthen the action of other herbs in a formula and carry its effects to all 12 meridians, making it a unique assistant drug, although it is not compatible with all toxic herbs (Guo *et al.*, 2014b; Kao *et al.*, 2014). The harmonizing and detoxifying effects can be explained by the interaction with cytochrome P450 enzymes and permeability glycoprotein (Wang *et al.*, 2013b). Its indications, additionally to the common indications with European medicine, include sores and boils, spasmodic abdominal pain, and detoxification (Qian, 2007). Newer studies have found a potential in neuroprotection and enhancing memory for *G. glabra* (Dhingra *et al.*, 2004; Parle *et al.*, 2004; Shen *et al.*, 2013) and in protection against Aβ toxicity for *G. uralensis* (Ahn *et al.*, 2006; Kanno *et al.*, 2013).

Roots of *Glycyrrhiza* spp. contain triterpene saponins, flavonoids, coumarines, and some other minor compounds (Fig. 1.7) (Nassiri-Asl & Hosseinzadeh, 2008). The most abundant compounds in all three medicinally used species are the triterpene saponin glycyrrhizic acid (GA), and flavonoid glycosides liquiritin apioside and isoliquiritin apioside (Farag *et al.*, 2012; Kondo *et al.*, 2007a; Montoro *et al.*, 2011). Most triterpenes found are derivatives of GA or its aglycon glycyrrhetinic acid (GRA). *G. uralensis* has higher content of liquiritin, isoliquiritin, and liquiritigenin than the other two species and a higher ratio of flavanones to chalcones. Some compounds like glycycoumarin, glabridin, and licochalcone A are specific to *G. uralensis*, *G. glabra*, and *G. inflata*, respectively (Kondo *et al.*, 2007a; Simmler *et al.*, 2015). The roots also contain considerable amounts of sugars and starch, the aerial parts of the plant contain high amounts of flavonoids (Hayashi *et al.*, 2000; Lutomski, 1983).

GA as the most abundant compound is also responsible for many of the therapeutic effects of *Glycyrrhiza* species. The best studied of those is the anti-inflammatory effect, that arises from the competitive inhibition of 11β-hydroxysteroid dehydrogenase and consequent rise in cortisol activity (Krähenbühl *et al.*, 1994; Monder *et al.*, 1989). The aglycon GRA, that is produced from GA by intestinal bacteria (Hattori *et al.*, 1983), is thereby a stronger inhibitor than GA. Other mechanisms mediating the effects of GA and GRA are modulation of phosphoinositide 3-kinase (PI3K) activity and consequent suppression of transcription factors like nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB). Modulation of PI3K activity leads to changes in different pathways and contributes to the anti-

inflammatory and antioxidant activities (Kao *et al.*, 2009, 2013; Wang *et al.*, 2011). This, inhibition of the NF-κB, as well as an anti-excitotoxic effect also lead to neuroprotection in models for AD, Parkinson's disease and epilepsy (Kao *et al.*, 2009; Luo *et al.*, 2013; Zhao *et al.*, 2013). The effect of GRA on NF-κB has also been suggested as a protective mechanism in liver diseases (Chen *et al.*, 2014). Furthermore, GRA has been shown to be beneficial in metabolic disorders, since it can improve glucose tolerance, increase insulin secretion, and has an anti-adiposity effect (Ko *et al.*, 2007; Park *et al.*, 2014). Recent studies have shown that GRA can also activate protective autophagy which contributes to the anti-viral activity of this compound (Laconi *et al.*, 2014; Tang *et al.*, 2014). Some additional effects of GA and GRA as well as their side effects (mostly arising from 11β-hydroxysteroid dehydrogenase inhibition) are reviewed in Ming & Yin (2013).

The major phenolic constituents liquiritigenin (LG) and isoliquiritigenin (ILG) have a number of biological activities, too. Both of them are phyto-oestrogens, whereby LG is a selective agonist of the β type of oestrogen receptor (Mersereau *et al.*, 2008). They are also antioxidants via inducing NRF2 (Gong *et al.*, 2015; Wang *et al.*, 2015). The antioxidant activity and protection against mitochondrial dysfunction prevents cell damage and apoptosis in different models, including glutamate induced neuronal toxicity (Choi *et al.*, 2014; Denzer *et al.*, 2016; Teng *et al.*, 2014; Yang *et al.*, 2012, 2013a; Zeng *et al.*, 2013). ILG is furthermore an antagonist of NMDAR (Kawakami *et al.*, 2011). Liquiritin and isoliquiritin both have antidepressant properties, mediated by antioxidant effects and modulation of neurotransmitters (Wang *et al.*, 2008; Zhao *et al.*, 2008). Additionally, LG has beneficial effects on learning and memory and can attenuate Aβ-induced neurotoxicity (Liu *et al.*, 2011, 2010, 2009; Zeng *et al.*, 2013). ILG has anti-diabetic properties and improved memory in a diabetes model (Gaur *et al.*, 2014; Ma *et al.*, 2015b). It is also protective against Aβ toxicity and inhibits Aβ aggregation (Chen *et al.*, 2013; Lee *et al.*, 2012b). The anti-inflammatory properties of ILG have been shown to involve NF-κB and toll-like receptor 4 suppression (Kim *et al.*, 2008b; Park & Youn, 2010; Wang *et al.*, 2015). Due to reduction of prostaglandin E_2 and interleukin 6 signalling, ILG can induce apoptosis in different cancer cells (Li *et al.*, 2013a; Takahashi *et al.*, 2004; Zhao *et al.*, 2014).

1.2.3 *Carlina acaulis*

Carlina acaulis L. (Asteraceae) is a perennial plant that can be found in central and southern Europe. It has a fleshy taproot and pinnatilobate, spiny leaves, arranged as a rosette. The common name 'silver thistle' derives from the white silvery involucral bracts that surround the flowerhead (Fig. 1.8). The bracts react to changes in air humidity and close to protect the flowers from rain—a feature that has been used to predict weather (Junghans, 2003). The flowerhead is sessile (*C. acaulis* ssp. *acaulis*) or with a short stem (*C. acaulis* ssp. *simplex*), which has given the plant another common name: stemless carline thistle.

The medically used part is the root, *Carlinae radix*. It is traditionally used as diuretic, diaphoretic and anthelmintic drug (Meusel & Kästner, 1990). The essential oil from the root and also traditional decocts have antibacterial activity, especially against *Staphylococcus aureus* (Đorđević *et al.*, 2012; Herrmann *et al.*, 2011a; Jović *et al.*, 2012; Schmidt-Thomé, 1950). Furthermore, antioxidant (Đorđević *et al.*, 2007, 2012), anti-inflammatory, anti-ulcer (Đorđević *et al.*, 2012), and anti-trypanosomal effects (Herrmann *et al.*, 2011a) have been reported. The drug *Carlinae radix* is supposed to contain *C. acaulis*, but often also the roots of *Carlina acanthifolia* are sold (Schilcher & Hagels, 1990; Stojanović-Radić *et al.*, 2012). The roots from these two plants have a similar morphology, chemical composition, and pharmaceutical properties (Đorđević *et al.*, 2012; Schilcher & Hagels, 1990; Stojanović-

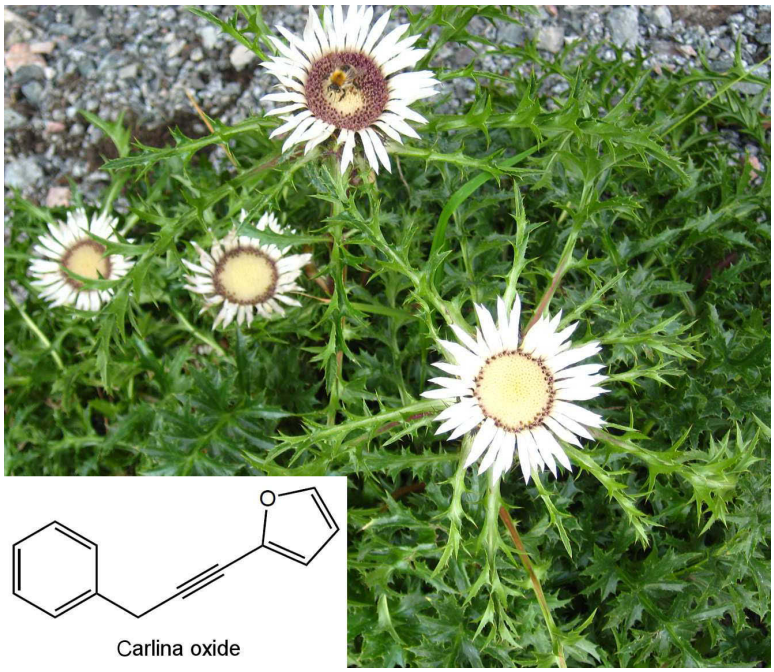

Figure 1.8: *Carlina acaulis* flower head and leaf rosette, and the main active compound Carlina oxide. The picture was taken in botanical garden of Heidelberg University, July 2014.

Radić *et al.*, 2012), therefore *C. acanthifolia* is considered a substitute drug.

Carlinae radix contains 1–2 % essential oil with the main compound Carlina oxide (Fig. 1.8). This alkyne was first isolated by Semmler (1906), who identified the benzyl and furan groups but considered it unlikely to have a triple bond in a natural product. His attempts to synthesize the 1-phenyl-3-α-furyl-allene that he believed to be Carlina oxide failed, so the theoretical structure could not be proven (Semmler & Ascher, 1909). The correct structure, 2-(3-phenylprop-1-ynyl)furan, was discovered almost 30 years later by Gilman *et al.* (1933). The biosynthesis of Carlina oxide results from the fatty acid metabolism (Bohlmann & Mannhardt, 1957; Bohlmann & Rode, 1967; Meusel & Kästner, 1990). Next to Carlina oxide the essential oil contains the sesquiterpenes ar-curcumene, farnesene, and β-sesquiphellandrene (Chalchat *et al.*, 1996; Meusel & Kästner, 1990; Semmler, 1906; Stojanović-Radić *et al.*, 2012). Additionally, the roots contain inulin as a reserve polysaccharide (Vitkova & Evstatieva, 2002).

The aerial parts of the plant contain flavonoids such as the C-glycosylflavones vitexin, isoschaftoside, orientin, and isoorientin (Đorđević *et al.*, 2012; Raynaud & Rasolojaona, 1979), apigenin, apigenin-7-O-glucoside (Đorđević *et al.*, 2012), and chlorogenic acids (Jaiswal *et al.*, 2011). Herb extracts of *C. acaulis* and *C. acanthifolia* have higher antioxidant and anti-inflammatory activities than the respective root extracts (Đorđević *et al.*, 2012).

1.3 *Caenorhabditis elegans*

Caenorhabditis elegans is a free-living roundworm (phylum Nematoda) of the family Rhabditidae. It was first described by Maupas (1900), who gave it the name *Rhabditis elegans*. There are two sexes: hermaphrodites and males, although males occur only with a frequency of 1.5 per 1000 and the hermaphrodites are able to survive for many generations without mating. Figure 1.9 shows the anatomy of an adult hermaphrodite. Its about 1 mm long transparent body is filled with the intestine, starting at the pharynx that marks the head region, and reproductive organs. The males are slightly smaller and can be recognized by the bursa—a sensitive organ surrounding their tails that is needed for mating.

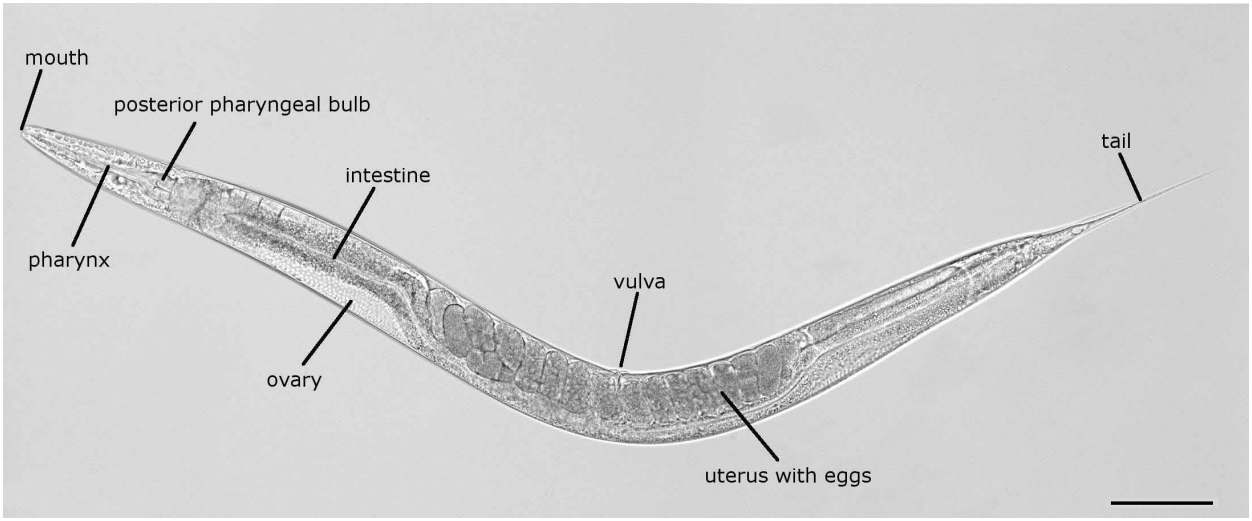

Figure 1.9: Anatomy of an adult *C. elegans* hermaphrodite. Picture taken with Keyence BZ9000, scale bar corresponds to 100 µm.

Brenner (1974) introduced it as a model organism for genetic studies on the development and function of nervous system. He chose this organism because of the simplicity of handling and a manageable amount of cells. Together with H. R. Horwiz and J. E. Sulston, Sidney Brenner became the Nobel Prize in Physiology or Medicine in 2002 for his work concerning organ development and programmed cell death that they had done on this model system. *C. elegans* became the first multicellular organism with completely sequenced genome (The *Caenorhabditis elegans* Sequencing Consortium, 1998) and mapped connectome (Albertson & Thomson, 1976; White *et al.*, 1986), and the entire cell lineage of this organism is known (Sulston *et al.*, 1983). The genome of *C. elegans* consists of five autosomal chromosomes and one sex chromosome with a constitution XX in hermaphrodites and OX in males. The haploid genome contains 97 megabase DNA with over 19,000 genes. 36 % of the proteins in *C. elegans* have a homologue in *Homo sapiens* (The *Caenorhabditis elegans* Sequencing Consortium, 1998). The availability of the complete sequence and high homology to human genome together with the ease of handling make this nematode an excellent model organism.

The normal lifespan of *C. elegans* is about three weeks, depending on temperature and food concentration (Klass, 1977). After hatching from the egg, the larvae pass through four moults (larval stages L1–L4) before they become fertile adults (Fig. 1.10) (Cassada & Russell, 1975). Each moult is preceded by a short period called lethargus, during which the larvae stop feeding and are less agile. The time between moults is dependent on temperature. At lower temperatures the larvae grow slower and therefore need more time to reach the size at which the moult occurs (Byerly *et al.*, 1976; Cassada & Russell, 1975). The young adult hermaphrodites produce sperm during the first hours of adulthood, then eggs begin to form. The sperm remains stored in spermatheca. It fertilizes the ripe eggs as they pass from ovary to uterus. One hermaphrodite can lay about 280 eggs before all the sperm is used, unless it mates with a male and gets additional sperm (Byerly *et al.*, 1976). After that it can lay unfertilized eggs. The eggs are laid shortly before the larvae hatch. In some cases the larvae hatch already in the uterus where they feed on unfertilized eggs and eventually break through in the body cavity of their mother, thereby killing it (Maupas, 1900). This phenomenon is referred to as 'bagging', because the dead hermaphrodite looks like a bag of larvae.

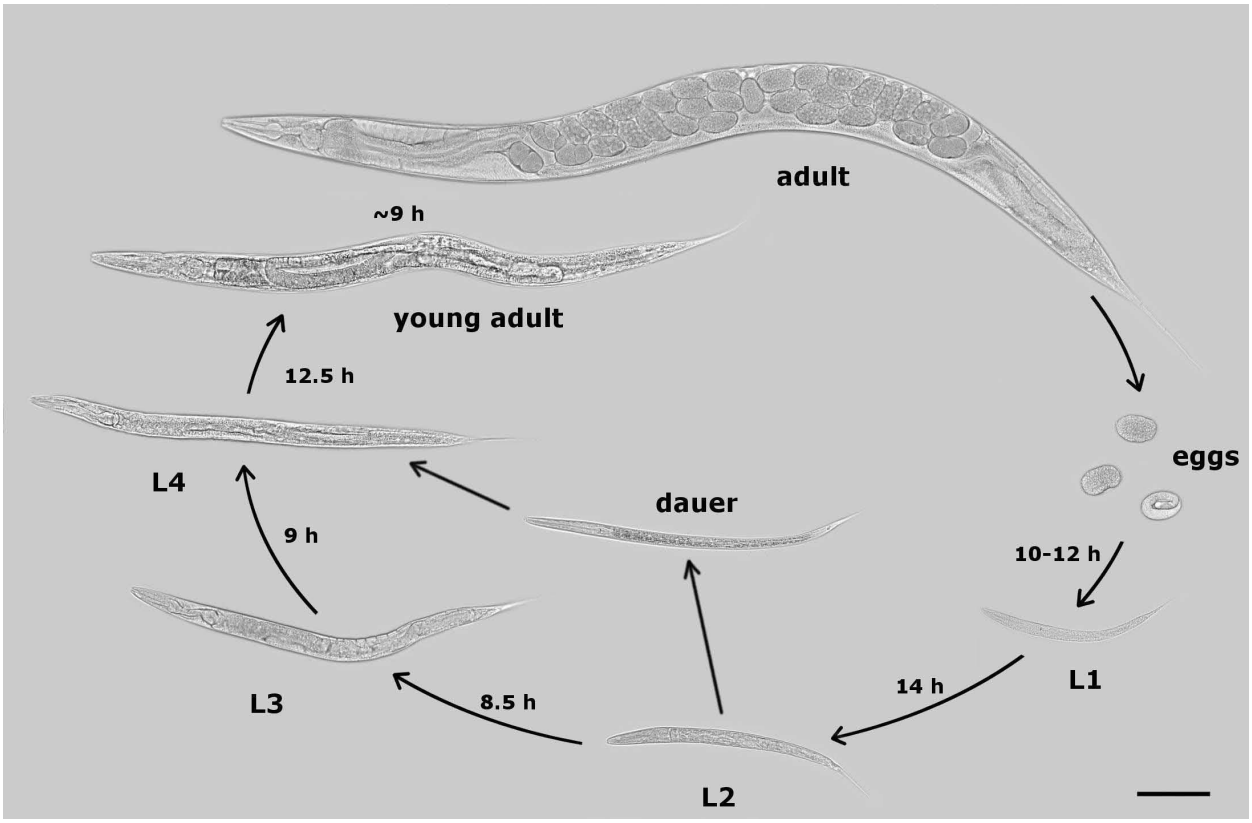

Figure 1.10: Lifecycle of *C. elegans* at 20 °C. Pictures taken with Keyence BZ9000, moulting times after Byerly *et al.* (1976), scale bar corresponds to 100 µm.

When the food is limited or the population density too high, the L2 larvae can form a dormant survival stage called dauerlarva. This stage has similar length as the L2 larva but is much thinner. The dauers do not feed, their pharynx is closed, and they have an altered, thicker cuticle. This stage is more resistant to desiccation, high temperatures, and harmful chemicals than normal worms (Cassada & Russell, 1975). In convenient environmental conditions the dauerlarvae can resume normal development by a moult to L4 larvae. The time spent in dauer stage does not affect the residual lifespan of the worm, so that going through a harsh period can more than double its lifespan.

1.3.1 Insulin-like signalling pathway

Genes involved in regulating dauer formation in *C. elegans* were named *daf* (*abnormal dauer formation*) in an early genetic study (Riddle *et al.*, 1981). The same genes were later observed to play a role in ageing and regulation of lifespan. The gene *daf-2* was described by Kimura *et al.* (1997) as coding for a receptor tyrosine kinase homologous to human insulin and IGF-1 receptors. Mutations in this gene lead to dauer formation or increased lifespan in worms. The life lengthening activity of *daf-2* is dependent on DAF-16 (Kenyon *et al.*, 1993), a transcription factor homologous to human FOXO1, FOXO4 (Lin *et al.*, 1997; Ogg *et al.*, 1997), and FOXO3 (Lee *et al.*, 2001). DAF-16 can translocate to the nucleus under stressful conditions such as starvation, oxidative stress, and heat (Henderson & Johnson, 2001). Together *daf-2*, *daf-23* that was later described as *ageing alteration 1* (*age-1*), coding for a homologue to PI3K catalytic subunit p110 (Morris *et al.*, 1996), and *daf-16* form a distinct pathway among the daf-genes (Gottlieb & Ruvkun, 1994)—the insulin-like signalling (ILS) pathway (Fig. 1.11).

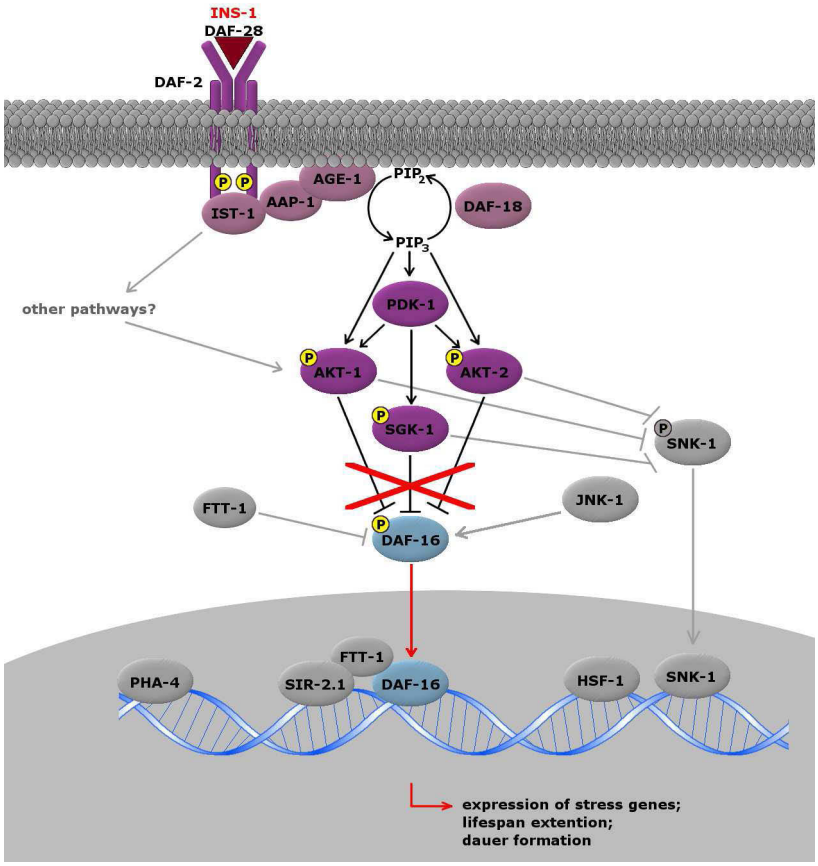

Figure 1.11: The ILS pathway in *C. elegans*. Activation of DAF-2 leads to auto-phosphorylation and recruits IST-1. IST-1 interacts with AAP-1 that recruits AGE-1 to the plasmamembrane where it can produce PIP_3. This is antagonized by the phosphatase DAF-18. PIP_3 activates PDK-1 and together they activate AKT-1 and 2. SGK-1 is also activated by PDK-1. AKT-1, AKT-2, and SGK-1 phosphorylate DAF-16, thereby inhibiting its translocation into nucleus. DAF-2 antagonists lead to nuclear localization of DAF-16. ILS and DAF-16 interact with other pathways and transcription factors to achieve differential gene expression.

The ILS pathway is conserved throughout evolution. Most of the genes involved have homologues in humans and other organisms (Tab. 1.2) (Papatheodorou *et al.*, 2014). Additionally to the three named genes, others have been found to be associated with this pathway. The proteins insulin receptor substrate (IST-1) (homologue to human insulin receptor substrate (IRS)) and AGE-1 adaptor protein (AAP-1) (homologue to human PI3K adaptor subunit p55) help to recruit AGE-1 to plasma membrane upon DAF-2 activation (Wolkow *et al.*, 2002). The phosphatidylinositol-3,4,5-trisphosphat (PIP_3) produced by AGE-1 leads to activation of 3-phosphoinositide-dependent kinase-1 (PDK-1) (Paradis *et al.*, 1999), protein kinase B (AKT)-1 and AKT-2 (Paradis & Ruvkun, 1998). This process is antagonized by DAF-18, a PIP_3 phosphatase homologous to human phosphatase and tensin homologue (PTEN) (Ogg & Ruvkun, 1998). Serum- and glucocorticoid-inducible kinase 1 (SGK-1) is activated through phosphorylation by PDK-1 and forms a possibly temporary complex with AKT-1 and 2 (Hertweck *et al.*, 2004). These three kinases are responsible for phosphorylating DAF-16 and preventing its translocation to nucleus.

DAF-16 activation is regulated by environmental signals via sensory neurons (Apfeld & Kenyon, 1999), by reproductive system (Hsin & Kenyon, 1999), and by stress such as heat shock (Lin *et al.*, 2001b). Insulin related peptides (INSs) (Pierce *et al.*, 2001) and DAF-28 (Li *et al.*, 2003) are produced in neurons and possibly released upon sensory input. Some

Table 1.2: Homologous genes of the *C. elegans* ILS pathway in *Drosophila melanogaster*, *Mus musculus*, and *Homo sapiens*.

C. elegans	***D. melanogaster***	***M. musculus***	***H. sapiens***
daf-2	*InR*	*Insr, Insrr, Igf1r*	*INSR, INSRR, IGF1R*
ist-1	*chico*	*Irs1, Irs2, Irs3, Irs4*	*IRS1, IRS2, IRS4*
aap-1	*Pi3K21B*	*Pik3r1, Pik3r2, Pik3r3*	*PIK3R1, PIK3R2, PIK3R3*
age-1	*Pi3K68D, Pi3K92E*	*Pik3cd, Pik3cb, Pik3ca, Pik3cg*	*PIK3CA, PIK3CG, PIK3CB, PIK3CD*
daf-18	*Pten*	*Pten*	*PTEN*
pdk-1	*Pk61C*	*Pdpk1*	*PDPK1*
akt-1, akt-2	*Akt1*	*Akt1, Akt2, Akt3*	*AKT1, AKT2, AKT3*
sgk-1	–	*Sgk1, Sgk2, Sgk3*	*SKG1, SKG2, SKG3*
daf-16	*foxo*	*Foxo1, Foxo3, Foxo4, Foxo6*	*FOXO1, FOXO3, FOXO4, FOXO6*

of these peptides like INS-1 are antagonists of DAF-2, others like DAF-28 act as agonists. Some of the INSs are also found in other tissues and may have different targets. ILS is an important pathway in regulation of ageing and growth, but apparently there are other mediators whose signals converge on DAF-16 such as c-Jun N-terminal kinase 1 (JNK-1) that can phosphorylate DAF-16 and thereby promote its translocation into nucleus (Oh *et al.*, 2005). Also, different signals result in different activation patterns of DAF-16 (Lin *et al.*, 2001b). This can be explained by the fact that there are three isoforms of DAF-16 (Kwon *et al.*, 2010; Lin *et al.*, 2001b), that have different expression patterns in specific tissues and differential response to the upstream kinases (Kwon *et al.*, 2010; Lee *et al.*, 2001).

ILS affects lifespan and stress resistance by regulating the expression of stress related genes, genes involved in metabolism, development, intra- and extracellular signalling, and also genes for antimicrobial proteins (Lee *et al.*, 2003; McElwee *et al.*, 2003; Murphy *et al.*, 2003; Oh *et al.*, 2006). For expression of some genes interactions between DAF-16 and other transcription factors are necessary. Small HSPs can lengthen the lifespan and possibly protect for age related diseases. To activate these genes both DAF-16 and HSF-1 are needed (Hsu *et al.*, 2003; Morley & Morimoto, 2004) and the activity of HSF-1 is modulated by ILS (Chiang *et al.*, 2012). Oxidative stress is involved in ageing and age related diseases. The transcription factor skinhead 1 (SKN-1) (orthologous to human NRF2) is required for detoxification of free radicals (An & Blackwell, 2003) and is regulated via ILS by the same kinases that phosphorylate DAF-16 (Tullet *et al.*, 2008). Together these transcription factors regulate the expression of partly the same, partly different genes. Dietary restriction can increase lifespan independent of DAF-16 via defective pharynx development 4 (PHA-4), although those two transcription factors have overlapping binding sites and both of them can regulate the expression of some SOD proteins (Panowski *et al.*, 2007). Yeast silent information regulator related 2.1 (SIR-2.1) is also involved in the ageing regulation (Tissenbaum & Guarente, 2001) by interacting with 14-3-3 family protein 1 (FTT-1) and DAF-16, thereby

activating the stress related gene expression in a DAF-16 dependent manner (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006). FTT-1 can also bind the phosphorylated form of DAF-16 in the cytoplasm, inhibiting its entry into the nucleus (Berdichevsky *et al.*, 2006).

Taken together the ILS is a central pathway in a complex network that regulates ageing and stress resistance. The evolutionary conservation of this pathway suggests that it is important for normal function of a multicellular organism. It also opens the possibility to investigate these signals and the impact of modifications to them in a simple model organism such as *C. elegans*.

1.3.2 Nervous system

The nervous system of *C. elegans* consists of 302 neurons. Their cell bodies are organized into 11 ganglia and connected by processes that are concentrated in the circumpharyngeal nerve ring and the ventral and dorsal chords (Ware *et al.*, 1975; White *et al.*, 1986). The ganglia and process bundles are highly organized and symmetrical. The neurons can be divided into sensory, moto-, and interneurons. They have a simple morphology with few branches and connect to each other via gap junctions or chemical synapses. As neurotransmitters acetylcholin (Lewis *et al.*, 1987), biogenic amines (Alkema *et al.*, 2005; Horvitz *et al.*, 1982; Sulston *et al.*, 1975), γ-aminobutyric acid (GABA) (McIntire *et al.*, 1993), glutamate (Arena *et al.*, 1992; Schaeffer *et al.*, 1990), and various neuropeptides are described. The pharynx and its 20 neurons form a separated, nearly self-contagious system, which gets input from somatic nervous system only by one pair of interneurons (Albertson & Thomson, 1976). Additionally to the neurons, 56 glial cells support the functions of the nervous system (Oikonomou & Shaham, 2011).

The body wall muscles of *C. elegans* are obliquely striated and allow dorso-ventral contractions of the body, leading to the sinuous movement of the worms. The muscle cells send arms towards motoneurons in the nerve ring or ventral chord to meet their innervating neural processes that make synapses *en passant*. Gap junctions between the muscle cells might ensure the uniform distribution of a signal in adjacent cells (White *et al.*, 1986). Some motoneurons have also sensory properties that mediate a withdrawal in response to a touch or help to maintain the oscillating body movement by monitoring the stretch of one side of the body (McIntire *et al.*, 1993; White *et al.*, 1986). In contrast to the rest of the body, the head can also be moved in lateral directions. The head muscles are innervated by neurons from the nerve ring which probably allows for finer adjustment of movements than the relatively simple ventral and dorsal chords. One sensory neuron is also found to synapse directly on a muscle cell in the head (Ware *et al.*, 1975; White *et al.*, 1986).

C. elegans is able to sense different chemical and mechanic stimuli. The main chemosensors are organized in the amphids, that are located on the lateral lips of the mouth (Fig. 1.12). Each amphid contains 12 neurons and two glial cells that form the amphid channel. Most of the neuronal endings lie in this channel and are in contact with the outer medium through a pore in the cuticula, some are buried in one of the glial cells called the sheath cell. The nerve ending of AFD, a thermosensory neuron (Mori & Ohshima, 1995), is also buried in the sheath cell, but outside of the amphid channel (Ward *et al.*, 1975). The tree neurons which are not directly exposed to the environment—AWA, AWB, and AWC—are responsible for the chemotaxis to volatile molecules, therefore they can be considered olfactory neurons (Bargmann *et al.*, 1993). The exposed neurons can sense water-soluble molecules like cAMP and several ions, ASE being the most important chemosensory neuron in this set (Bargmann & Horvitz, 1991; Dusenbery, 1974; Ward, 1973). Several of these neurons play a role in dauer formation (Schackwitz *et al.*, 1996; Vowels & Thomas, 1992).

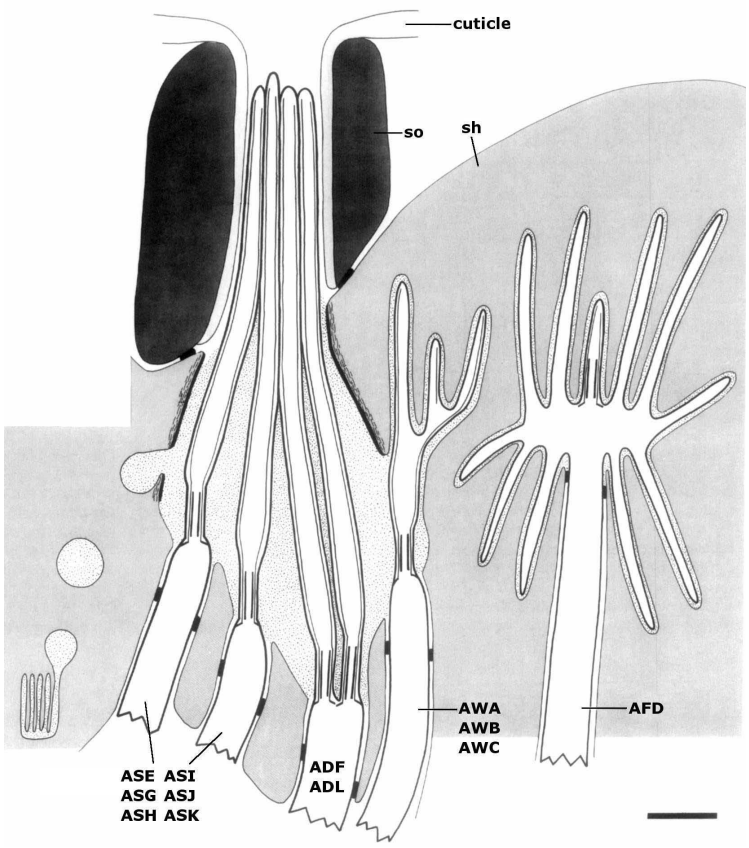

Figure 1.12: Scematic longitudinal section through an amphid. The sensory neurons are surrounded by a sheath cell (sh) and socket cell (so) that form the amphid channel. Five of the 12 neurons are shown. The cilia of the neurons sensitive for water-soluble substances are in direct contact with surrounding medium. They have either a single cilium (ASE, ASG, ASH, ASI, ASJ, ASK) or a branched dendrite with two cilia (ADF, ADL). The olfactory neurons AWA, AWB, and AWC have their dendrites buried in the sheat cell. AFD has its dendrites outside the amphid channel. The scale bar corresponds to 1 µm. Adapted from (Perkins *et al.*, 1986).

Detection of chemicals can help the worms find possible food sources (microorganisms), but also some mechanic stimuli are involved in feeding behaviour. The mechanosensory dopaminergic neurons CEP, ADE, and PDE are required for basal slowing response—the behaviour observed when a *C. elegans* encounters food. Due to slower movement the worm will stay longer at the food source. If a worm has been deprived from food for some time, it reacts even stronger by completely stopping its movements. This behaviour—the enhanced slowing response—is mediated by a pair of serotonergic neurosecretory neurons called NSM (Sawin *et al.*, 2000). The NSM neurons are located in the pharynx and have mechanosensory endings under the cuticle of pharyngeal lumen (Albertson & Thomson, 1976). Exogenous dopamine and serotonin can trigger the respective behaviours in mutants defective in catecholamine synthesis (Sawin *et al.*, 2000). In wildtype animals these neurotransmitters also inhibit locomotion (Horvitz *et al.*, 1982; Schafer & Kenyon, 1995).

1.3.3 *Caenorhabditis elegans* as a model for AD

C. elegans has a homologue to human APP called APP-like 1 (APL-1) (Daigle & Li, 1993) that is involved in sensory responses and plasticity, development and metabolism of the worms by interacting with ILS and other related signalling pathways (Ewald *et al.*, 2012a,b). There are also homologues for α- and γ-secretases, but no BACE1 activity has been found in the worms and APL-1 lacks the Aβ sequence (reviewed in Alexander *et al.*, 2014). Therefore, the *C. elegans* strains used as models for AD are mostly transgenic. The strains best characterized are CL2006 and CL4176 that express a human Aβ peptide in their muscle cells, and CL2355 that expresses the same peptide in nervous system (see Tab. 2.2 for detailed genotype information). A strain with Aβ expression in glutamatergic neurons and three strains expressing tau have also been described (reviewed in Lublin & Link, 2013).

CL2006 was the first transgenic *C. elegans* strain expressing human Aβ. It was made in the lab of Link (1995). This strain expresses Aβ constitutively in its body wall muscles, where it aggregates and forms plaques. The transgenic construct contains a signal peptide for secretion, so the model was expected to produce extracellular $A\beta_{1–42}$. Further examination of this model, however, showed that Aβ fibrils were formed intracellularly and the peptide produced was $A\beta_{3–42}$, probably due to cleavage of the signal peptide at a different site than expected (Link *et al.*, 2001; McColl *et al.*, 2009). The aggregates can be stained with anti-Aβ antibodies or amyloid specific dyes like thioflavin S, Congo red, or X-34 (Fay *et al.*, 1998; Link, 1995; Link *et al.*, 2001). These dyes can also be used to stain senile plaques in AD brains, indicating that the plaques in worms have a similar structure to the plaques found in humans. This was also confirmed by electron microscopy (Link *et al.*, 2001). Therefore, this model is suitable for investigating Aβ aggregation and possible modulators of this process. Aβ expression and aggregation in muscles of these worms leads to progressive paralysis. This phenotype is more pronounced in the strain CL4176 that also expresses Aβ in muscles, but the expression can be induced by temperature change (Link *et al.*, 2003). These worms paralyse and suffer under oxidative stress without the formation of fibrillar amyloid deposits, suggesting that the toxicity of Aβ in this strain is mediated by oligomers (Drake *et al.*, 2003). Hence, this model can be used to study the effects on oligomers and oligomer toxicity. Both of these strains express Aβ in their muscles, representing rather inclusion body myositis (a disease with Aβ aggregates in muscles) than AD. To better recapitulate the neuronal disease, the strain CL2355 with pan-neuronal Aβ expression was developed. These worms have deficits in chemotaxis, associative memory, and serotonin signalling (Dosanjh *et al.*, 2010; Wu *et al.*, 2006), being therefore useful for testing effects on the nervous system.

In addition to the Aβ aggregation and toxicity, these models share some similarities with the human disease. As already mentioned, Aβ dependent oxidative stress is observed in these worms (Drake *et al.*, 2003). This is accompanied by higher levels of iron (Wan *et al.*, 2011), copper can have protective or enhancing activity on the toxicity depending on its concentration as also seen in other models (Luo *et al.*, 2011; Rebolledo *et al.*, 2011). Oxidation of specific proteins involved in several cellular functions corresponding to defects in AD have been identified (Boyd-Kimball *et al.*, 2006). Aβ causes synaptic dysfunction and mislocalisation of ACh receptors (Rebolledo *et al.*, 2011). It induces expression of HSPs in worms and also in AD brain (Link *et al.*, 2003). HSP-16.2 can interact with Aβ oligomers and reduce their toxicity in *C. elegans* model (Fonte *et al.*, 2002, 2008; Wu *et al.*, 2010). Aβ toxicity is also reduced by a proteasomal protein, also upregulated by Aβ expression, and its human homologue (Hassan *et al.*, 2009). Defective autophagy has also been observed in the *C. elegans* model of Aβ toxicity as well as in AD neurons (Florez-McClure *et al.*, 2007). Aβ is degraded through autophagy in this model. The degradation can be enhanced and Aβ toxicity reduced by decreasing the activity of the ILS pathway (Cohen *et al.*, 2006, 2010; Florez-McClure *et al.*, 2007). The same pathway in humans is impaired in diabetes mellitus, a risk factor for AD, and is also involved in Aβ clearance (see section 1.1.4). The first metabolomic study in a *C. elegans* AD model could also draw parallels to human disease (Van Assche *et al.*, 2015).

Despite these similarities, *C. elegans* is obviously not a perfect model for AD. Due to the lack of BACE1 activity this model has no relevance to APP processing and Aβ production. It also lacks processes comparable to neuroinflammation, several pathways relevant to Aβ clearance in humans, and even cell types and organs like astrocytes, microglia, or the blood brain barrier. These models are still useful for studying the aggregation of Aβ in an *in vivo* environment, the response of a single cell to Aβ toxicity, or the effect of compounds

interacting directly with Aβ, independent of an vertebrate organism. In the pan-neuronal Aβ strain also effects on neurons, neurotransmission, and simple behaviour can be studied. In contrast to mammalian models like mice or rats *C. elegans* has a short life span, making it a good model organism for ageing and age related diseases. The short reproduction cycle and easy handling of these worms make it possible to carry out high throughput screens, that would not be possible in mammalian models. On the other hand, this is an *in vivo* system, having the advantage over cell culture where usually only one cell type is kept in an artificial medium. Hence, *C. elegans* can be seen as a bridge between *in vitro* experiments and more complex vertebrate models.

1.4 Objectives

Neurodegenerative diseases are a growing social and medical problem in our ageing society. Although neurodegeneration can be caused by different mechanisms, the diseases tend to share many features like protein aggregation, oxidative stress, neuroinflammation, or defects of lysosomal degradation. For this work AD was chosen as a representative neurodegenerative disease, since it is becoming a major health problem and new medications for its treatment are desperately needed. Phytotherapy can be beneficial in such complex disorders because of the multitarget action and good tolerability, which is important in old, fragile patients. Identifying active extracts and secondary metabolites is therefore imperial. *C. elegans* was chosen as a model organism for this study because of its easy handling and availability of several strains that can model aspects of AD.

The objectives of the present work are:

- **Screening different extracts of selected plants for their activity on Aβ aggregation to identify active extracts**

  25 potentially effective plants from TCM were identified via literature research. Methanol and dichloromethane extracts of these plants will be studied on the *C. elegans* strain CL2006. Most active extracts will be chosen for further analysis.

- **Testing the chosen extracts for their ability to counteract Aβ-induced toxicity**

  An effect on Aβ aggregation in CL2006 does not verify a reduction of Aβ toxicity, if the toxic species are oligomers, not fibrils. To study the effect on oligomers, two other strains will be used: CL4176, to identify the general potential to counteract Aβ-induced toxicity, and CL2355, to study the effect on neurons.

- **Phytochemical characterization of active extracts**

  In order to identify active constituents, the extracts will be analysed via HPLC, LC-MS, or GLC-MS. Major constituents will be tested for their activity on Aβ aggregation and toxicity in the systems mentioned before.

- **Evaluation of *in vivo* antioxidant activity**

  Since oxidative stress is an important feature in neurodegenerative disease, the antioxidant activity of the extract and of the major compounds will be studied. The *C. elegans* strain TJ375 will be used to detect HSP expression, a marker for general stress resistance.

- **Testing effects on ILS pathway and lifespan**

  ILS pathway is highly conserved throughout evolution, which makes it possible to draw conclusions about the effects in humans based on the study in *C. elegans*. Insulin signalling in disturbed in AD, therefore the extracts and compounds will be tested for their ability to activate DAF-16, the downstream transcription factor of ILS. Activation of DAF-16 leads to lengthened lifespan in *C. elegans*, hence this effect will be also tested.

2 Material and methods

2.1 Material

2.1.1 Instruments

Bioblock Scientific	Thermolyne Corp.
BioDoc-It™ Imaging System	UVP
Biofuge pico	Heraeus
Certoclav EL	Certoklav Sterilizer GmbH
Centrifuge Rotina 380R	Hettich
Cooling incubator IPP	Memmert
DS-Qi1Mc black and white camera	Nikon
Degasser ERC-3215α	Shodex
Electrophoresis Power Supply E452	Consort
Fluorescence microscope Ni-E	Nikon
Fluorescence microscope BZ9000	Keyence
Freezer –20 °C	Liebherr
Freezer –80 °C	Fryka
GLC system HP5890 Series II	Hewlett Packard
HPLC system 9100 (YL9110 quaternary pump, YL9101 vacuum degasser, YL9160 photo diode array detector)	Young Lin
HPLC system Gold (125P solvent module, 168 detector)	Beckman
Incubating Mini Shaker	VWR
Incubator	Heraeus
Ion trap mass spectrometer LCQ-Duo	Finnigan
LAS 3000 Imaging system	Brennan & Company
Marathon autosampler	Spark
Microplate reader Asys UVM340	Anthos Mikrosysteme GmbH
MP120 pH meter	Mettler-Toledo

NanoPhotometer	Implien
PerfectBlue™ Dual Gel Twin S	Peqlab
Quadrupole mass spectrometer SSQ 7000	Finnigan
Refrigerator	Liebherr
Rotary evaporator	Heidolph
Sartoblot® II	Satorius
Shaker	GFL
Stereomicroscope SM 22-AD 24	Hund
Sterile bench	Labortechnik Fröbel
Thermocycler TGradient	Biometra

2.1.2 Laboratory material

8-Chambre slides	BD Biosciences
96-Well plates	Greiner Bio-One
Cellstar® tubes (15, 50 ml)	Greiner Bio-One
Cover slips	Marienfeld
GLC column Zebron (30 m × 0.25 mm, film thickness 0.25 µm)	Phenomenex
LiChroCART (4-4) RP-18e (5 µm) guard column	Merck
LiChroCART RP-18 column (250 × 4 mm, 5 µm)	Merck
Object slides	Menzel-Gläser
Petri dishes (35 × 10, 60 × 15, 100 × 20 mm)	Greiner Bio-One
pH indicator	Macherey-Nagel
Preparative TLC plates (30 × 30 cm, silica gel 60, F_{254} 0.5 mm)	Merck
Round filter	Schleicher & Schuell
Safe-lock tubes (0.1, 0.5, 1.5, 2 ml)	Eppendorf
Semi-micro cuvettes 10 × 4 mm	Sarstedt

2.1.3 Chemicals

2,2-Diphenyl-1-picrylhydrazyl	Sigma-Aldrich
β-Mercaptoethanol	Sigma-Aldrich
λ-DNA	Fermentas
Acetic acid	J.T. Baker

Acetonitrile (ACN)	J.T. Baker
Acrylamide/bisacrylamide (30:1)	Roth
Actin antibody C11	Santa Cruz Biotechnology
Agarose	Sigma-Aldrich
Ammonium acetate (NH_4Ac)	Grüssing
Ammonium persulfate (APS)	Amersham Biosciences
Bacto™ Agar	Beckton, Dickinson and Company
Bacto™ Tryptone	Beckton, Dickinson and Company
Bacto™ Yeast Extract	Beckton, Dickinson and Company
Benzaldehyde	Merck
Bovine serum albumin (BSA)	Sigma-Aldrich
Beta-amyloid 1-42, human	PeptaNova
Beta-amyloid monoclonal antibody 6E10, horse radish peroxidase (HRP) labelled	Signet
Bromphenolblue	Fulka
Calcium chloride ($CaCl_2$)	Merck
Cholesterol	Sigma-Aldrich
Citric acid monohydrate	Applichem
Complete PCR reaction buffer, 10 ×	Bioron
Coomassie blue G-250	Fulka
Copper sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$)	Grüssing
Cyclohexane	Grüssing
Deoxynucleotides	Bioron
Dichlormethane	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	VWR
Dipotassium hydrogen phosphate (K_2HPO_4)	Merck
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate	Applichem
(–)-Epigallocatechin gallate (EGCG)	Sigma-Aldrich
Ethanol	Berkel
Ethidium bromide	Serva
Ethylacetate	University Heidelberg

Formic acid (HCOOH)	Merck
Glycerin	AppliChem
Glycin	AppliChem
Glycyrrhizic acid	Northeast Forestry University, China
Glycyrrhizic acid monoammonium	Northeast Forestry University, China
Glycyrrhetinic acid	Northeast Forestry University, China
Hydrochloric acid (HCl)	AnalaR Normapur
Isoliquiritigenin	Phytolab
Iron sulphate septahydrate ($FeSO_4 \cdot 7H_2O$)	Sigma-Aldrich
Juglone (5-hydroxy-1,4-naphthochinon)	Sigma-Aldrich
Liquiritigenin	Phytolab
Methanol	Sigma-Aldrich
Magnesium sulphate ($MgSO_4$)	Merck
Milk powder	Granovita
Manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	Merck
Nitocellulose blotting membrane	Satorius
Nitrogen	Air Liquide
Paraformaldehyde (PFA)	Sigma-Aldrich
Phosphoric acid	Roth
Poly-L-lysin	Sigma-Aldrich
Primers ITS4, ITS5	NWG
Protease inhibitor cocktail Complete Mini	Roche
PST I	Fermentas
Potassium chloride (KCl)	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	Applichem
Sodium azide	Applichem
Sodium chloride (NaCl)	Applichem
Sodium dodecyl sulphate (SDS)	Merck
Sodium hydroxide (NaOH)	Sigma-Aldrich
Sodium hypochlorite (NaClO)	Grüssing
Spectra™ Multicolor low range protein ladder	Fermentas
Taq polymerase	Bioron

Tetramethylethylenediamine (TEMED)	Roth
Thioflavin S	Sigma-Aldrich
Thioflavin T	Sigma-Aldrich
Tricine	Roth
Tri-potassium citrate monohydrate	Roth
Tris(hydroxymethyl)aminomethane (Tris)	Roth
Triton® X-100	Fulka
Tween® 20	Roth
Water HiPerSolv Chromanorm®	VWR
WEST-ZOL™ Plus Western blot detection system	iNtRON Biotechnology
Whatman™ blotting paper, 0.34 mm	GE Healthcare
Zinc sulphate septahydrate ($ZnSO_4 \cdot 7H_2O$)	Merck

2.1.4 Buffers, solutions, media

Phosphate buffered saline (PBS), 1 ×

- 140 mM NaCl
- 2.7 mM KCl
- 10 mM Na_2HPO_4
- 1.8 mM KH_2PO_4

pH was adjusted to 7.4 with HCl.

4 % Paraformaldehyde (PFA) solution

50 ml H_2O was heated to about 60 °C. 4 g PFA was added and stirred, to get a suspension. Then 1 M NaOH was added until the PFA was dissolved and the solution turned transparent. It was let to cool down and filtered. 10 ml of 10 × PBS was added to the solution and it was then filled up to 100 ml with H_2O. The pH value was checked using a pH indicator and adjusted with HCl to be between 7 and 8.

S-basal

- 5.85 g NaCl
- 1 g K_2HPO_4
- 6 g KH_2PO_4
- 1 ml 5 mg/ml Cholesterol in ethanol
- H_2O to 1 litre

The solution was autoclaved for 15 min at 125 °C .

Trace metals solution

- 1.86 g Disodium EDTA
- 0.69 g $FeSO_4 \cdot 7H_2O$
- 0.2 g $MnCl_2 \cdot 4H_2O$
- 0.29 g $ZnSO_4 \cdot 7H_2O$
- 0.025 g $CuSO_4 \cdot 5H_2O$
- H_2O to 1 litre

The solution was autoclaved for 15 min at 125 °C.

1 M Potassium citrate pH 6

- 20 g Citric acid monohydrate
- 293.5 g Tri-potassium citrate monohydrate
- H_2O to 1 litre

The solution was autoclaved for 15 min at 125 °C.

S-Medium

- 1 l S-Basal
- 3 ml 1 M $CaCl_2$
- 3 ml 1 M $MgSO_4$
- 10 ml 1 M Potassium citrate pH 6
- 10 ml Trace metals solution

1M Potassium phosphate (kpo_4), pH 6

- 35.6 g K_2HPO_4
- 108.3 g KH_2PO_4
- H_2O to 1 litre

The solution was autoclaved for 15 min at 125 °C.

NGM

- 17 g Agar
- 3 g NaCl
- 2.5 g Tryptone
- 975 ml H_2O

The solution was autoclaved for 15 min at 125 °C. After cooling down to 55 °C the following ingredients were added:

- 1 ml 1 M $CaCl_2$
- 1 ml 1 M $MgSO_4$
- 25 ml 1 M KPO_4
- 1 ml 5 mg/ml Cholesterol in ethanol

Test-agar for chemotaxis assay

- 19 g Agar
- 975 ml H_2O

The solution was autoclaved for 15 min at 125 °C. After cooling down to 55 °C the following ingredients were added:

- 1 ml 1 M $CaCl_2$
- 1 ml 1 M $MgSO_4$
- 25 ml 1 M KPO_4

M9-Buffer

- 5 g NaCl
- 6 g Na_2HPO_4
- 3 g KH_2PO_4
- 1 ml 1 M $MgSO_4$
- H_2O to 1 litre

The solution was autoclaved for 15 min at 125 °C.

LB Medium

- 10 g Tryptone
- 5 g Yeast extract
- 10 g NaCl
- 950 ml H_2O

The solution was autoclaved for 15 min at 125 °C.

LB Agar

- 10 g Tryptone
- 5 g Yeast extract
- 10 g NaCl
- 15 g Agar
- 950 ml H_2O

The solution was autoclaved for 15 min at 125 °C and poured into 100 mm petri dishes.

TE Buffer

- 10 mM Tris
- 1 mM EDTA

Sample buffer for agarose gel electrophoresis

- 500 µl Glycerin
- 2.5 mg Bromphenolblue
- 500 µl H_2O

TAE-Buffer, 50 ×

- 2420 g Tris
- 186 g EDTA
- 571 ml Acetic acid
- H_2O to 10 litre

Bradford reagent

- 25 mg Coomassie blue G-250
- 12.5 ml Ethanol
- 25 ml Phosphoric acid
- H_2O to 250 ml

Coomassie solution

- 0.5 % Coomassie blue G-250
- 50 % Ethanol
- 10 % Acetic acid
- 39.5 % H_2O

Destaining solution

- 20 % Ethanol
- 10 % Acetic acid
- 70 % H_2O

4 × Laemmli buffer

- 5 ml 1 M Tris, pH 6.8
- 40 ml Glycerin
- 5 g SDS
- 5 mg Bromphenolblue
- H_2O to 100 ml

10 % β-Mercaptoethanol were added prior to use.

Gels for SDS-PAGE

	15 % resolving gel	**5 % stacking gel**
H_2O	2.3 ml	3.4 ml
30 % Acrylamide mix	5 ml	0.8 ml
1.5 M Tris (pH 8.8)	2.5 ml	-
1 M Tris (pH 6.8)	-	0.6 ml
10 % SDS	0.1 ml	0.05 ml
10 % APS	0.1 ml	0.05 ml
TEMED	4 µl	5 µl

Cathode buffer

- 12.11 g Tris
- 17.92 g Tricine
- 1 g SDS
- 1 l H_2O

Final concentrations: 0.1 M Tris, 0.1 M tricine, 0.1 % SDS.

Anode buffer, 5 ×

- 121.1 g Tris
- 500 ml H_2O

The pH was adjusted to 8.9 with HCl, then water was added to 1 l.

Transfer buffer

- 25 mM Tris
- 196 mM glycin
- 20 % methanol

2.1.5 Plants

Most of the plants investigated in the present study are used in TCM. They were obtained commercially in China and kindly provided to our institute by Prof. Thomas Efferth. The unripe fruits of *G. biloba* were gathered on university campus in Heidelberg, Germany. Other suppliers are indicated in Table 2.1. Voucher specimens of the plants are deposited at IPMB, Department of Biology, Heidelberg University, Germany.

Table 2.1: Plant material used in this work including plant part, supplier and respective IPMB accession numbers (IPMB-nr).

Name	**Family**	**Used part**	**IPMB-nr.**	**Supplier**
Alpinia galanga	Zingiberaceae	Fruit	P6837	China
Alpinia oxyphylla	Zingiberaceae	Fruit	P6917	China
Areca catechu	Arecaceae	Seed	P6840	China
Capsella bursa-pastoris	Brassicaceae	Herb	P6846	China
Carlina acaulis	Asteraceae	Root	P8212	Caesar & Loretz
Centella asiatica	Apiaceae	Herb	P6849	China
Cnidium monnieri	Apiaceae	Seed	P6854	China
Coptis chinensis / *deltoidea* / *teeta*	Ranunculaceae	Root	P6855	China
Eleutherococcus senticosus	Araliaceae	Root	P6919	Russia
Epimedium brevicornum / *sagittatum* / *pubescens* / *wushanense* / *koreanum*	Berberidaceae	Leaves	P6865	China
Evodia rutaecarpa / *rutaecarpa* var. *officinalis* / *rutaecarpa* var. *bodinieri*	Rutaceae	Fruit	P6870	China
Ginkgo biloba	Ginkgoaceae	Leaves	P8075	Heinrich Klenk

Continued on next page

Table 2.1 – *Continued from previous page*

Name	**Family**	**Used part**	**IPMB-nr.**	**Supplier**
Ginkgo biloba	Ginkgoaceae	Nuts	P6872	China
Ginkgo biloba	Ginkgoaceae	Unripe seeds	P8517	Im Neuenheimer Feld, Heidelberg
Ginkgo biloba	Ginkgoaceae	EGb761®		Dr. Willmar Schwabe
Glycyrrhiza uralensis	Fabaceae	Wood	P6873	China
Magnolia officinalis / *officinalis* var. *biloba*	Magnoliaceae	Bark	P6882	China
Berberis bealei	Berberidaceae	Wood	P6883	China
Paeonia lactiflora / *veitchii*	Paeoniaceae	Root	P6886	China
Panax ginseng	Araliaceae	Root	P8077	Kräuter Schulte
Panax notoginseng	Araliaceae	Root	P6887	China
Phellodendron chinense / *amurense*	Rutaceae	Wood	P6890	China
Polygonum multiflorum	Polygonaceae	Root	P6895	China
Punica granatum	Lythraceae	Fruit	P6897	China
Rhodiola rosea	Crassulaceae	Root	P6920	Kräuterparadies Lindig
Rosa laevigata	Rosaceae	Fruit	P6900	China
Sanguisorba officinalis / *officinalis* var. *longifolia*	Rosaceae	Root	P6901	China
Scutellaria baicalensis	Lamiaceae	Wood	P6903	China
Verbena officinalis	Verbenaceae	Herb	P6910	China

2.1.6 *Caenorhabditis elegans* strains

The *C. elegans* strains used in this work (Tab. 2.2) and their food source *Escherischia coli* OP50 were obtained from Caenorhabditis Genetics Center (CGC) at University of Minnesota, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). An exception are the strains CL2355 and PD8120 that were a kind gift from Dr. Christopher D. Link, University of Colorado.

Table 2.2: *C. elegans* strains used in the present work.

Strain	Genotype	Description from CGC
N2	wildtype	*C. elegans* var. *bristol*. Isolated from mushroom compost near Bristol, England by L.N. Staniland.
CL2006	*dvIs2*	*dvIs2 [pCL12(unc-54::Aβ_{1-42}) + pRF4(rol-6(su1006))]*. Adult onset paralysis and egg-laying deficiency when raised at 20 °C.
CL4176	*smg-1(cc546) I; dvIs27 X*	*dvIs27 [pAF29(myo-3::Aβ_{1-42}::let UTR) + pRF4(rol-6(su1006))]*. Temperature sensitive: needs to be propagated at 16 °C. At 25 °C expression of human Aβ in muscle cells, leading to paralysis.
CL802	*smg-1(cc546) I; rol-6(su1006) II*	Temperature sensitive with roller phenotype.
CL2355	*smg-1(cc546) I; dvIs50 I*	*dvIs50 [pCL45(snb-1::Aβ_{1-42}::let UTR + mtl-2::GFP]*. Temperature sensitive: needs to be propagated at 16 °C. At 23 °C pan-neuronal expression of human Aβ. Shows deficits in chemotaxis, associative learning, and thrashing in liquid. Also has incomplete sterility due to germline proliferation defects and embryonic lethality.
PD8120	*smg-1(cc546) I*	Temperature sensitive.
TJ375	*gpIs1*	*gpIs1 [hsp-16.2::GFP]*. Insertion not mapped. GFP fluorescence after > 1 hour heat shock at 35 °C.
TJ356	*zIs356 IV*	*zIs356 [daf-16::GFP]*. Roller strain, increased resistance to heat and UV. Grows and reproduces slowly. Integrated by gamma irradiation of extrachromosomal (*Ex daf-16::GFP*) line.

2.1.7 Software

32 Karat™ 5.0	Beckman Coulter, Inc.
Bioedit	Ibis Biosciences
DigiRead V 1.8.0.2	Biochrom
ImageJ 1.43u	public domain
MEGA 5.1	Tamura *et al.* 2011
NIS-Elements	Nikon
SigmaPlot 13.0	Systat Software, Inc.
StatView 5.0.1	SAS Institute Inc.
YL-Clarity	Young Lin
Xcalibur™ 2.0	Thermo Scientific

2.2 Methods

2.2.1 Preparation of extracts

Extraction of TCM plants

The dried plant material from plants listed in Table 2.1 (with exception of *C. acaulis*) was extracted with methanol and dichloromethane. For *G. uralensis* also a water extract was prepared. 50 g of the plant material was heated under reflux with the respective solvent for 4 h, then filtrated. The extracts were concentrated in a rotary evaporator and stored at –20 °C. Small amounts of these crude extracts were dried in a heating block and dissolved in methanol or DMSO to the desired concentration.

Some of these extracts were prepared earlier in the same manner by Dorothea Kaufmann and Florian Hermann during their PhD thesis at Heidelberg University, IPMB, Department of Biology.

Extraction of *Carlina acaulis*

This extract was prepared by Frank Sporer, a scientific assistant at Heidelberg University, IPMB, Department of Biology. The dried roots of *C. acaulis* were extracted with dichlormethane in a Soxhlet extractor. The crude extract was dried under nitrogen flow and dissolved in methanol for further experiments.

2.2.2 Isolation of Carlina oxide

Carlina oxide was isolated from the dichloromethane extract of *C. acaulis* using preparative thin layer chromatography (TLC) plates with silica gel and a mixture of 90 % ethylacetate and 10 % cyclohexane as eluent. The Carlina oxide fraction was separated from all other fractions. Both the Carlina oxide fraction and the residual extract were extracted from silica gel with methanol. The amount of obtained Carlina oxide and residual extract from three preparative TLC plates was 7.4 and 5.2 mg, respectively. The isolation was carried out by Kevin Roth, a Master of Science student of Molecular Biotechnology at Heidelberg University, during his laboratory practical at IPMB, Department of Biology.

2.2.3 HPLC analysis

The *G. uralensis* extracts were analysed with a high pressure liquid chromatography (HPLC) system from Young Lin on a reversed phased column using gradient elution of 3 % acetic acid in water and ACN (Zeng *et al.*, 1990). The gradient is shown in Table 2.3. Detection was performed at different wavelengths: 245, 310, 365, 276, 231, and 520 nm and evaluated using the YL-Clarity software. The substances LG, ILG, GA, and GRA were identified by comparison of the retention time (RT) and absorption pattern with external standards. Calibration curves for quantification of these compounds were prepared with following concentrations: 150, 100, 50, 25, and 17.5 µg/ml for GA; 60, 40, 20, 10, and 5 µg/ml for LG and GRA; 30, 20, 10, 5, and 2.5 µg/ml for ILG. GA, GRA, and ILG were quantified at 254 nm, LG at 310 nm.

2.2.4 LC-MS/MS analysis

For LC-MS analysis of water and methanol extracts of *G. uralensis* the HPLC system Gold from Beckman was used. A gradient of ACN with 0.1 % formic acid (A) and 0.1 % formic

Table 2.3: Gradient used for HPLC analysis of *G. uralensis* extracts.

Time in min	% H_2O with 3 % acetic acid	% ACN	Flow rate in ml/min
0	80	20	1
13	80	20	1
15	50	50	1.2
25	20	80	1.2
30	20	80	1.2

acid in water (B) was used at a constant flow rate of 1 ml/min. The gradient from 80 % B to 20 % B in 20 min was applied, followed by 10 min at 20 % B and a 10 min equilibration at 80 % B. The run was detected at 254 nm and 365 nm using 32 Karat™ software and with the ion trap mass spectrometer (MS) LCQ-Duo equipped with an electrospray ionisation source. The spectra were acquired in negative ion mode over a mass range of 50–2000 m/z with highly purified nitrogen as sheath and auxiliary gas, capillary voltage –10 V, capillary temperature 200 °C. The Xcalibur™ software was used to analyse the data.

2.2.5 GLC-MS analysis

Work on gas-liquid chromatography (GLC)-MS was carried out by Frank Sporer, a scientific assistant at Heidelberg University, IPMB, Department of Biology. For the GLC analysis of *C. acaulis* extract following temperature gradient was applied: isotherm at 40 °C for 2 min, 8 °C/min to 300 °C, isotherm at 300 °C for 10 min. For the Carlina oxide and extract without Carlina oxide the temperature gradient was started at 100 °C and increased in 3 °C/min for better resolution. Data acquisition was started after 3 min. The injector temperature was 250 °C, head pressure 15 hPa, carrier gas helium, split 1:100. The separated substances were ionized by electron ionisation with an electron energy of 70 eV and source temperature 175 °C. Detection followed via SSQ 7000 MS. The spectra were analysed using Xcalibur™ software.

2.2.6 Identification of *Glycyrrhiza uralensis* via DNA barcoding

Variable sites in four DNA regions have been described in *Glycyrrhiza* species by Kondo *et al.* (2007b). Here one of them, the *internal transcribed spacer (ITS)* sequence, was examined. DNA was extracted from the dried plant material of gān cǎo earlier by Florian Hermann during his work on his PhD thesis at Heidelberg University, IPMB, Department of Biology. It was stored in Tris-EDTA (TE) buffer at 4 °C. The ITS sequence containing ITS1, 5.8S ribosomal ribonucleic acid (rRNA) gene, and ITS2 was amplified by polymerase chain reaction (PCR) with the primers ITS4 (TCC TCC GCT TAT TGA TAT GC) and ITS5 (GGA AGT AAA AGT CGT AAC AAG G). 0.5 µl of each primer, 1.5 µl of mixed deoxynucleotides, 2.5 µl 10 × reaction buffer, 0.5 µl 20 mg/ml BSA, 0.2 µl Taq polymerase, 16.3 µl H_2O, and 3 µl DNA were pipetted together in a PCR tube. The PCR program is shown in Table 2.4.

The amplified DNA was tested for the right size by agarose gel electrophoresis. 6 µl of the PCR product with 1.5 µl sample buffer were loaded on a 1 % agarose gel in TAE buffer with 0.01 % ethidium bromide. The separation was performed at 90 V for 40 min. The bands

Table 2.4: PCR program for amplifying ITS.

Temperature	**Duration**
94 °C	5 min
38 cycles:	
94 °C	45 s
45 °C	50 s
72 °C	50 s
72 °C	5 min
4 °C	5 min

were compared to a λ-marker (λ-DNA cut by PST I enzyme).

Finally the samples containing correct PCR product were purified and sequenced. For the purification DNA was precipitated with NH_4Ac and ethanol and centrifuged for 30 min at 13,000 rpm. The solvent was discarded, the pellet washed with 70 % ethanol. The solvent was completely removed and the pellet dried at 56 °C. The dry pellet was then dissolved in sterile H_2O and sent for sequencing to StarSEQ® GmbH. The raw sequences were edited using Bioedit and a divergence analysis was performed using MEGA 5.1. For the divergence analysis the sample P6873 was compared to sequences retrieved from GenBank, *Glycyrrhiza astragalina* was used as an outgroup (Tab. 2.5).

2.2.7 DPPH• assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH•) is a free radical that can be reduced by hydrogen donors. This reaction can be quantified photometrically, since DPPH• has a strong absorbance at 517 nm but the reduced form, hydrazine, does not. DPPH• free radical scavenging assay was performed using a modified version of the method described by Blois (1958). DPPH• was prepared as a 0.2 mM solution in methanol. Equal volumes of the tested substance and DPPH• solution were mixed and incubated for 30 min at room temperature. After the incubation the absorbance of each sample in duplicates was measured at 517 nm in a microplate reader. For each tested substance concentration a blank without DPPH• was prepared and measured in the same manner. DPPH• free radical scavenging effect was calculated using the following equation:

$$\%\ \text{DPPH}^{\bullet}\ \text{scavenging effect} = \frac{A_0 - A_1}{A_0} \times 100$$

A_0: absorbance of DPPH•
A_1: absorbance of DPPH• with the tested compound

2.2.8 SDS-PAGE and Western blot analysis of Aβ

To separate the different forms of Aβ present in the worms and evaluate the effect of the extracts on the amount of oligomers and monomers of this peptide, a Western blot analysis was conducted. CL2006 worms were treated the same way as for the quantification of

Table 2.5: Sequences used for the divergence analysis.

GenBank accession nr.	Species	Encoded region
JF778868	*G. inflata*	5.8S rRNA, partitial sequence; ITS2; 26S rRNA, partitial sequence
AB280740	*G. inflata*	ITS1; 5.8S rRNA; ITS2
AY065623	*G. glabra*	18S rRNA, partitial sequence; ITS1; 5.8S rRNA; ITS2; 26S rRNA, partial sequence
JF778867	*G. glabra*	5.8S rRNA, partitial sequence; ITS2; 26S rRNA, partitial sequence
HE602402	*G. glabra*	ITS1; 5.8S rRNA; ITS2
AB280739	*G. glabra*	ITS1; 5.8S rRNA; ITS2
JF421504	*G. uralensis*	5.8S rRNA, partitial sequence; ITS2; 26S rRNA, partitial sequence
JF778869	*G. uralensis*	5.8S rRNA, partitial sequence; ITS2; 26S rRNA, partitial sequence
HQ229003	*G. uralensis*	5.8S rRNA, partitial sequence; ITS2; 26S rRNA, partitial sequence
GQ246129	*G. uralensis*	ITS1; 5.8S rRNA; ITS2
AB280738	*G. uralensis*	ITS1; 5.8S rRNA; ITS2
EU418258	*G. uralensis*	ITS1, partitial sequence; 5.8S rRNA; ITS2, partitial sequence
AB649775	*G. uralensis*	18S rRNA, partitial sequence; ITS1; 5.8S rRNA; ITS2; 26S rRNA, partitial sequence
JF421503	*G. uralensis*	5.8S rRNA, partitial sequence; ITS2; 26S rRNA, partitial sequence
GQ246133	*G. astragalina*	ITS1; 5.8S rRNA; ITS2
GQ246134	*G. astragalina*	ITS1; 5.8S rRNA; ITS2

plaques (see section 2.2.10). Instead of fixing the worms at the end of the treatment, they were collected in a Eppendorf tube, washed twice with 1 × PBS, and resuspended in 100 µl PBS with protease inhibitor cocktail. These samples were frozen and kept at –80 °C until further use.

The samples were thawed on ice and homogenized. The protein concentration was measured after Bradford (1976). Briefly, 2 µl of the sample were mixed with 1 ml diluted Bradford reagent (1:5 in water) and the absorption was measured at 595 nm after 5 min incubation at room temperature. A standard curve was prepared using BSA in a concentration range of 0.5–3 mg/ml.

A SDS-tricine polyacrylamide gel electrophoresis (PAGE) was performed with 15 % resolving gel and 5 % stacking gel. The samples (final concentration of 50 µg/ml protein) were heated at 100 °C for 3 min in Laemmli buffer prior to loading on the gel. A low range protein ladder was used as marker. 0.5 µg Aβ were used as positive control. The gel was run for approximately 1 h at 100 V in tricine containing cathode buffer and anode buffer without tricine.

For detection either a Coomassie staining or Western blot were used. For the Coomassie staining the gel was covered with Coomassie solution and stained on shaker for several hours followed by destaining with the destain solution. For Western blot analysis the proteins were blotted on a nitrocellulose membrane in transfer buffer for 2 h at 48 mA . Then the membrane was blocked with 5 % milk powder in phosphate buffered saline containing 0.05 % Tween® 20 (PBST) over night at 4 °C. On the next day the membrane was incubated with either anti-Aβ or anti-actin antibodies coupled with HRP (1:1000 dilution in 2.5 % milk powder in PBST) for 2 h. The membrane was washed 3× 10 min in PBST and detected with WEST-ZOL™ Western blot detection system. The blots were documented by LAS 3000 Imaging system.

2.2.9 *Caenorhabditis elegans* culture conditions

The temperature sensitive strains CL4176, CL802, CL2355, and PD8120 were cultivated at 16 °C, all others at 20 °C. The worms grew on nematode growth medium (NGM) and were fed with *E. coli* OP50, an uracil deficient *E. coli* strain (Brenner, 1974). The bacteria were cultured in liquid LB medium at 37 °C overnight. This overnight culture was stored at 4 °C for further use. Aliquots of it were spread on LB agar plates and left at 37 °C overnight prior to resuspension in S-medium. Most of the experiments were conducted in S-medium containing 10^9 $cells/ml$ *E. coli* OP50 ($OD_{600} \approx 1$).

To obtain eggs for the experiments the hypochlorite treatment described earlier (Sulston & Hodgkin, 1988) was used. Briefly, the worms were treated with 0.5 M NaOH and 1 % NaClO for 10 minutes or until the worms were dissolved. The eggs were pelleted by centrifuging at 1300 × g for 40 s and washed with sterile water.

2.2.10 Quantification of Aβ aggregates in CL2006

To see the effect of the treatments on Aβ aggregation, the *C. elegans* strain CL2006 was used. These worms constitutively express human Aβ in their muscle cells where the peptides aggregate causing difficulties in movement (Link, 1995). The eggs of CL2006 worms were isolated, placed in S-medium with *E. coli* OP50 and kept at 20 °C. The worms were treated on day 2 after hatching. 100 µg/ml of the methanolic TCM extracts were used, except for *Magnolia officinalis*, *Rhodiola rosea*, and *Ginkgo biloba* unripe seeds, where 50 µg/ml was used. For the dichloromethane extracts different concentrations were used depending on the solubility of the extracts: 100 µg/ml for *Ginkgo biloba* nuts, *Berberis bealei*, *Panax notoginseng*, *Phellodendron* spp., and *Alpinia oxyphylla*; 50 µg/ml for *Alpinia galanga*, *Coptis* spp., *Epimedium* spp., *Evodia rutaecarpa*, *Rosa laevigata*, *Sanguisorba officinalis*, *Ginkgo biloba* leaves and unripe seeds; 25 µg/ml for *Capsella bursa-pastoris*, *Centella asiatica*, *Glycyrrhiza uralensis*, *Magnolia officinalis*, *Paeonia* spp., *Punica granatum*, *Scutellaria baicalensis*, *Panax ginseng*, and *Rhodiola rosea*; 10 µg/ml for *Areca catechu*, *Cnidium monnieri*, *Polygonum multiflorum*, *Verbena officinalis*, and *Eleutherococcus senticosus*. As a positive control the worms were treated with 100 µg/ml EGCG, which has been shown to be effective in this assay (Abbas & Wink, 2010). The negative controls were

treated with the respective amount of solvent, maximum 1 % as final concentration. Additionally, the compound thioflavin T (ThT), that has been shown to decrease the number of Aβ aggregates in *C. elegans* by Alavez *et al.* (2011), was tested.

For *G. uralensis* and the pure compounds GA, glycyrrhizic acid monoammonium (GAA), GRA, LG, and ILG dose-dependence curves were prepered by testing different concentrations of these substances. The *G. uralensis* extract was tested in a range from 0 to 500 µg/ml, the pure compounds from 0 to 100 µg/ml. This experiment was repeated several times at different time points with the *G. uralensis* extract.

On day 6 after hatching the worms were collected and fixed in 4 % PFA in 1 × PBS over night. Then the PFA was removed and the worms were permeabilized in a solution containing 1 % Triton® X-100 and 5 % β-mercaptoethanol in 125 mM Tris-buffer for 24 h at 37 °C. The permeabilization solution was removed by washing with 1 × PBS twice. The permeabilized worms were transferred in a well of a 8-chamber microscope slide coated with poly-L-lysin and air-dried. The Aβ aggregates were stained with 0.0125 % thioflavin S (ThS) solution in 50 % ethanol for 1 minute, the excess stain was washed away with 50 % ethanol, 2 × 1 minute. The Aβ aggregates were quantified on fluorescence microscope Ni-E (excitation wavelength 480 nm, emission wavelength 525 nm) by counting the aggregates in the head region of 25 worms per sample.

2.2.11 Paralysis assay in CL4176

The paralysis assay was described by Dostal & Link (2010) as a method for assaying Aβ toxicity. For this assay the strains CL4176 and CL802 are used. CL4176 expresses human Aβ peptide in its muscle cells, similar to CL2006 (section 2.2.10), but the expression is temperature inducible and the paralysis phenotype is more penetrative than in the latter strain. The temperature sensitivity is accomplished via a mutation in the smg-1 (suppressor with morphogenetic effect on genitalia) gene and a long 3' untranslated region on the messenger ribonucleic acid (mRNA) of the Aβ minigene. SMG-1 is a key component of the non-sense mediated mRNA decay (NMD) pathway. At 16 °C it functions normally despite the mutation and the Aβ mRNA is degraded. At temperatures higher then 20 °C the SMG-1 protein loses its function and so in this strain Aβ can be expressed. The control strain CL802 has the temperature sensitive mutation in the smg-1 gene and the roller transfection marker but no transgenic Aβ.

Eggs from the worms of the strains CL4176 and CL802 were isolated and placed on small NGM plates containing the test substances and *E. coli* OP50. In this assay the used concentration for the methanol extract of *A. catechu* and the control EGCG was 100 µg/ml. The methanol extract of *G. uralensis* was used in concentrations of 200 and 500 µg/ml, for the water extract of *G. uralensis* and methanol extract of *A. oxyphylla* a concentration of 200 µg/ml was used. For the pure substances GA, GRA, LG, and ILG the concentration was 50 µg/ml. The dichloromethane extract of *C. acaulis*, the residual extract without Carlina oxide, and Carlina oxide were tested in concentrations of 25 and 50 µg/ml each. As a negative control the respective amount of methanol or water was used.

The worms were cultivated at 16 °C for 36 h. Then the temperature was risen to 25 °C. After 24 h the worms on each plate were counted, and paralysed worms were scored under stereomicroscope every 2 h for 12 h in total. Worms were counted as paralysed when they failed to react to a touch of platinum wire or only moved their heads creating a halo in the bacterial lawn.

2.2.12 Chemotaxis assay in CL2355

CL2355 worms have a similar genotype as CL4176 but with pan-neuronal expression of the transgene. The Aβ in this strain interferes mostly with behaviours like chemotaxis, enhanced slowing response or egg laying. In chemotaxis assay these worms fail to respond to the attractant benzaldehyde. The control strain PD8120 has the temperature sensitive mutation but no transgene, and therefore it has no behavioural impairments.

CL2355 and PD8120 worms were raised from eggs in S-medium at 16 °C for 36 h. Then the temperature was risen to 23 °C and kept there for 36 h. 24 h after experiment start the test substances GA, GRA, LG, and ILG in a concentration of 50 µg/ml, or the methanol extract of *G. uralensis* in concentrations of 200 or 500 µg/ml were added to the medium. As positive control EGCG in a concentration of 100 µg/ml was used, as negative control 1 % methanol or water. After 72 h in total the worms were washed twice with M9 buffer and placed in the middle of a 100 mm test plate. The test agar was prepared without NaCl, tryptone, and cholesterol to avoid undesired chemotactic effects. 1 µl of 1 M sodium azide in water was added to two spots on opposite sides of the plate. To one side 1 µl of ethanol and to the other side 1 µl of 0.1 % benzaldehyde in ethanol was added. The plates were incubated at 23 °C for 1 h. Afterwards the number of worms was counted on both sides in 2 cm radius of the benzaldehyde and ethanol spots. The chemotaxis index (CI) was calculated with following formula:

$$CI = \frac{\text{worms on the side of benzaldehyde} - \text{worms on the side of ethanol}}{\text{total number of worms on the plate}}$$

2.2.13 Serotonin sensitivity assay in CL2355

C. elegans can take up exogenous serotonin, and it can trigger physiological effects like the enhanced slowing response. The CL2355 worms are reported to be hypersensitive to serotonin due to the transgenic Aβ expression in the nervous system (Wu *et al.*, 2006). The control strain PD8120 shows a wildtype reaction.

CL2355 and PD8120 worms were raised from eggs in S-medium at 16 °C for 36 h. Then the temperature was risen to 23 °C and kept there for 36 h. 24 h after experiment start the test substances GA, GRA, LG, and ILG in a concentration of 50 µg/ml, or the methanol extract of *G. uralensis* in concentrations of 200 or 500 µg/ml were added to the medium. The dichloromethane extract of *C. acaulis* was tested in concentrations of 25, 10, and 5 µg/ml, the residual extract without Carlina oxide with 25 µg/ml and Carlina oxide with 10 µg/ml. Additionally, 200 µg/ml ascorbic acid was tested. As positive control EGCG in a concentration of 100 µg/ml was used, as negative control 1 % methanol or water.

After 36 h at 23 °C the worms were washed twice with M9 buffer to remove all of the bacteria. They were placed in 1 mM serotonin solution in M9 buffer and the relative number of active worms was determined after 5 min.

2.2.14 Heat shock protein expression

Small HSPs are molecular chaperones that are expressed under stress conditions. Here low doses of the pro-oxidant juglone are used to induce oxidative stress. The worm strain TJ375 with the transgenic construct *hsp-16.2::GFP* was used to visualize oxidative stress. Treatment with an antioxidant, that can reduce the oxidative stress induced by juglone, leads to decreased expression of HSP-16.2 and therefore lower fluorescence in this strain.

Eggs of TJ375 worms were raised in S-medium at 20 °C for 5 days. On day 1 after hatching the test substances GA, GRA, LG, and ILG in a concentration of 50 µg/ml, the methanol

extract of *G. uralensis* in concentrations of 200 or 500 µg/ml, the dichloromethane extract of *C. acaulis*, 50, 10, or 5 µg/ml, residual *Carlina* extract without Carlina oxide or Carlina oxide, 25, 10, or 5 µg/ml each, were added. As positive controls EGCG (100 µg/ml) and ascorbic acid in concentrations of 100, 200, and 500 µg/ml were used, as negative controls 1 % methanol or water.

On day 4 after hatching 20 µM juglone in ethanol were added. 24 h after adding juglone the worms were collected in an Eppendorf tube, the medium was removed, and the worms were washed with 1 × PBS. After removing the PBS, 20 µl 10 mM sodium azide in PBS was added to the worms, they were transferred to an object slide and covered with a cover slip. The worms were observed on flourescence microscope Ni-E at excitation wavelength 480 nm, emission wavelength 525 nm. Pictures were taken with the DS-Qi1Mc black and white camera of 25 worms per sample. For the experiments with *C. acaulis* BZ9000 from Keyence was used. The fluorescence intensity in the head region was measured using ImageJ.

2.2.15 Survival assay

Eggs of wildtype (N2) worms were cultivated in S-medium at 20 °C. On day 1 after hatching the test substances GA, GRA, LG, and ILG in a concentration of 50 µg/ml, or the methanol extract of *G. uralensis* in concentrations of 200 or 500 µg/ml were added to the medium. As positive controls EGCG (100 µg/ml) and ascorbic acid (200 µg/ml) were used, as negative controls 1 % methanol or water. On day 3 after hatching 80 µM juglone was added—a dose that usually kills the worms. 24 h after that the survivors were counted.

2.2.16 DAF-16 delocalisation

DAF-16 is a transcription factor that triggers the expression of many stress related genes. The strain TJ356 carries the transgenic construct *daf-16::GFP*, which makes it possible to monitor the stress level in *C. elegans*. Under stress the DAF-16::GFP protein delocalises from cytoplasm into the nuclei, which then become visible under microscope as fluorescing dots (Henderson & Johnson, 2001).

Eggs of TJ356 worms were cultivated in S-medium at 20 °C. On day 1 after hatching the larvae were treated with the test substances for one hour. GA, GRA, LG, and ILG in a concentration of 50 µg/ml, the methanol extract of *G. uralensis* in concentrations of 200 or 500 µg/ml, the dichloromethane extract of *C. acaulis*, residual *Carlina* extract without Carlina oxide, and Carlina oxide, 25, 10, and 5 µg/ml each, or EGCG, 100 µg/ml (dissolved in water or in methanol), were tested. As positive controls 20 µM juglone or a heat shock at 37 °C for 15 min were used, as negative controls 1 % methanol or water. Then the worms were collected, anesthesized with 10 mM sodium azide and transferred to an object slide. 25 worms per sample were evaluated under fluorescence microscope, excitation wavelength 480 nm, emission wavelength 525 nm. Worms with green fluorescent protein (GFP) in the cell nuclei were counted as positive, worms with fluorescence spread through the whole body as negative.

2.2.17 Lifespan assay

In the lifespan assay the effect of methanol extract from *G. uralensis* and the pure substances GA, GRA, LG, and ILG on longevity of the worms was tested. The test was carried out with the wildtype (N2) and the Aβ expressing strain CL2006 to see possible effects related to Aβ. Eggs were placed in S-medium and cultivated at 20 °C. On day 2 after hatching

methanolic *G. uralensis* extract in concentrations of 50, 200, or 500 µg/ml or the pure substances in a concentration of 50 µg/ml were added to the worms. On day 3 100 worms per sample were transferred to a new culture dish with fresh S-medium and same treatment as before. From days 3–10 after hatching the worms were transferred to a new dish every day to exclude larvae from the experiment. Afterwards the worms were transferred every second day. While transferring, the number of living and dead animals, that did not move when touched with a platinum wire, was counted. Worms that had died from bagging or unnatural causes like wounds taken from the platinum wire were censored.

2.2.18 Statistical analysis

Statistics were carried out using the StatView software. Treatments were compared to their respective solvent controls in an unpaired Students t-test. Results were considered significant when $p < 0.05$. In case of the dose-dependence curves one-way analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) test with significance level $p < 0.05$ were used. For survival analysis in paralysis and lifespan assays a life table analysis was performed. The determined median values were compared to the respective solvent controls using an unpaired Students t-test with $p < 0.05$ as significance level. For the determination of relative mean effective concentration (EC_{50}) (Sebaugh, 2011) values in the $DPPH^{\bullet}$ assay the measured values were normalized, so that the highest gave 100 % effectiveness and the lowest 0 %. Then standard curve analysis in the software SigmaPlot was used. All experiments, except the lifespan assay, were repeated at least 3 times.

3 Results

3.1 Screening the TCM extracts against Aβ aggregation

To find plants that can interact with Aβ, a screening of extracts of TCM plants was conducted. 25 probably interesting plants were identified through literature research. Metha-

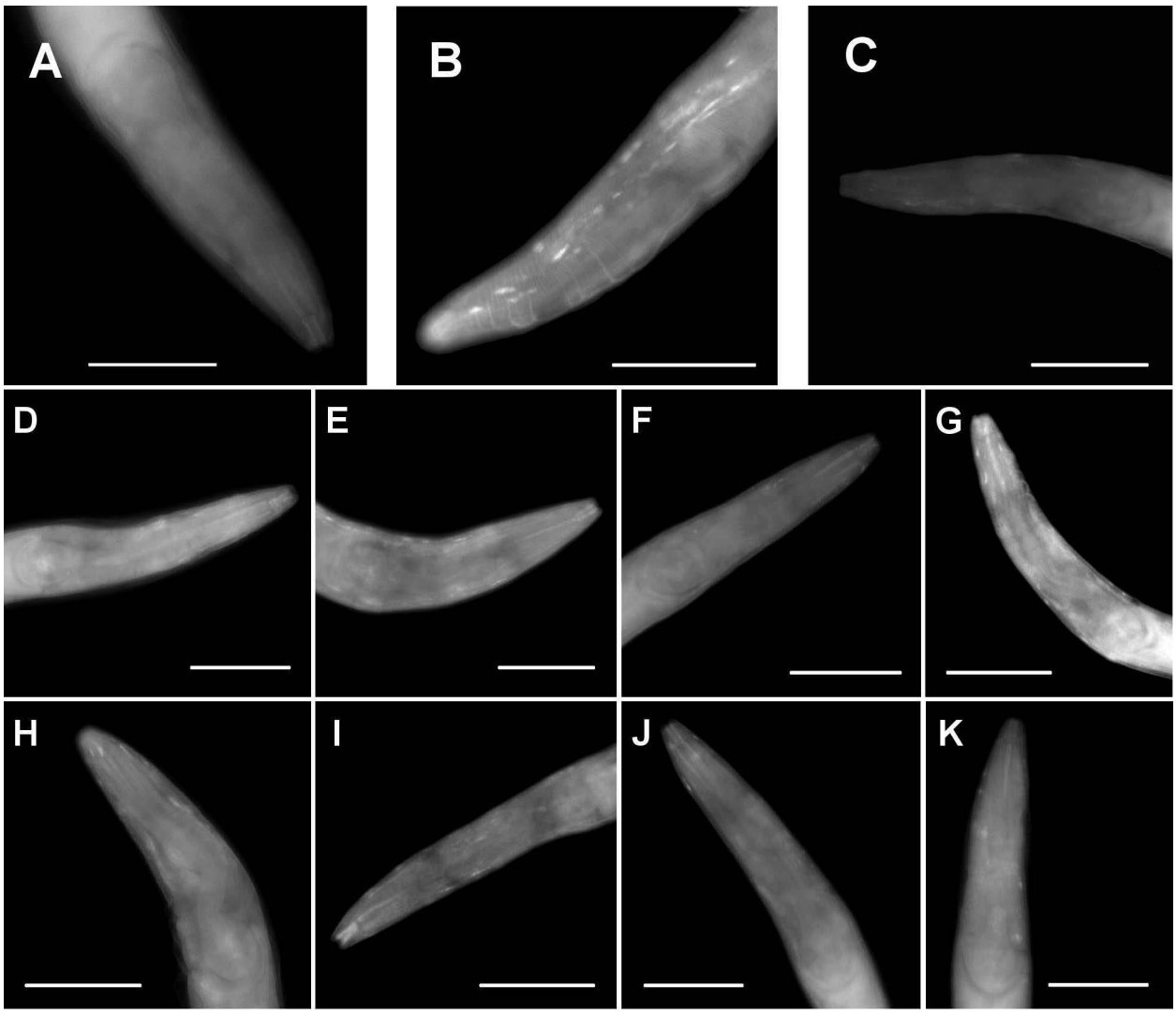

Figure 3.1: Microscopic fluorescence pictures of *C. elegans* stained with thioflavin S. A) wildtype worm; B–K) CL2006 worms treated with 1 % methanol (B), 100 µg/ml EGCG (C), methanol extract of *Areca catechu* (D), methanol extract of *Alpinia oxyphylla* (E), methanol extract of *Cnidium monnieri* (F), methanol extract of *Glycyrrhiza uralensis* (G), 10 µg/ml dichloromethane extract of *Areca catechu* (H), 100 µg/ml dichloromethane extract of *Alpinia oxyphylla* (I), 10 µg/ml dichloromethane extract of *Cnidium monnieri* (J), or 25 µg/ml dichloromethane extract of *Glycyrrhiza uralensis* (K). The scale bar indicates 50 µm. Pictures were taken with DS-Qi1Mc black and white camera on a Ni-E fluorescence microscope using FITC filter and 40× magnification.

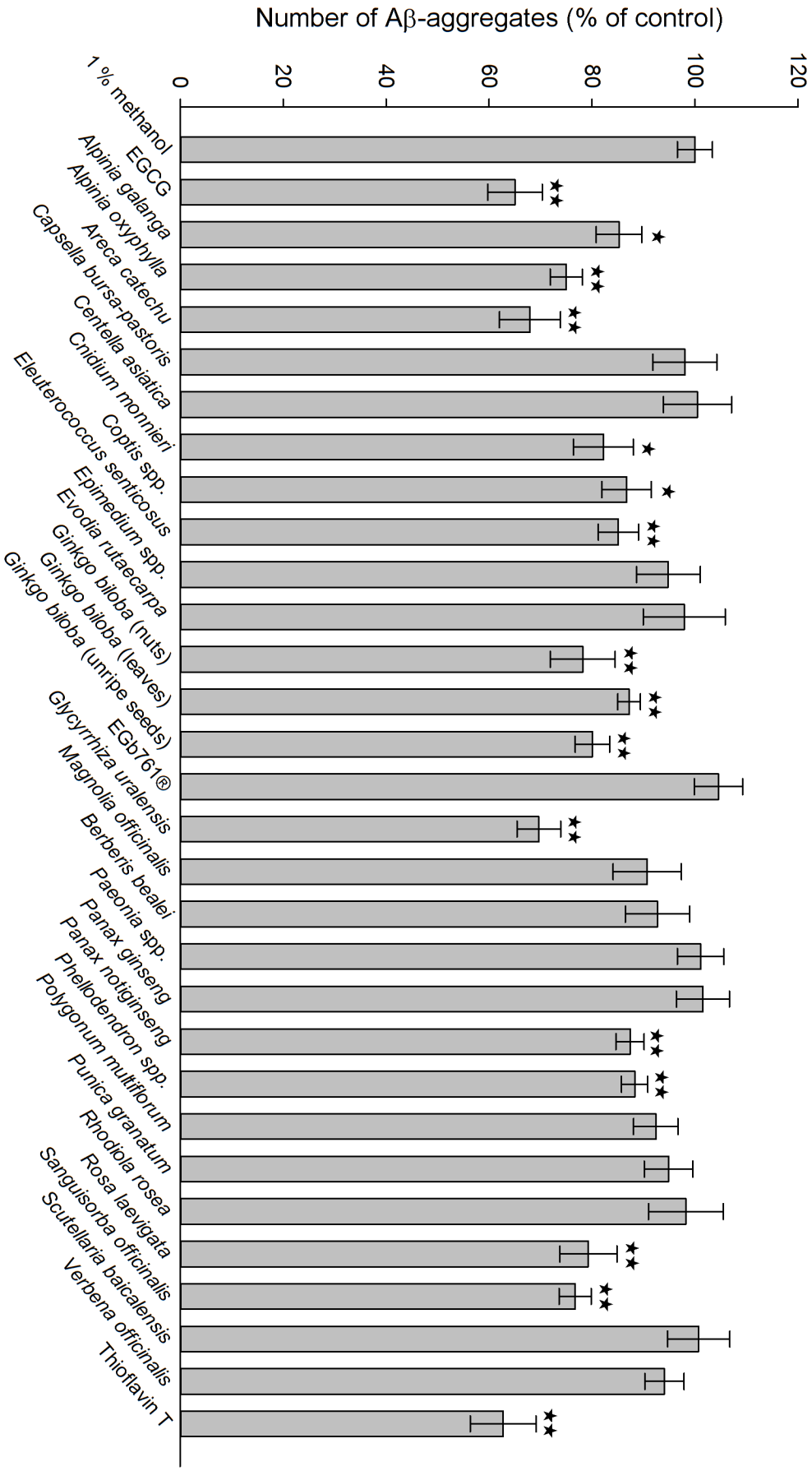

Figure 3.2: Effects of the methanolic TCM extracts on the Aβ aggregation in CL2006. 100 µg/ml of the TCM extracts were used, except for *Magnolia officinalis*, *Rhodiola rosea*, and *Ginkgo biloba* unripe seeds, where 50 µg/ml was used. 1 % Methanol was used as control. * $p < 0.05$; ** $p < 0.01$ as compared to control.

nol and dichloromethane extracts of these plants were tested on their ability to inhibit Aβ aggregation (see section 2.2.10). Aβ aggregates are only seen in the transgenic CL2006 worms but not in the wildtype (Fig. 3.1 A & B). EGCG, that was used as positive control, decreased the number of Aβ aggregates by 35 % ($p < 0.01$) (Fig. 3.1 C & Fig. 3.2), which is comparable to the results of Abbas & Wink (2010). Some methanol extracts like *Areca catechu* (32 % decrease, $p < 0.01$) and *Glycyrrhiza uralensis* (30 % decrease, $p < 0.01$) showed a similar effect. Several other extracts were effective to a lesser extent: *Alpinia oxyphylla* with 25 % decrease ($p < 0.01$), *Sanguisorba officinalis* with 23 % ($p < 0.01$), *Rosa laevigata* 21 % ($p < 0.01$), *Cnidium monnieri* 18 % ($p = 0.01$), *Eleutherococcus senticosus* 15 % ($p < 0.01$), *Alpinia galanga* 15 % ($p = 0.01$), *Panax notoginseng* 13 % ($p < 0.01$), *Coptis* spp. 13 % ($p = 0.03$), *phellodendron* spp. 12 % ($p < 0.01$). All tested methanol extracts of *Ginkgo biloba* were effective (22 % decrease in the number of Aβ aggregates for nuts, 20 % for unripe seeds, and 13 % for leaves, respectively, $p < 0.01$), but the special extract EGb761® had no effect. Thioflavin T decreased the number of plaques by 37 % ($p < 0.01$). Representative pictures for some treatments are shown in Figure 3.1 and the results are summarised in Figure 3.2.

The dichloromethane extracts were poorly soluble in the aqueous culture medium, so that for some extracts smaller concentrations had to be used (Fig. 3.4). Even so, the extracts of *Areca catechu* and *Cnidium monnieri* had a strong effect decreasing the Aβ aggregates by 32 % and 35 %, respectively ($p < 0.01$). *Polygonum multiflorum* (21 % decrease, $p = 0.01$) and *Eleutherococcus senticosus* (15 % decrease, $p = 0.04$) also showed an effect in the 10 µg/ml treatment group (Fig. 3.4 A). Among the extracts used in the concentration of 25 µg/ml (Fig. 3.4 B) only *Magnolia officinalis* had an significant effect (14 % decrease, $p = 0.02$). Raw seeds of *Ginkgo biloba* could significantly decrease (by 20 %, $p < 0.01$) the number of Aβ aggregates at 50 µg/ml (Fig. 3.4 C), whereas *Sanguisorba officinalis* increased the number by 14 % ($p = 0.04$). *Panax notoginseng* and *Alpinia oxyphylla* showed an effect at a concentration of 100 µg/ml (Fig. 3.4 D), decreasing Aβ aggregation by 17 % and 14 %, respectively ($p < 0.01$).

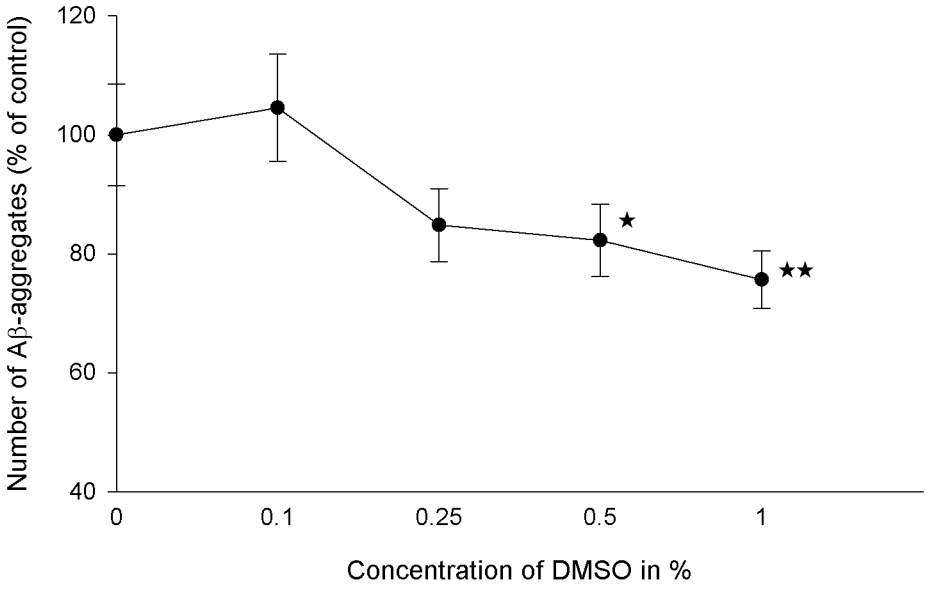

Figure 3.3: Effect of DMSO on the Aβ aggregation in CL2006. * $p < 0.05$; ** $p < 0.01$ as compared to untreated control.

All values were compared to respective solvent controls. For methanol no dose-dependent effect was observed but DMSO, that was used to solve the dichloromethane extracts, had

an effect on the number of Aβ aggregates as shown in Figure 3.3. Concentrations above 0.25 % were significantly different from untreated worms with 18 % decrease in number of Aβ aggregates for 0.5 % DMSO ($p = 0.02$) and 24 % for 1 % DMSO ($p < 0.01$).

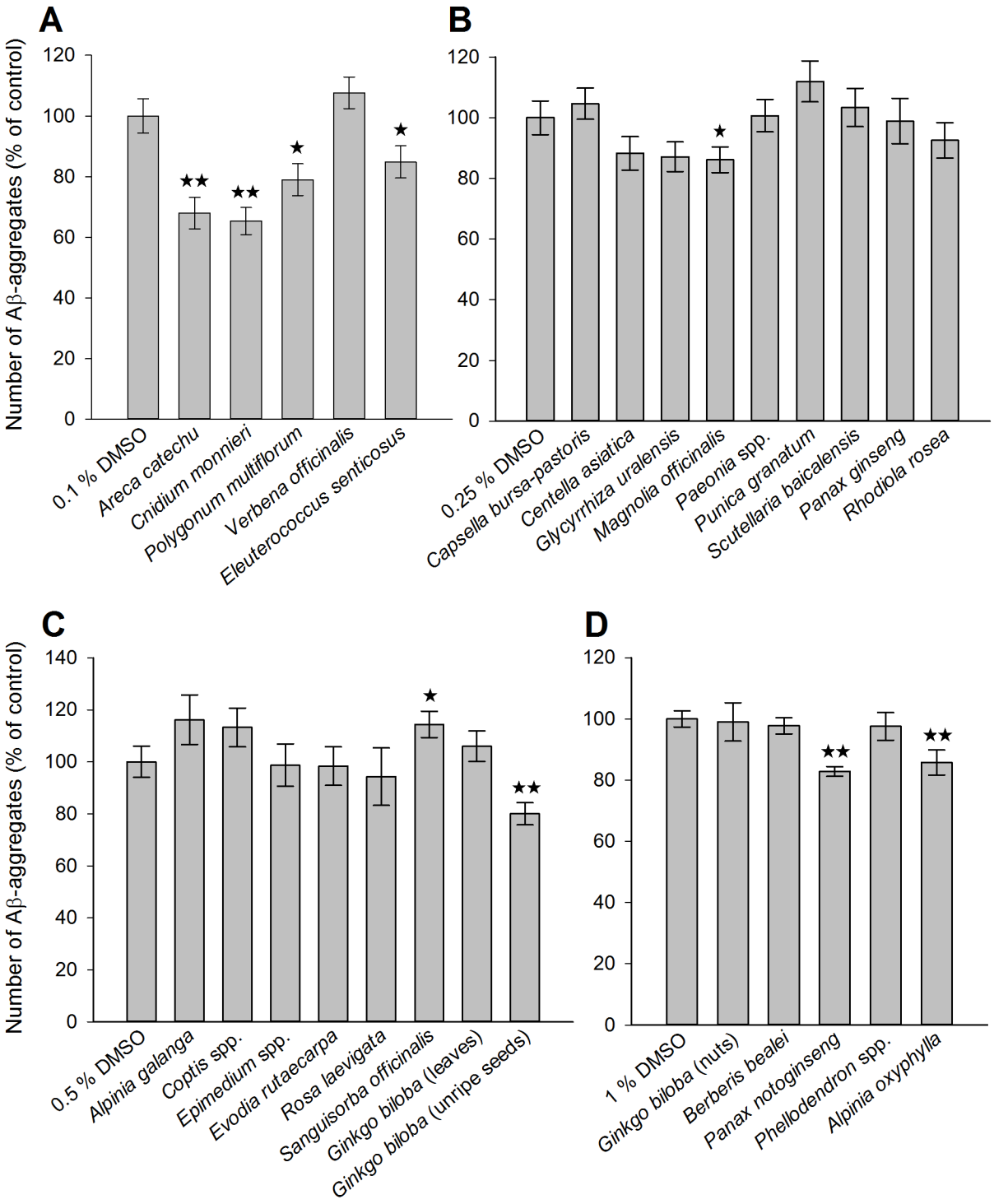

Figure 3.4: Effects of the dichloromethane extracts of TCM plants on the Aβ aggregation in CL2006. A) 10 µg/ml of the TCM extracts solved in DMSO were used; B) 25 µg/ml of the TCM extracts solved in DMSO were used; C) 50 µg/ml of the TCM extracts solved in DMSO were used; D) 100 µg/ml of the TCM extracts solved in DMSO were used. * $p < 0.05$; ** $p < 0.01$ as compared to respective controls.

3.2 *Areca catechu*

3.2.1 Dose-dependence

Dose-dependence of the Aβ aggregation reducing effect in CL2006 was tested for *A. catechu* in the concentration range from 0 to 100 µg/ml. The two highest tested concentrations of 75 µg/ml and 100 µg/ml significantly reduced the number of aggregates by 17 % ($p = 0.03$) and 32 % ($p < 0.01$), respectively (Fig. 3.5). Higher concentrations could not be tested, because they proved to be toxic for the worms.

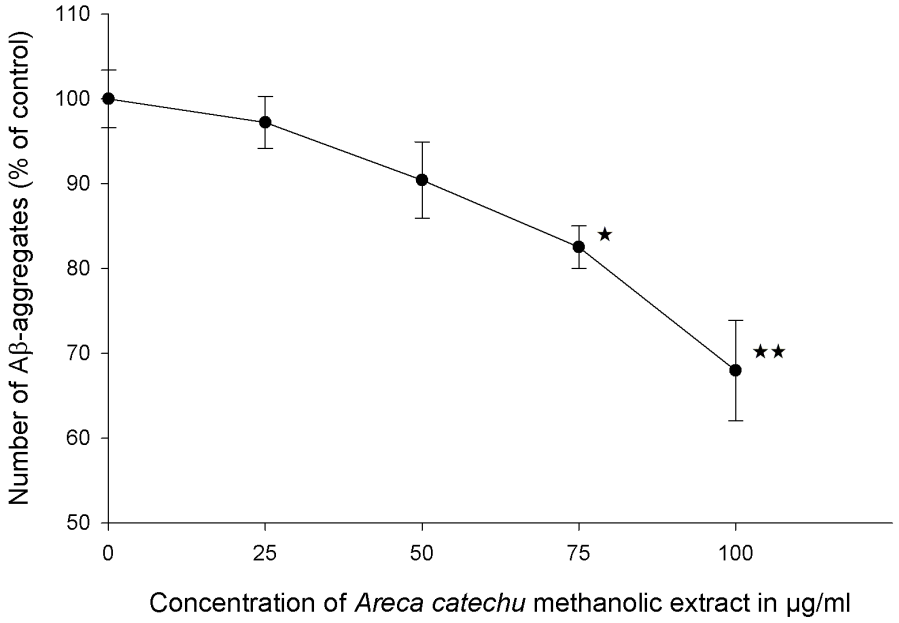

Figure 3.5: Dose-dependence of the effect of methanol extract from *A. catechu* on Aβ aggregation in CL2006. * $p < 0.05$; ** $p < 0.01$ as compared to control.

3.2.2 Paralysis assay

Instead of counteracting Aβ toxicity, the methanol extract of *A. catechu* proved to be rather toxic itself in the paralysis assay (see section 2.2.11). 100 µg/ml, that were not problematic in the Aβ aggregation assay, shortened the mean time of paralysis (PT_{50}) significantly by 1.4 h ($p < 0.01$) (Fig. 3.6).

3.3 *Alpinia oxyphylla*

3.3.1 Paralysis assay

The paralysis assay with *A. oxyphylla* was carried out by Mariam Baalbaki, a pharmacy student at Heidelberg University, during her laboratory practical at IPMB, Department of Biology, as described in section 2.2.11. No effect on the Aβ toxicity can be seen for the methanol extract (Fig. 3.7). Also the positive control, EGCG (solved in methanol), showed no significant difference compared to the solvent control, although there is a slight tendency to a delay (0.4 h difference in the PT_{50} values).

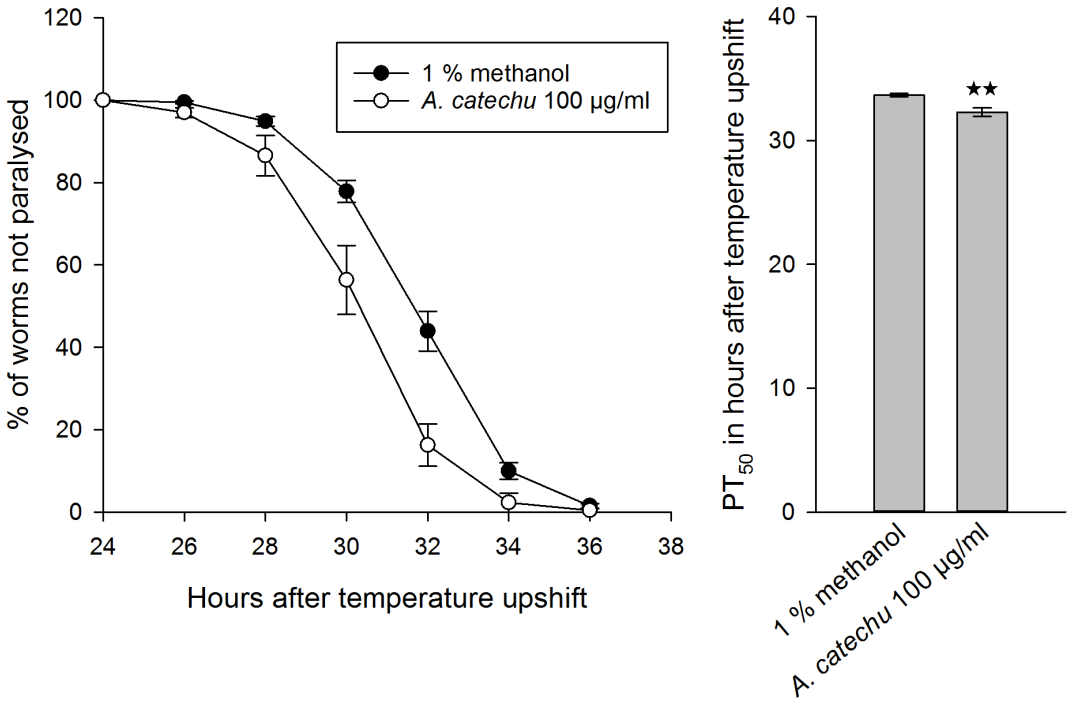

Figure 3.6: Results of the paralysis assay for methanol extract of *A. catechu*. ** $p < 0.01$ as compared to control.

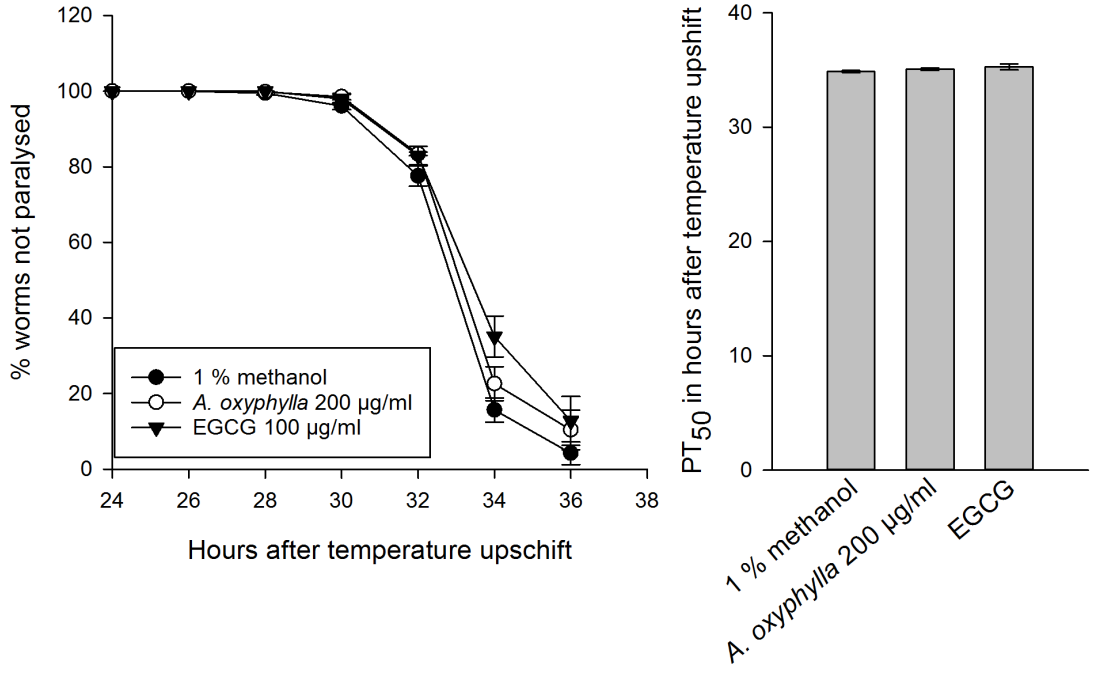

Figure 3.7: Results of the paralysis assay for methanol extract of *A. oxyphylla*.

3.4 *Glycyrrhiza uralensis*

3.4.1 Identification of the species

In TCM three different species of *Glycyrrhiza* can be medically used under the drug name gān cǎo. Therefore, it had to be determined from which species the material used in this work, P6873, originated (see section 2.2.6).

The estimated evolutionary divergence (Tab. 3.1) between all three species is quite low. The highest values are 1.2–1.5 % between the outgroup *G. astragalina* and the other species. The ITS sequence is very similar between *G. glabra* and *G. inflata* with 0.05 % divergence. The same value was observed between *G. uralensis* and the sample P6873, whereas it has a divergence of 0.4 % to the other two species. The same divergence was observed between *G. uralensis* and the other species *G. glabra* and *G. inflata* (0.4 and 0.3 %, respectively). This suggests that the plant material used here comes from *Glycyrrhiza uralensis*.

The obtained sequence is available in GenBank, number KM588200.

Table 3.1: Estimates of evolutionary divergence over sequence pairs between the *Glycyrrhiza* species calculated from the *ITS* sequence data retrieved from NCBI database and compared to the sample P6873. Data was calculated by MEGA software using the Maximum Composite Likelihood model.

P6873				
G. uralensis	0.0005			
G. glabra	0.0044	0.0037		
G. inflata	0.0036	0.0032	0.0005	
G. astragalina	0.0128	0.0124	0.0147	0.0141

3.4.2 Phytochemical analysis of the extracts

HPLC analysis

The three extracts of *G. uralensis* were analysed via HPLC (see section 2.2.3) and compared to the standards GA, GRA, LG, and ILG (Fig. 3.8). The LG standard eluates first with a RT of 17.1 min and can be best detected at 276 nm. GA follows with the RT 19.1 min and is detected at 254 nm as is GRA that has the highest RT with 27.5 min. ILG has a RT of 19.5 min and shows a strong absorption at 365 nm. LG and ILG both also absorb at 310 nm in contrast to the other standards. The wavelenghts 231 and 520 nm did not contribute any additional information, therefore they are not included in the analysis.

The methanol and water extracts give a similar chromatogram, but the intensity of the peaks is higher in methanol extract. Both extracts contain peaks that can be assigned to LG, GA, and ILG based on their RT and absorption behaviour. Only traces of GRA were found in these extracts. The dichloromethane extract has a completely different chromatogram. Only trace amounts of the standard substances could be detected in this extract.

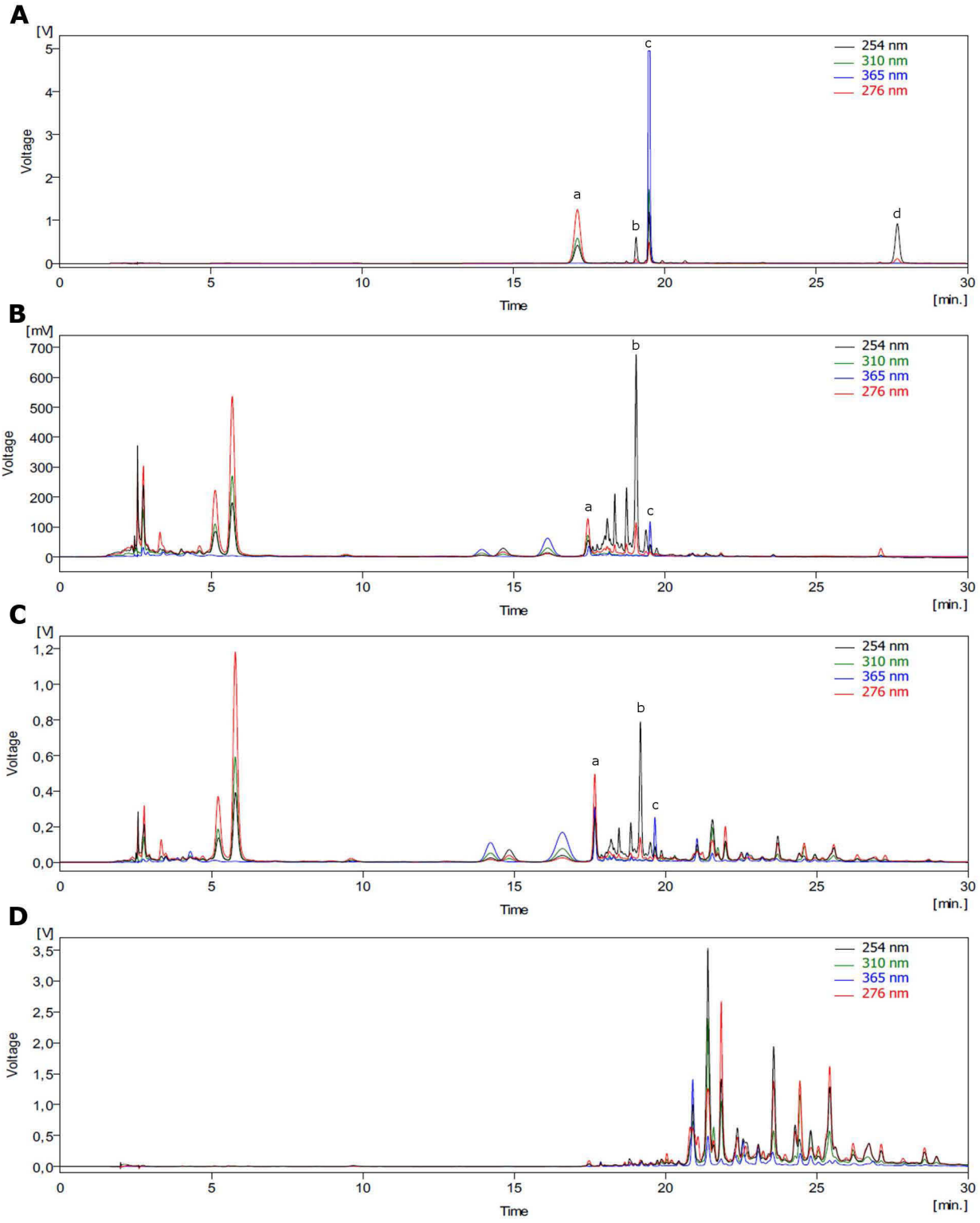

Figure 3.8: HPLC analysis of *G. uralensis* extracts. A) Standards: 50 µg/ml LG (a), 50 µg/ml GA (b), 25 µg/ml ILG (c) and 50 µg/ml GRA (d); B) water extract, 1 mg/ml; C) methanol extract, 1 mg/ml; D) dichloromethane extract, 1 mg/ml.

Quantification of selected compounds

The content of GA, GRA, LG, and ILG in the *G. uralensis* extract was quantified via HPLC using an external standard. The results are summarised in Table 3.2. The highest amounts of GA, LG, and ILG were found in the methanol extract. All the amounts are quite low, the highest being 64.2 µg/ml for GA in the methanol extract.

Table 3.2: Quantification of the compounds detected in *G. uralensis* extracts. LG: liquiritigenin; ILG: isoliquiritigenin; GA: glycyrrhizic acid; GRA: glycyrrhetinic acid. Values given in µg/ml.

	LG	**ILG**	**GA**	**GRA**
Water extract	3.0	0.9	54.1	0.2
Methanol extract	9.4	1.8	64.2	0.4
Dichlormethane extract	2.9	1.5	4.6	4.1

LC-MS/MS analysis

LC-MS/MS analysis was carried out for water and methanol extracts of *G. uralensis* (see section 2.2.4). Figure 3.9 shows the HPLC profiles detected at 254 and 365 nm. In methanol extract 18 peaks were detected, in water extract 15 of them could be found. Some more lipophilic substances seem to be missing. Also, the intensity of the detected peaks is lower in the water extract, suggesting that most of the substances are better soluble in methanol. The detected peaks were further analysed via MS (Fig. 3.10). The results are summarised in Table 3.3. Some of the substances could be identified by comparing the MS and MS/MS spectra with published data (Farag *et al.*, 2012; Montoro *et al.*, 2011).

The first two peaks eluting at 5.4 and 5.8 min, respectively, could be assigned to liquiritin apioside (Tab. 3.3 **1**) and liquiritin (Tab. 3.3 **2**). The MS spectrum for liquiritin apioside (Fig. 3.11 **1**) shows a negatively charged molecular ion ($[M-H^+]^-$) peak with the m/z = 549, a peak with m/z = 1099 which corresponds to a dimer, and peaks with m/z = 617 and 662 which are probably adducts of the $[M-H^+]^-$ with formic acid and sodium ($[M-2H^+ + Na^+ + HCOOH]^-$, m/z = 617; $[M-2H^+ + Na^+ + HCOOH + {}^{\bullet}COOH]^{\bullet -}$, m/z = 662). Also, a small peak corresponding to a sodium adduct can be detected ($[M-2H^+ + Na^+]^-$, m/z = 571). The fragmentation via MS/MS splits the molecule in liquiritin (m/z = 417) and further in its aglycon liquiritigenin (m/z = 255). The split off sugars could not be detected. Furthermore the molecule can be split in the middle, at the unconjugated ring, resulting in the fragments with m/z = 429 ($[M-C_7O_2H_5^+]^-$) and m/z = 297 due to additional loss of the apiose residue ($[M-C_7O_2H_4^+ - C_5O_4H_8]^-$). For liquiritin (Fig. 3.11 **2**) also a $[M-H^+]^-$ peak and a dimer (m/z = 835) were detected, accompanied by formic acid and sodium adducts ($[M-2H^+ + Na^+ + HCOOH]^-$, m/z = 485; $[M-3H^+ + 2\,Na^+ + 2\,HCOOH]^-$, m/z = 553). The MS/MS spectrum shows only one peak for the aglycon liquiritigenin.

The spectra for isoliquiritin apioside (Tab. 3.3 **3**) and isoliquiritin (Tab. 3.3 **4**) are similar to the ones of their isomers, but they can be distinguished by their absorption spectra. Isoliquiritigenin and its derivatives have a much stronger absorption at 365 nm than liquiritigenin derivatives (compare peaks **1** and **2** with **3** and **4** in Fig. 3.9). The MS spectrum for isoliquiritigenin apioside (Fig. 3.12 **3**) shows $[M-H^+]^-$ peak (m/z = 549) and the adducts with sodium (m/z = 571), formic acid ($[M-H^+ + HCOOH]^-$, m/z = 595) and combinations of both

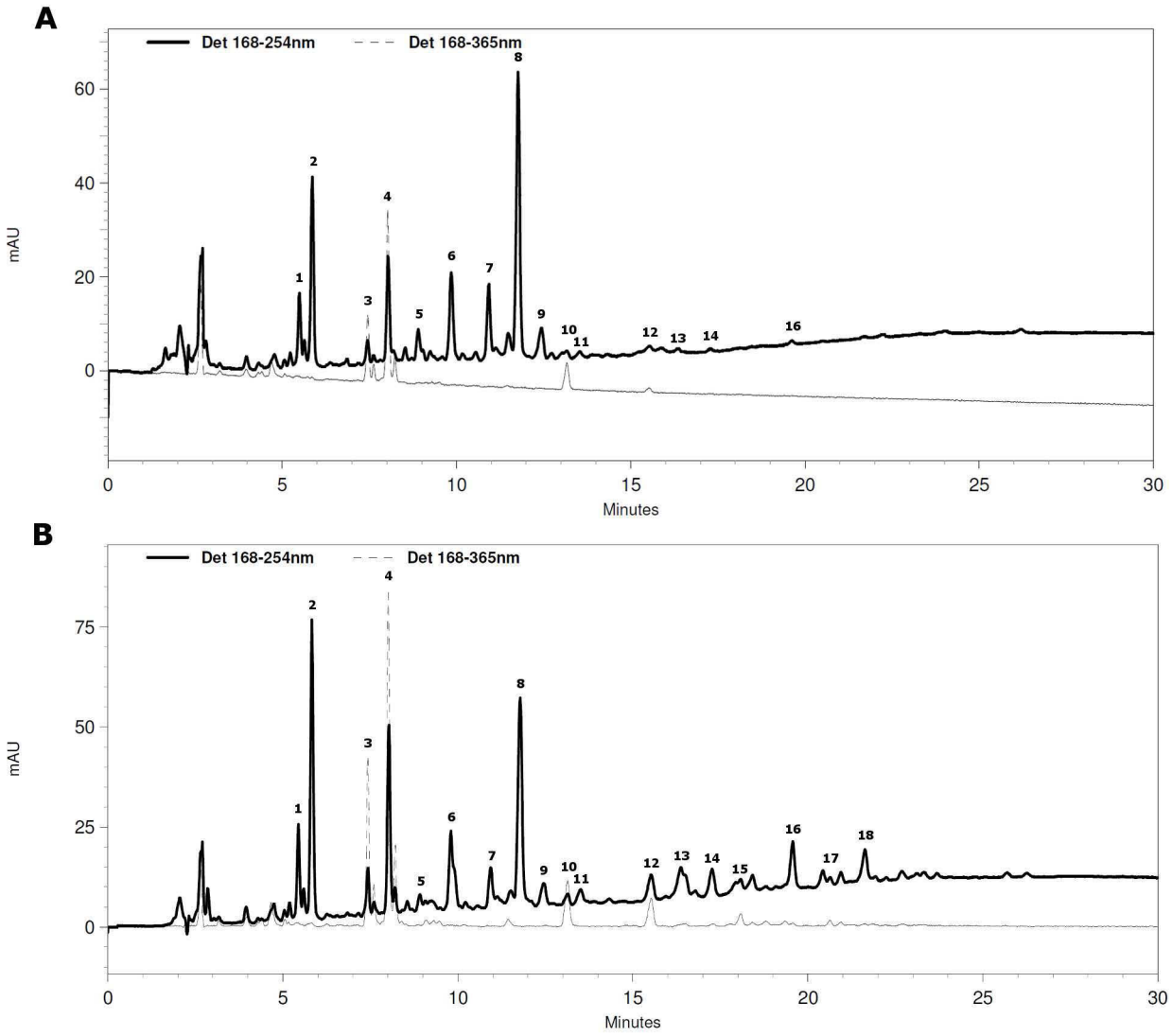

Figure 3.9: HPLC chromatograms of the LC-MS/MS runs. A) *G. uralensis* water extract, 1 mg/ml; B) *G. uralensis* methanol extract, 1 mg/ml. The peak numbers correspond to compounds described in Table 3.3.

(m/z = 662 and 617 as above). The peak with m/z = 662 is much higher for **3** than for **1**, probably because of the exposed double bond that can react with the formic acid radicals. The fragmentation pattern is the same as for liquiritin apioside, but the fragment with m/z = 429 is much less intense. This is probably due to the different structure at the site of fragmentation. For isoliquiritin the same adducts are observed as for liquiritin. The fragmentation results in the aglycon and the fragment without apiose as described above ($[M-C_7O_2H_5^+]^-$, m/z = 297). The absorption and fragmentation pattern of these four substances concur with those published by Farag *et al.* (2012).

The substances **5**–**11** seem to have a similar structure. Their retention times lie within 4 min, they have similar masses and fragmentation patterns, and all but number **10** absorb more light at 254 nm then at 365 nm (Tab. 3.3). Substance number **8** could be identified as glycyrrhizic acid by comparing its RT and mass to a standard. That suggests, that all substances in this group are saponins. The MS spectrum of glycyrrhizic acid (Fig. 3.13 **8**) shows $[M-H^+]^-$ peak with m/z = 821, sodium adducts ($[M-2H^++Na^+]^-$, m/z = 843; $[M-3H^++2\,Na^+]^-$, m/z = 865), adducts with formic acid ($[M-H^++2\,^\bullet COOH]^-$, m/z = 911), combinations of

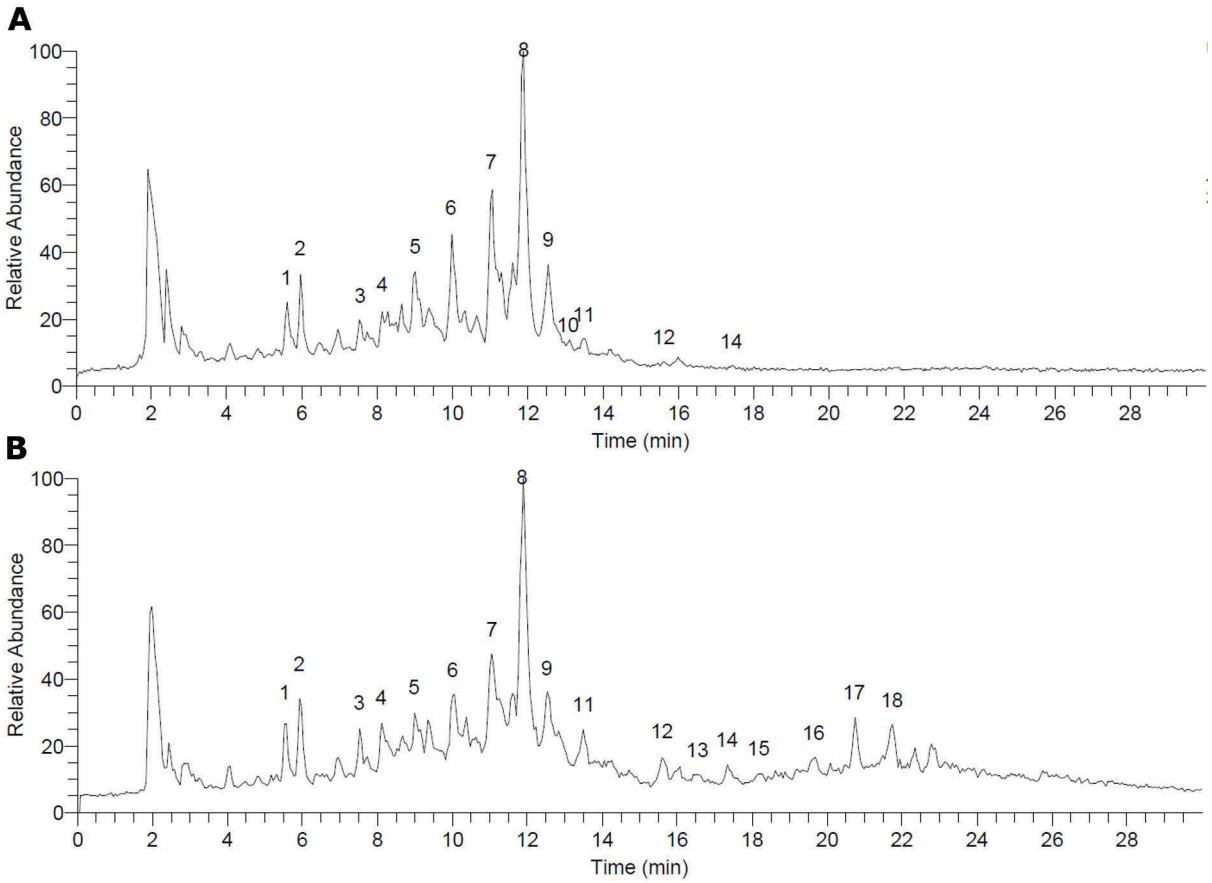

Figure 3.10: Total ion current in MS, ESI negative. A) *G. uralensis* water extract, 1 mg/ml; B) *G. uralensis* methanol extract, 1 mg/ml. The peak numbers correspond to compounds described in Table 3.3.

both kinds of adducts ($[M-2H^+ + Na^+ + {}^\bullet COOH]^{\bullet -}$, m/z = 888; $[M-2H^+ + Na^+ + 2\,{}^\bullet COOH]^-$, m/z = 933; $[M-2H^+ + Na^+ + 2\,{}^\bullet COOH^+ HCOOH]^-$, m/z = 979), and a dimer peak (m/z = 1643). The MS/MS spectrum is dominated by a fragment with m/z = 351, which corresponds to the negatively charged ion of the two glucuronic acid molecules ($C_{12}O_{12}H_{15}^-$), the sugars linked to the aglycon of glycyrrhizic acid. This fragment can be found in all detected saponins, suggesting they all have the same sugar moiety. The same fragment was reported in all detected saponins by Farag *et al.* (2012). The remaining triterpene fragment is poorly ionized, there is only a very small peak with m/z = 469. The other visible peaks belong to $[M-H^+-H_2O]^-$ (m/z = 803), $[M-H^+-H_2O-CO_2]^-$ (m/z = 759) and the fragment without one glucuronic acid moiety ($[M-C_6O_6H_9^+]^-$, m/z = 645).

Substance **9** has the same molecular mass as glycyrrhizic acid, a very similar RT and almost identical fragmentation pattern (Tab. 3.3 & Fig. 3.13 **9**). Based on this data it could be licorice saponin H2, a stereoisomer of glycyrrhizic acid. The fragments correspond to those reported by Montoro *et al.* (2011). The fragment with m/z = 583, that was not present in the MS/MS spectrum of glycyrrhizic acid, belongs to $[M-H_2O-CO_2-C_6O_6H_9^+]^-$.

Substance number **5** could be identified as licorice saponin A3 by comparison of the data with Farag *et al.* (2012) and Montoro *et al.* (2011) (Tab. 3.3 **5**). It has a $[M-H^+]^-$ with m/z = 983 (Fig. 3.14 **5**) and adducts with sodium ($[M-2H^+ + Na^+]^-$, m/z = 1005; $[M-3H^+ + 2\,Na^+]^-$, m/z = 1027) and formic acid ($[M-2H^+ + Na^+ + HCOOH]^-$, m/z = 1051; $[M-3H^+ + 2\,Na^+ + HCOOH]^-$, m/z = 1073) can be seen in the MS spectrum as for the other substances. The MS/MS spectrum shows a base peak with m/z = 821 that corresponds to glycyrrhizic acid

Table 3.3: Tentative identification of the compounds detected in methanol and water extracts of *G. uralensis* via LC-MS/MS and comparison with the data of (a) Farag *et al.* (2012) and (b) Montoro *et al.* (2011).

Nr.	RT in min	λ_{max}	$[M-H]^-$	MS/MS	Tentative identification
1	5.4	198, 214, 277, 310	549	255, 429	liquiritin apioside [a]
2	5.8	199, 215, 277, 311	417	255	liquiritin [a]
3	7.4	206, 362	549	255, 417	isoliquiritin apioside [a]
4	8.0	202, 361	417	255	isoliquiritin [a]
5	8.9	214	983	821, 803, 351	licorice saponin A3 [a,b]
6	9.8	199, 215, 276, 311	879	351, 861, 703, 817	22-acetoxyglycyrrhizin
7	10.9	215	837	351, 661, 819, 775	licorice saponin G2 [a,b]
8	11.8	218, 250	821	351, 803, 645, 759	glycyrrhizic acid [a]
9	12.4	217	821	351, 803, 645, 759	licorice saponin H2 [b]
10	13.1	218, 370	821	351, 510, 645, 777	unknown (saponin)
11	13.5	218	823	351, 805, 647	licorice saponin J2 [a,b]
12	15.5	218, 348	367	298, 337	glycycoumarin
13	16.3	219	353	298, 284	unknown
14	17.3	221, 287	353	297, 285	unknown
15	18.1	221, 347	365	307, 295, 350	glycyrol
16	19.6	221	351	283, 265, 307	gancaonin G or A
17	20.6	219, 292	423	229, 193	unknown
18	21.6	222, 268	421	284	isoanguston A

(loss of the glucose moiety) and the fragments with m/z = 803 and m/z = 351 that were already discussed for glycyrrhizic acid.

The substance **6** is probably 22-acetoxyglycyrrhizin (Tab. 3.3 **6**). It has a $[M-H^+]^-$ with m/z = 879 (Fig. 3.14 **6**) that is by 58 units higher than the m/z for glycyrrhizic acid. This mass difference corresponds to a acetoxy group (CH_2COO^-). Other peaks in this spectrum correspond to sodium and formic acid adducts ($[M-2H^+ + Na^+]^-$, m/z = 901; $[M-3H^+ + 2\,Na^+]^-$, m/z = 923; $[M-2H^+ + Na^+ + {}^\bullet COOH]^{\bullet -}$, m/z = 946; $[M-3H^+ + 2\,Na^+ + HCOOH]^-$, m/z = 969; $[M-2H^+ + Na^+ + 2\,{}^\bullet COOH]^-$, m/z = 991). Also the fragments in MS/MS have masses that correspond to those of glycyrrhizic acid with a mass difference of 58 (861, 817, 703), except the glucuronic acid peak at 351. Farag *et al.* (2012) observed a fragment with m/z = 383 for 22-acetoxyglycyrrhizin, but this cannot be confirmed here. Still the data suggests that substance 6 is 22-acetoxyglycyrrhizin.

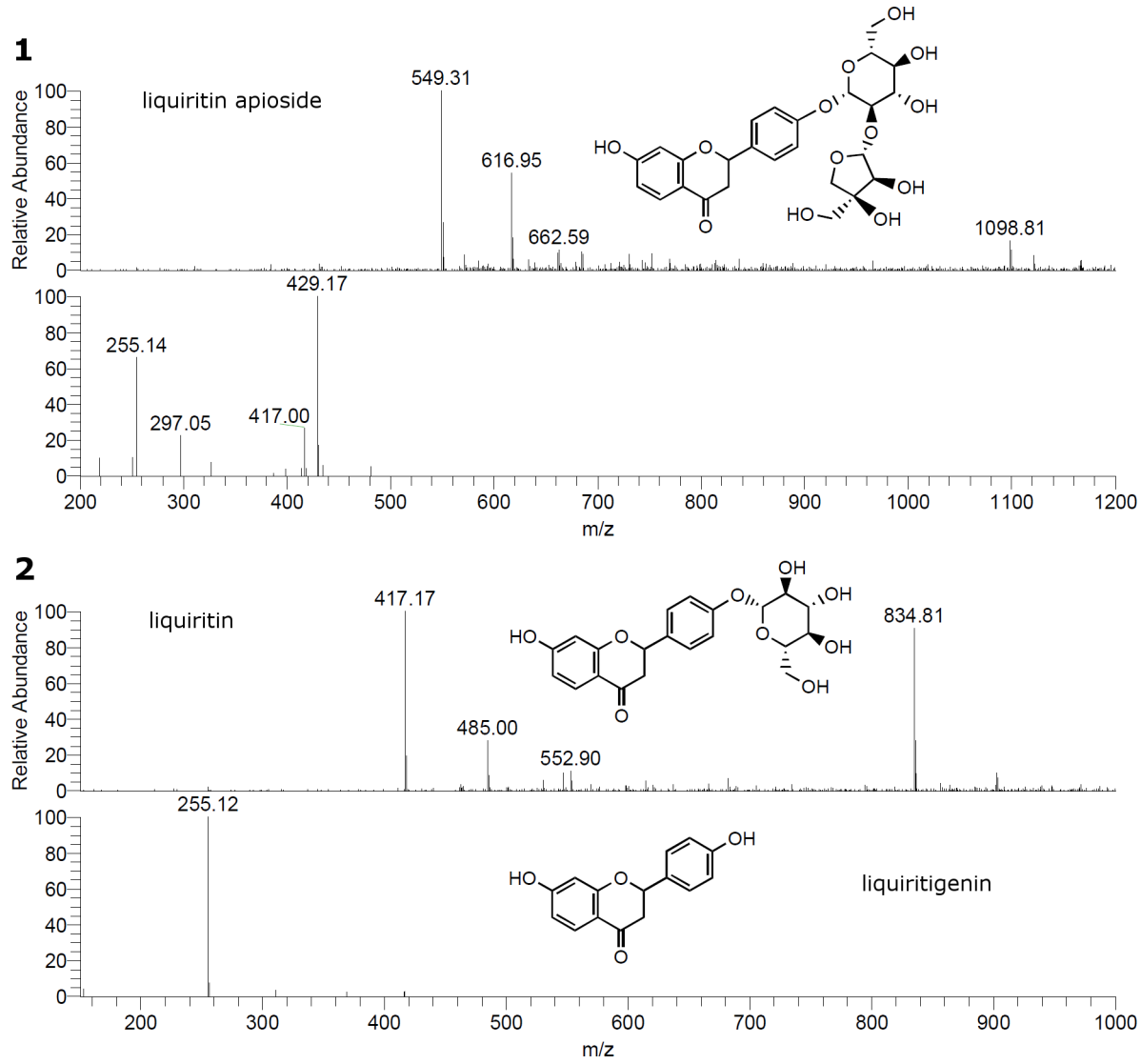

Figure 3.11: MS (upper part) and MS/MS (lower part) spectra for liquiritigenin derivatives in *G. uralensis* extract. The numbers correspond to compounds described in Table 3.3. All spectra are baseline substracted.

The next substance that could be identified is licorice saponin G2 (Tab. 3.3 **7**). In comparison to glycyrrhizic acid one of its methyl groups is hydroxylated and therefore the molecular mass rises by 16. In accordance to that, the $[M-H^+]^-$ seen in the MS spectrum has a m/z = 837 (Fig. 3.15 **7**). Again, adducts with sodium ($[M-2H^++Na^+]^-$, m/z = 859; $[M-3H^++2\,Na^+]^-$, m/z = 881) and formic acid ($[M-3H^++2\,Na^++HCOOH]^-$, m/z = 927; $[M-4H^++3\,Na^++2\,HCOOH]-$, m/z = 995) can be observed. The fragmentation pattern is the same as for glycyrrhizic acid with a sugar ion peak (m/z = 351), $[M-H^+-H_2O]^-$ (m/z = 819), $[M-H^+-H_2O-CO_2]^-$ (m/z = 775), and the fragment without one glucuronic acid moiety ($[M-C_6O_6H_9^+]^-$, m/z = 661). Farag *et al.* (2012) reported a fragment with m/z = 289 that might belong to $[C_{12}O_{12}H_{15}^--H_2O-CO_2]^-$. A small peak with that m/z was detected, too, as well as the fragment with m/z = 643 (corresponding to $[M-C_6O_6H_9^+-H_2O]^-$) reported by Montoro *et al.* (2011).

The last saponin, substance **11** (Tab. 3.3 **11**), is licorice saponin J2 which was also observed by Farag *et al.* (2012) and Montoro *et al.* (2011). Its MS spectrum (Fig. 3.15 **11**) shows a $[M-H^+]^-$ peak with m/z = 823, sodium ($[M-2H^++Na^+]^-$, m/z = 845; $[M-3H^++2\,Na^+]^-$,

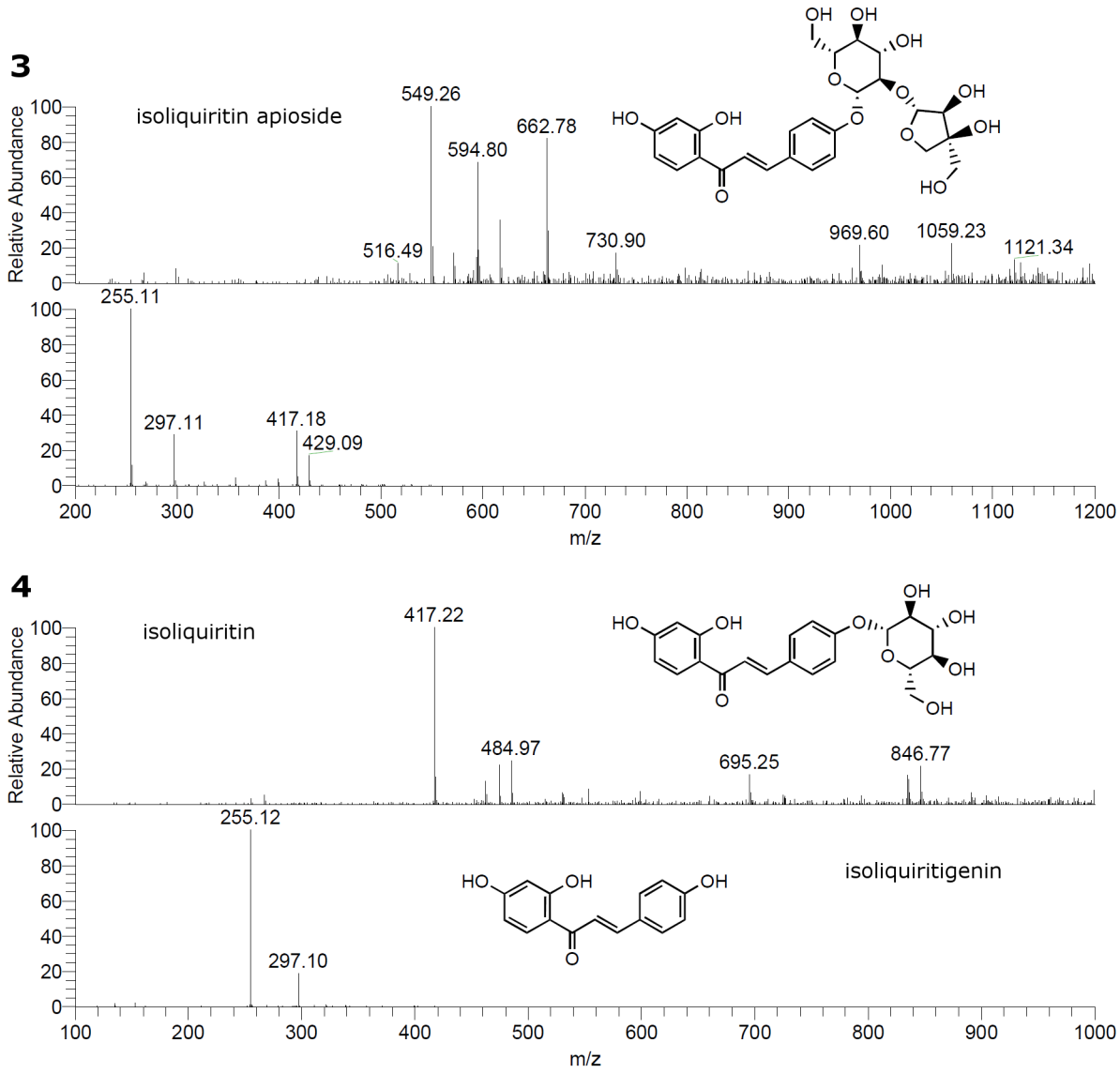

Figure 3.12: MS (upper part) and MS/MS (lower part) spectra for isoliquiritigenin derivatives in *G. uralensis* extract. The numbers correspond to compounds described in Table 3.3. All spectra are baseline substracted.

m/z = 867) and formic acid adducts ($[M-H^+ + HCOOH]^-$, m/z = 868; $[M-2H^+ + 2\,^{\bullet}COOH]^-$, m/z = 913), and combination of both ($[M-3H^+ + 2\,Na^+ + 2\,^{\bullet}COOH]^-$, m/z = 957). The fragments in the MS/MS spectrum have a by 2 units higher m/z (805, 761, 647) than the respective fragments for glycyrrhizic acid due to the differences in the triterpene structure. The peak at 351 remains same.

Substance **10** is probably a saponin, too, but it could not be identified with the given data (Tab. 3.3 **10**). It has a $[M-H^+]^-$ with m/z = 821 (Fig. 3.15 **10**), the same as for glycyrrhizic acid. Also the fragments in MS/MS have corresponding masses. A peak with m/z = 645 was observed for glycyrrhizic acid, 777 is the mass of decarboxylated glycyrrhizic acid ($[M-H^+-CO_2]^-$) and was also observed for other saponins, although in very small amounts. Likewise, the glucuronic acid peak at 351 is present for the unknown substance, suggesting that the difference lies in the triterpene structure and not in the sugar moiety. The fragment with m/z = 510 is new, it cannot be explained without having further structural information. The absorption is different for this substance as well compared to other saponins. It has a

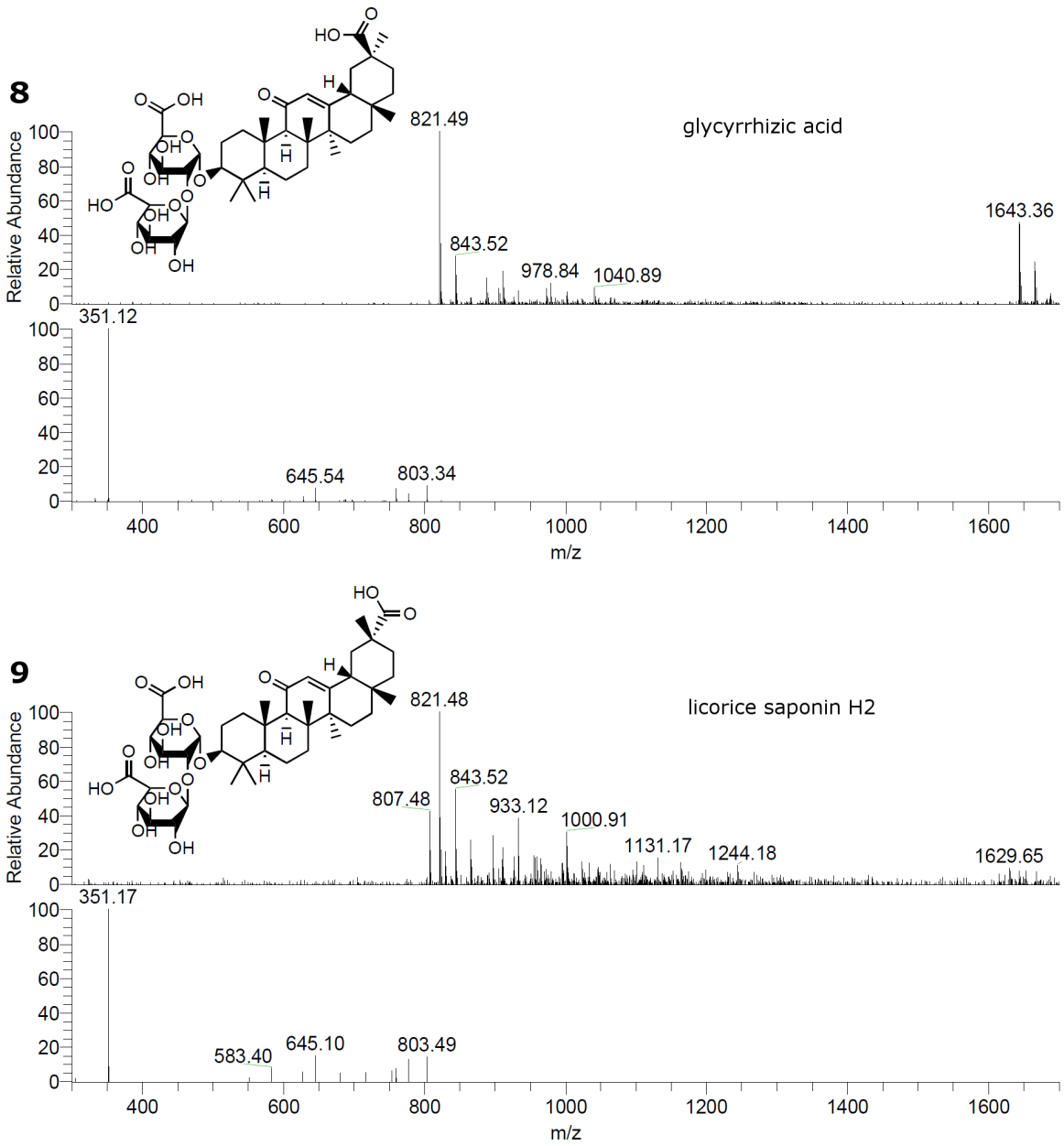

Figure 3.13: MS (upper part) and MS/MS (lower part) spectra for glycyrrhizic acid and licorice saponin H2. The numbers correspond to compounds described in Table 3.3. All spectra are baseline substracted.

strong absorption at 356 nm, pointing to a conjugated π-electron system.

Substance **12**, with the RT 15.5 min, probably belongs to glycycoumarin (Tab. 3.3 **12**). Its mass and absorption match earlier data (Farag *et al.*, 2012), although the fragmentation pattern is different. Still, the fragments observed can be explained using the molecular structure of glycycoumarin. The MS spectrum (Fig. 3.16 **12**) shows the $[M-H^+]^-$ peak with m/z = 367, an adduct with formic acid and sodium ($[M-2H^+ + Na^+ + HCOOH]^-$, m/z = 435), and a possible dimer (m/z = 734). The fragments arise from loss of the methoxy group ($[M-H^+ - {}^\bullet OCH_3 + H^\bullet]^-$, m/z = 337) or, to a lesser extent, only the methyl group ($[M-H^+ - {}^\bullet CH_3]^{\bullet -}$, m/z = 352), loss of carbon dioxide from the lactone group ($[M-H^+ - CO_2]^-$, m/z = 323), and loss of the isoprenyl group ($[M-H^+ - {}^\bullet C_5H_9]^{\bullet -}$, m/z = 298).

Substances **13** and **14** both have a $[M-H^+]^-$ with m/z = 353 and similar absorption (Tab. 3.3 **13** & **14**). The MS/MS fragments are similar but not identical (Fig. 3.17 **13** & **14**). Both have

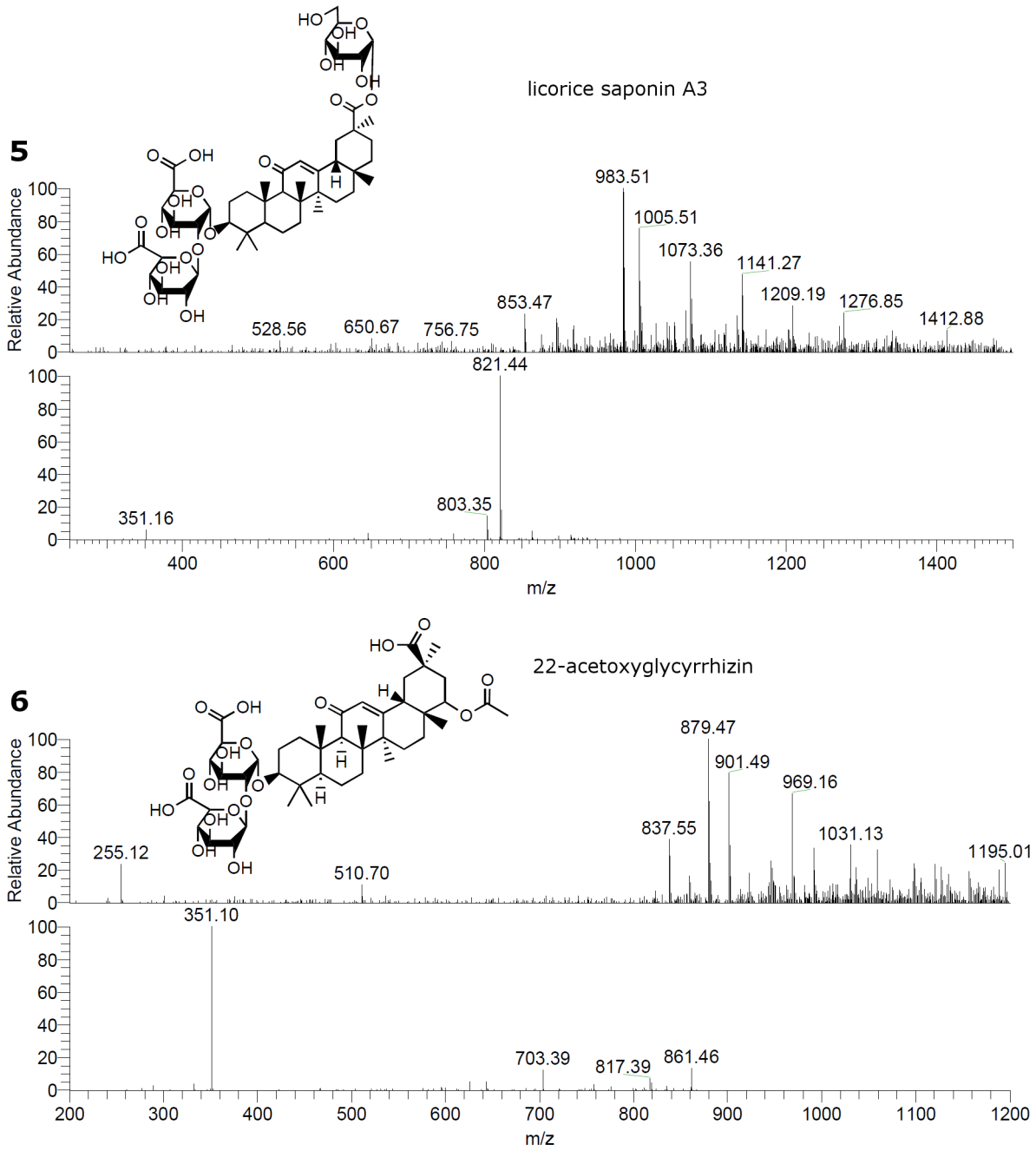

Figure 3.14: MS (upper part) and MS/MS (lower part) spectra for licorice saponin A3 and 22-acetoxyglycyrrhizin. The numbers correspond to compounds described in Table 3.3. All spectra are baseline substracted.

a small peak with m/z = 338 indicating the loss of a methyl group ($[M-H^+-{}^\bullet CH_3]^{\bullet-}$). A slightly higher peak appears with m/z = 298 for substance **13** and 297 for substance **14**, followed by m/z = 284 or 285, respectively. The masses 284 and 285 could indicate the loss of a isoprenyl group ($[M-H^+-{}^\bullet C_5H_9]^{\bullet-}$ or $[M-C_5H_9{}^+]^-$, respectively). The masses 298 and 297 could arise from a partitial loss of this group ($[M-H^+-{}^\bullet C_4H_7]^{\bullet-}$ and $[M-2H^+-C_4H_7{}^-]^-$, respectively). There are several substances with the corresponding molecular mass of 354, such as licochalcon D and G, licoflavonol, gancaonin I, dehydroglyasperin C, or glycybenzofuran. All of them have a isoprenyl group and all but licoflavonol have methoxy groups that might lose the methyl group. The first three substances all have the same conjugated π-electron system and absorb light at 365 nm, therefore these are probably not identical with

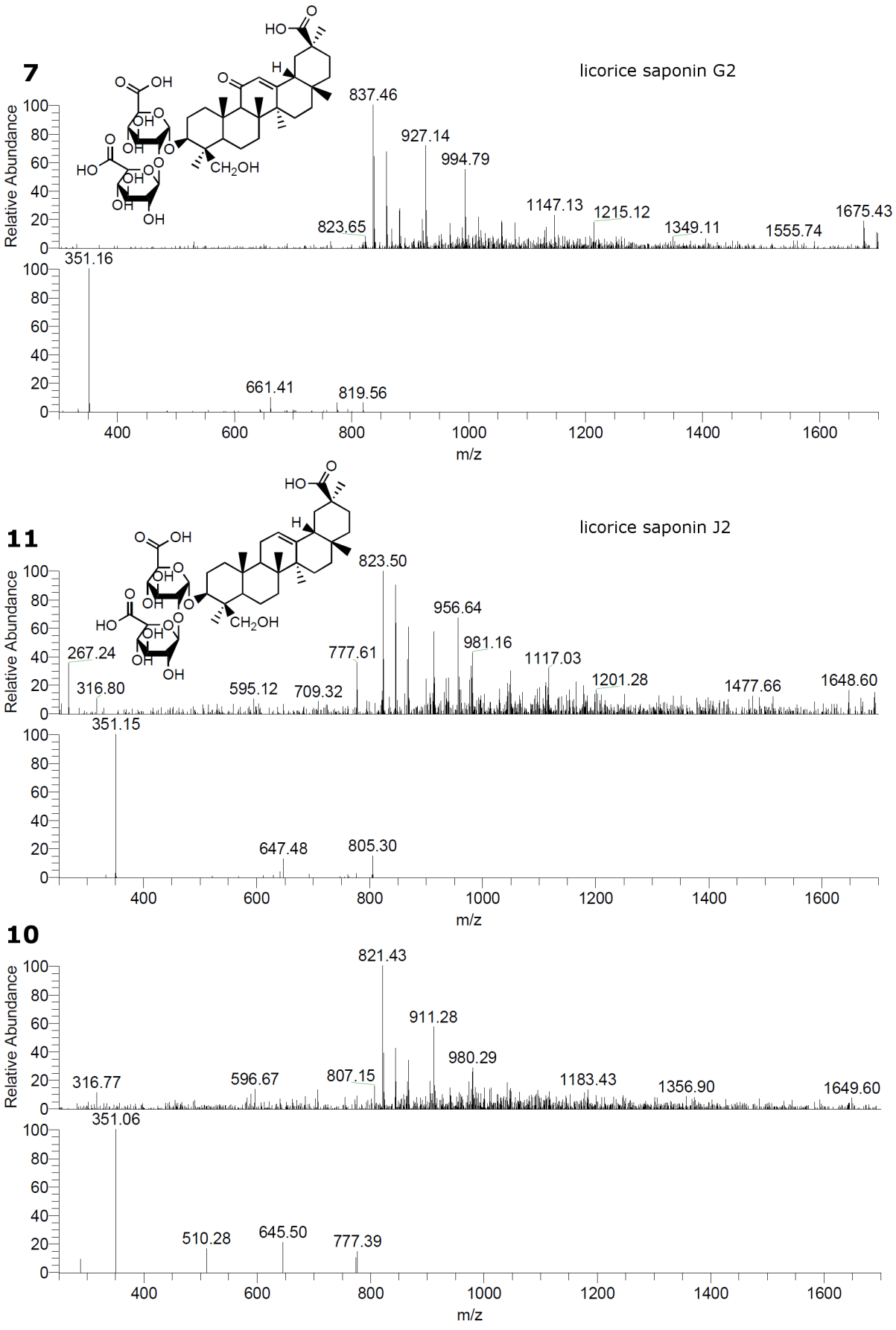

Figure 3.15: MS (upper part) and MS/MS (lower part) spectra for other saponins in *G. uralensis* extract. The numbers correspond to compounds described in Table 3.3. All spectra are baseline substracted.

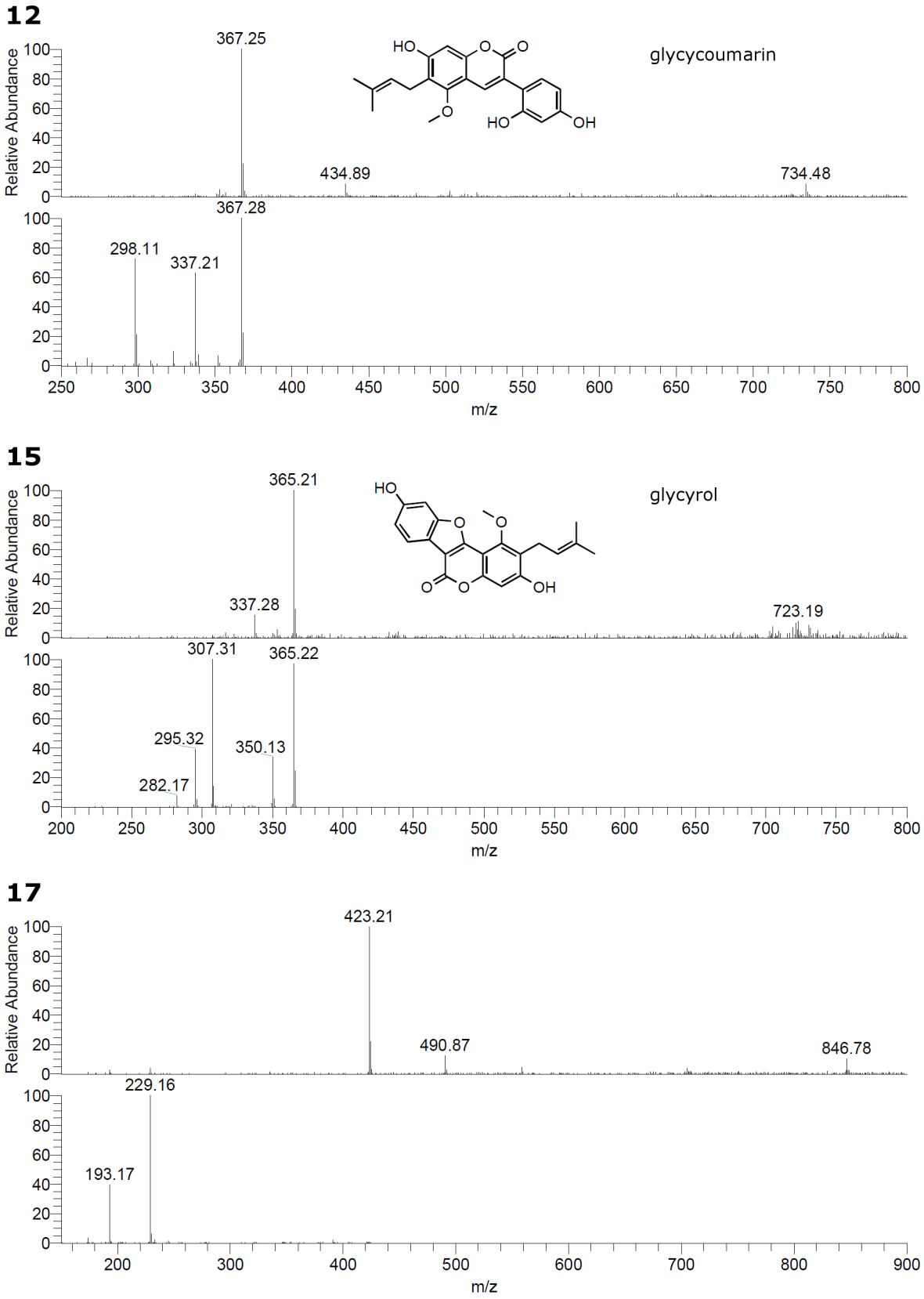

Figure 3.16: MS (upper part) and MS/MS (lower part) spectra for coumarins and an unknown substance in *G. uralensis* extract. The numbers correspond to compounds described in Table 3.3. All spectra are baseline substracted.

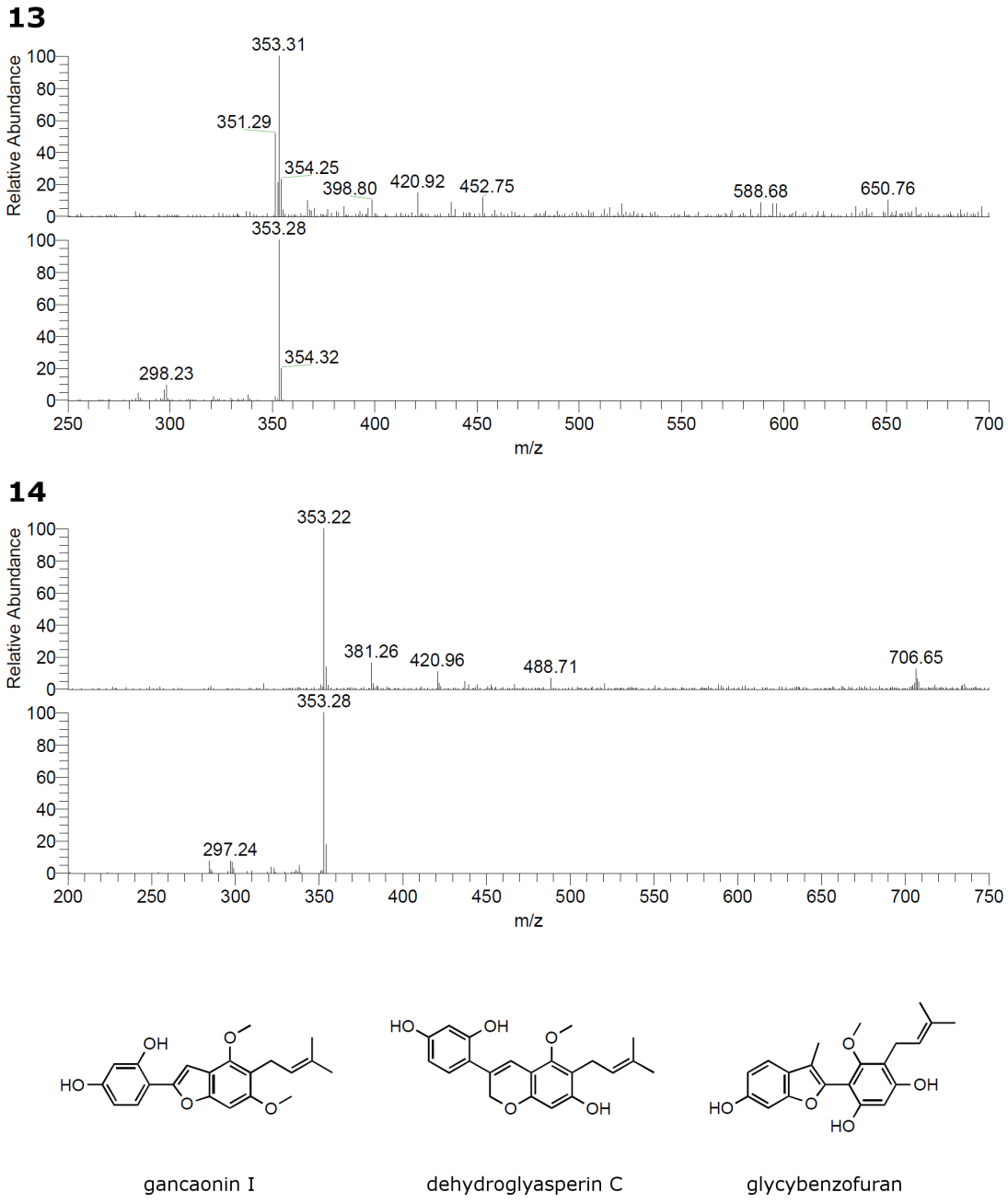

Figure 3.17: MS (upper part) and MS/MS (lower part) spectra for unknown substances in *G. uralensis* extract and some molecules with matching mass. The numbers correspond to compounds described in Table 3.3. All spectra are baseline substracted.

molecules detected here. Substances 13 and 14 could be gancaonin I, dehydroglyasperin C, glycybenzofuran, or similar prenylated methoxybenzofurans /-pyrans.

Substance **15** is probably glycyrol. The MS spectrum shows a $[M-H^+]^-$ peak with m/z = 365 (Fig. 3.16 & Tab. 3.3 **15**), which is fragmented into ions with m/z = 350 (loss of methyl group, $[M-H^+-{}^{\bullet}CH_3]^{\bullet-}$), 307 (additional loss of carbon dioxide, $[M-CH_3{}^+-CO_2]^-$), 295 (loss of a methyl and part of a isoprenyl group, $[M-H^+-{}^{\bullet}CH_3-{}^{\bullet}C_4H_7]^-$) and 282 (loss of a methyl and a isoprenyl group, $[M-CH_3{}^+-{}^{\bullet}C_5H_9]^{\bullet-}$). Glycyrol has a isoprenyl sidechain, a methoxy group able to split off the methyl group, and a lactone ring that can give rise to the carbon dioxide. Also, the observed absorption is consistent with the conjugated π-electron system of

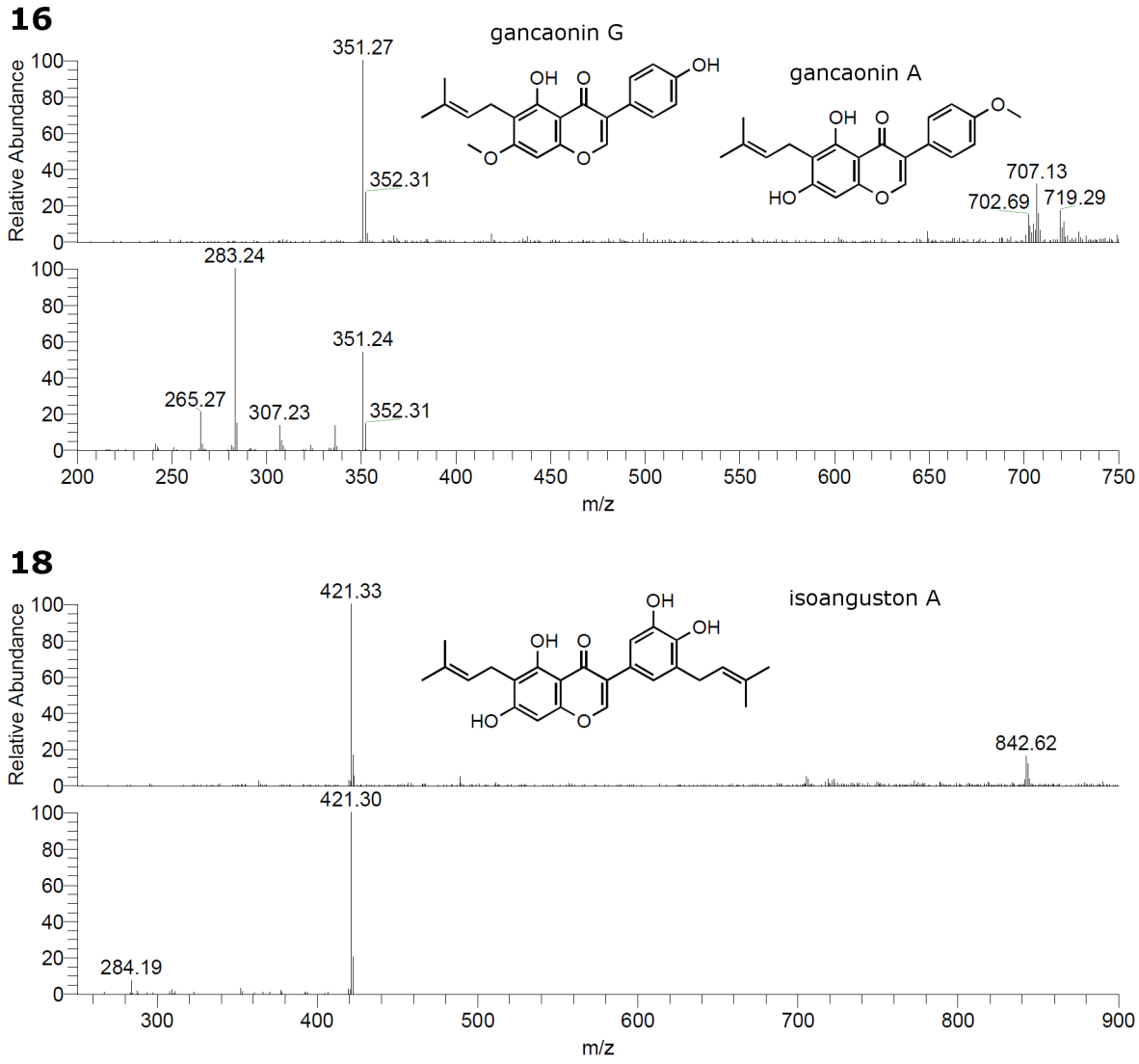

Figure 3.18: MS (upper part) and MS/MS (lower part) spectra for gancaonin and isoanguston A. The numbers correspond to compounds described in Table 3.3. All spectra are baseline substracted.

glycyrol. Montoro *et al.* (2011) reported this compound in *G. glabra*, although unfortunately no MS/MS data have been published.

Substance number **16** has a $[M-H^+]^-$ with m/z = 351 (Fig. 3.18 **16**), corresponding to a substance with a molecular mass of 352 like gancaonin A or G. The MS/MS fragments suggest a isoprenyl chain ($[M-C_5H_9^+]^-$, m/z = 283) and a methyl group ($[M-H^+-{}^{\bullet}CH_3]^{\bullet-}$, m/z = 336) that are present in both gancaonin isomers. The m/z = 265 suggest a loss of water, additionally to the loss of an isoprenyl group ($[M-C_5H_9^+-H_2O]^-$). The fragment with m/z = 307 could result from loss of three methyl groups: one of the methoxy group, the other two from the isoprenyl group, made possible by a ring formation between this group and the adjacent hydroxy group ($[M-C_3H_9^+]^-$). In case of gancaonin G there would be the oxygen radical of the methoxy group instead of a hydroxy group. That might facilitate the ring formation, making gancaonin G the more probable candidate for the identity of substance **16**, but there is no definite proof for either of the isomers.

The substance **17** could not be identified. It has a $[M-H^+]^-$ with m/z = 423 and the spectrum shows also a dimer with m/z = 847 (Fig. 3.16 **17**). This molecule is fragmented into

two parts with m/z = 193 and 229. Farag *et al.* (2012) reported a substance with m/z = 423 that was identified as kanzonol H, but the reported MS/MS fragments do not match with substance **17**.

Substance **18** has a $[M-H^+]^-$ with m/z = 421 and a dimer with m/z = 843 (Fig. 3.18 **18**). A compound with m/z = 421 was reported by Farag *et al.* (2012) but could not be identified. The molecular mass and absorption of this compound (Tab. 3.3 **18**) match isoanguston A (Lee *et al.*, 2010b). The fragment with m/z = 284 could result from loss of the two isoprenyl groups ($[M-^{\bullet}C_5H_9-C_5H_9^+]^{\bullet-}$).

3.4.3 Dose-dependence

Methanolic extract of *G. uralensis* showed an effect in the initial screening on CL2006 worms. The dose-dependence of this effect was tested as described in section 2.2.10. In the first test, that was conducted in May 2011, all treatments with concentrations above 50 µg/ml were significantly different from control ($p < 0.01$ for higher concentrations), but for some reason the effect of 500 µg/ml extract was lower than for 400 µg/ml (40 % decrease in number of Aβ aggregates vs. 53 % decrease, see Figure 3.19 A). In this experiment the methanol concentration was not constant but increased with rising extract concentration.

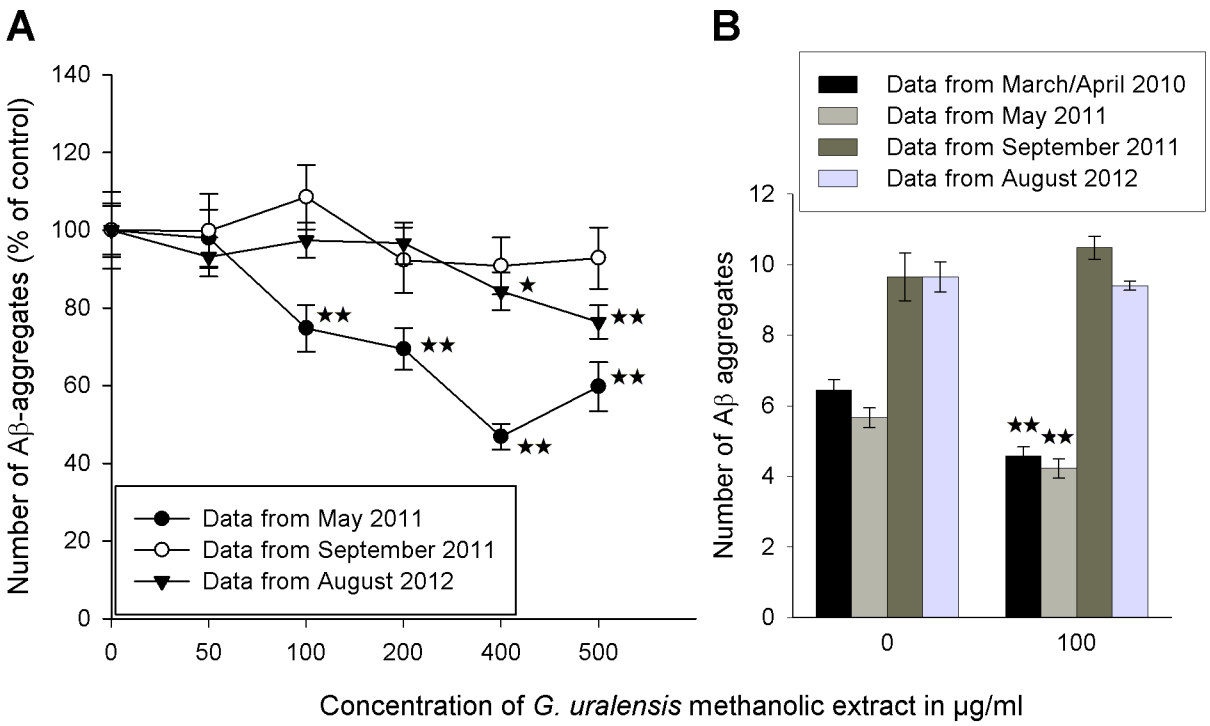

Figure 3.19: Dose-dependence of the effect of methanolic *G. uralensis* extract on Aβ aggregation in CL2006 measured at different time points. A) Dose-dependence as % of control; B) Number of Aβ aggregates in control and 100 µg/ml treatment in the different experiments. * $p < 0.05$; ** $p < 0.01$ as compared to solvent controls.

To test the possibility that high methanol concentrations may be responsible for the drop of effectiveness for high concentrations, the experiment was repeated in September 2011 with constant methanol concentration. Surprisingly the initial effect of 100 µg/ml extract could not be reproduced. In fact, none of the treatments had a significant effect. At the same time it was observed that the total number of Aβ aggregates in the control worms had increased from 6 to 10 in average (Figure 3.19 B).

Due to the different results gained in the previous experiments, the test was repeated again in August 2012. This time only 400 µg/ml and 500 µg/ml extract treatments had significant effects (16 %, $p = 0.01$ and 24 % decrease, $p < 0.01$, respectively). None of the treatments reached the effect observed for 100 µg/ml treatment in the initial screening. The total number of plaques was on the same level as a year ago.

In 2013 and 2014 more tests with the CL2006 strain were done to investigate the cause of the differences. New CL2006 worms were ordered from CGC together with new *E. coli* OP50. Fresh S-medium was prepared for these experiments. Even the incubator was changed. None of this changed the result that even the positive control EGCG was not effective any more. The number of plaques continued to rise up to 30 in untreated worms. This number is normal for CL2006 under standard conditions, but can be reduced at lower temperatures (Dr. Christopher D. Link, P. I. at University of Colorado, Boulder, Institute for Behavioural Genetics, personal communication). Since the rapid increase in the number of plaques coincided with the breakdown of our old incubator and bringing into service of a new one in May 2011, it is possible that the low numbers of plaques in earlier experiments resulted from a malfunction of temperature control in the old incubator.

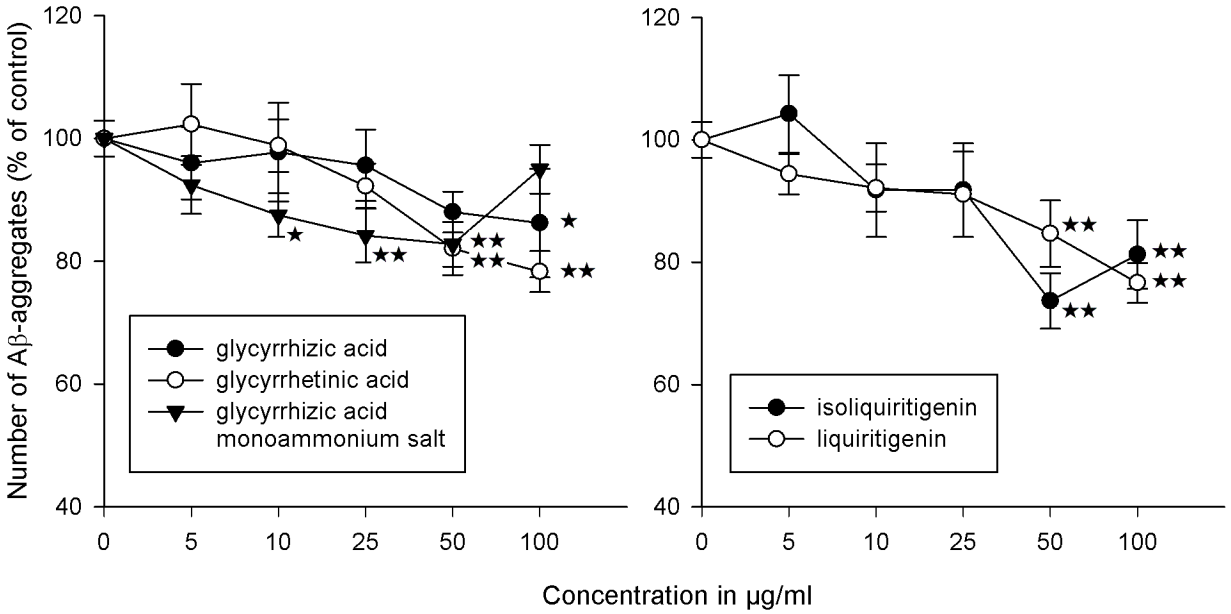

Figure 3.20: Dose-dependence of the effect of pure compounds from *G. uralensis* on Aβ aggregation in CL2006. * $p < 0.05$; ** $p < 0.01$ as compared to solvent controls.

The dose-dependence was also measured for pure compounds contained in *G. uralensis* extracts: GA, its aglycon GRA and the salt GAA, LG, and ILG (Fig. 3.20). The diammonium salt of glycyrrhizic acid showed no effect. GA had a significant effect only for the highest tested concentration of 100 µg/ml with 14 % decrease in number of Aβ aggregates ($p = 0.04$). GRA was active at 50 and 100 µg/ml with 18 % and 22 % decrease ($p < 0.01$), GAA had a significant effect already for 10 µg/ml (12 % decrease, $p = 0.01$), followed by 16 % and 17 % decrease for the following concentrations ($p < 0.01$), but the treatment with 100 µg/ml had no significant difference to control. The strongest effect was observed for ILG by 50 µg/ml treatment that decreased the number of Aβ aggregates by 26 % ($p < 0.01$), but 100 µg/ml of this substance had a somewhat lesser effect (19 % decrease, $p < 0.01$). LG had its strongest effect at 100 µg/ml with 23 % decrease and 15 % decrease at 50 µg/ml ($p < 0.01$). The treatment with 50 µg/ml was effective for most of the substances, therefore this concentration was used in following experiments.

3.4.4 Western blot analysis

The Western blot method was validated using pure $A\beta_{1-42}$ peptide as positive control (see section 2.2.8). It could be detected with both the Coomassie staining of the gel and with antibodies against Aβ (Fig. 3.21). Monomeric Aβ has a molecular weight of 4.5 kDa, so its band should appear near the last marker band. The band detected here appears slightly higher, so it could correspond to dimers. It has been shown that Aβ forms dimers and higher order oligomers in presence of SDS (Bitan *et al.*, 2005; Watt *et al.*, 2013), in which case it would be an artefact of the method. The two bands with higher molecular weight indicate the presence of tri- and tetramers that might have formed due to interactions with SDS or before the SDS-PAGE, since Aβ oligomerises rapidly in solution.

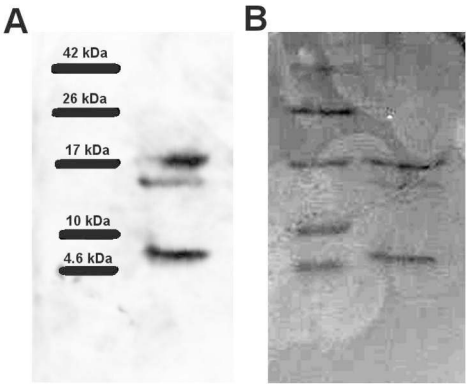

Figure 3.21: Validation of the Western blot analysis with pure Aβ. A) Detection with Aβ antibodies; B) Coomassie staining.

This positive control showed that the method is valid and can easily detect micromolar concentrations of Aβ. However, in the homogenates of *C. elegans* no Aβ could be detected. The control detection of actin was reproducibly positive. One possible explanation would be, that in the worms there is not enough monomeric Aβ or low weight oligomers that could be detected with this method. However, no bands were seen even when pure peptide was mixed in the homogenate to make sure that there was enough protein to detect. This indicates that something in the samples prevents the antibody from binding. Attempts to purify the sample with different methods failed. Removing the lipids from the samples with the purification method described by Wessel & Flügge (1984) restored the signal for Aβ added to the sample after purification, but failed to do so for samples with Aβ added prior to purification. This suggests, that Aβ in the samples interacts with lipids and is removed with them during the purification.

3.4.5 Paralysis assay

The ability of *G. uralensis* extracts and pure compounds to ameliorate Aβ toxicity was evaluated in a paralysis assay using the strains CL4176 and CL802 (see section 2.2.11). The control strain CL802 did not show paralysis for any treatments. The results with CL4176 are summarised in Figure 3.22.

Both the water extract and the methanol extract of *G. uralensis* could significantly prolong the PT_{50}. The effect for the methanol extract was dose-dependent, 200 µg/ml increased PT_{50} by 0.8 h, 500 µg/ml by 1.8 h ($p < 0.01$). The water extract (200 µg/ml) had a similar effect increasing the PT_{50} by 1.8 h ($p = 0.02$), but the control EGCG had the highest effect with 2.7 h increase in PT_{50} ($p = 0.03$). From the pure substances only ILG had a significant effect with 1.2 h increase in PT_{50} ($p < 0.01$). Notably, both controls treated either with water or with 1 % methanol had 2.7 h difference in their PT_{50} values.

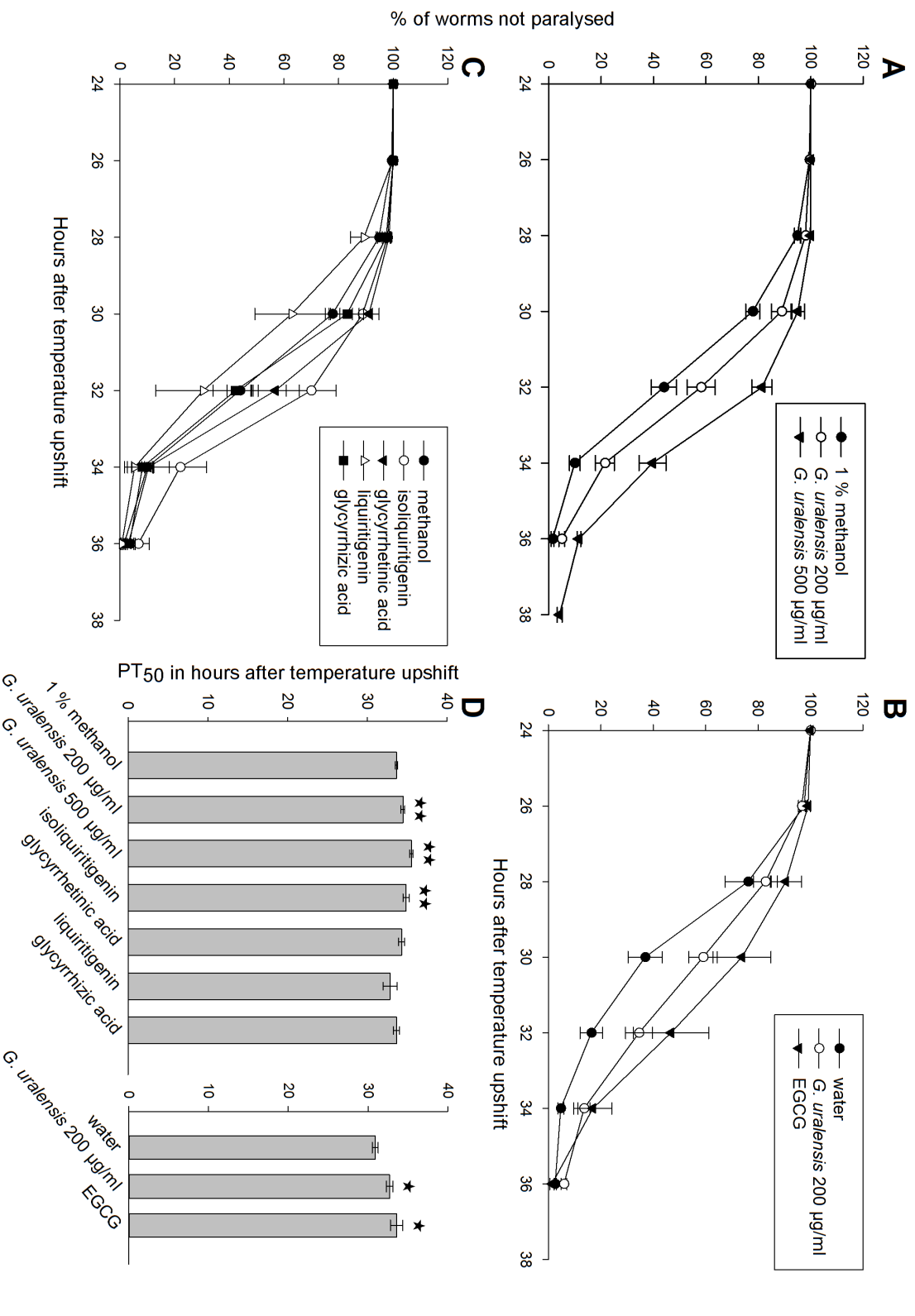

Figure 3.22: Results of the paralysis assay for *G. uralensis* and pure compounds. A) Paralysis curves for methanol extract of *G. uralensis*; B) Paralysis curves for water extract of *G. uralensis* and 100 µg/ml EGCG in water as positive control; C) Paralysis curves for 50 µg/ml of the pure compounds solved in methanol; D) PT_{50} values for all treatments. * $p < 0.05$; ** $p < 0.01$ as compared to controls.

3.4.6 Chemotaxis assay

The strain CL2355 has defective chemotaxis due to Aβ expression in nervous system. In this assay the ability of *G. uralensis* to restore the normal behaviour was studied (see section 2.2.12). Unfortunately the treatments had little effect (Fig. 3.23). Only GRA could significantly increase the CI towards benzaldehyde by 0.1 ($p = 0.05$). Some other treatments also seem to increase the CI, but due to the high standard error these values are not significantly different from control. Some treatments also decrease the CI of the control strain PD8120, pointing to unspecific effects on chemotaxis behaviour. This effect is significant for EGCG solved in water (0.17 decrease, $p = 0.02$). The solvent methanol changes the CI in both strains. Results for 200 µg/ml *G. uralensis*, ILG, and EGCG solved in methanol are not significantly different between the Aβ expressing worms and the control strain, probably due to the high standard error.

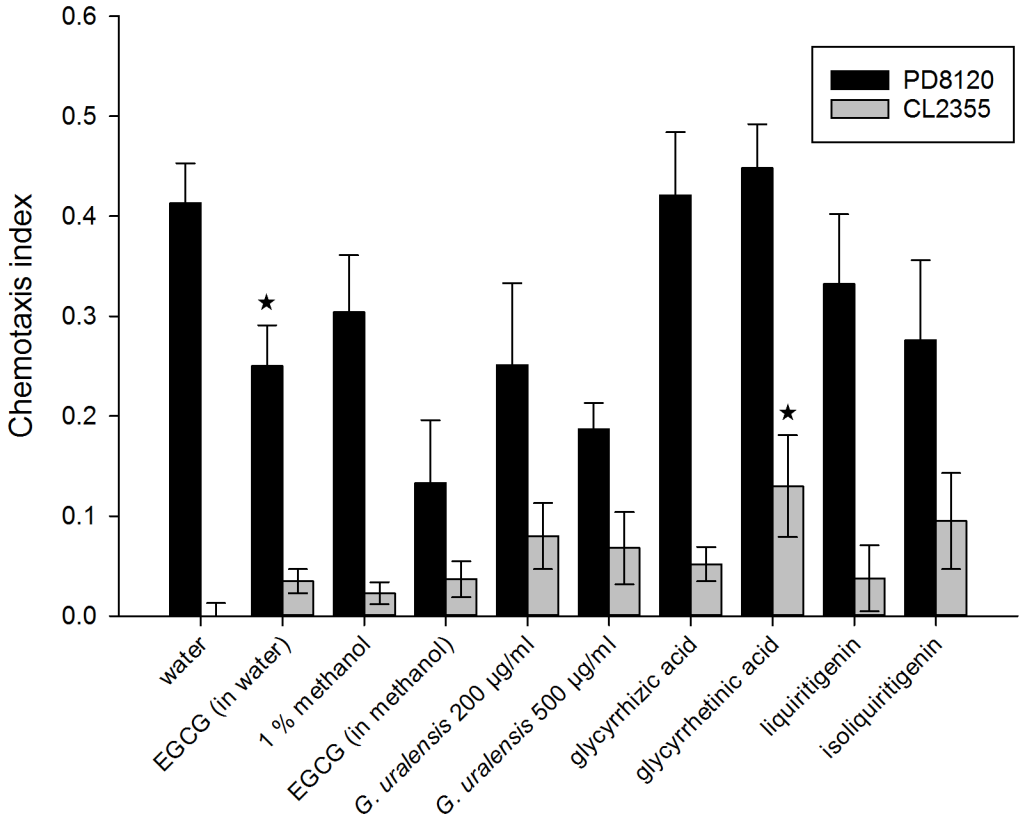

Figure 3.23: Results of the chemotaxis assay for *G. uralensis* and pure compounds. 50 µg/ml of the pure compounds solved in methanol were used and 100 µg/ml EGCG as positive control, the negative control was treated with water or 1 % methanol. * $p < 0.05$ as compared to the respective solvent control.

3.4.7 Serotonin sensitivity assay

Exogenous serotonin triggers the enhanced slowing response in normal *C. elegans* worms (see section 2.2.13). Although CL2355 worms are reported to be hypersensitive to serotonin (Wu *et al.*, 2006), no difference in behaviour compared to the control strain PD8120 was observed in the present work when the worms were treated with water or substances solved in water (Fig. 3.24 B). In presence of 1 % methanol the worms showed hyposensitivity (Fig. 3.24 A). The number of active CL2355 worms was significantly reduced after a treat-

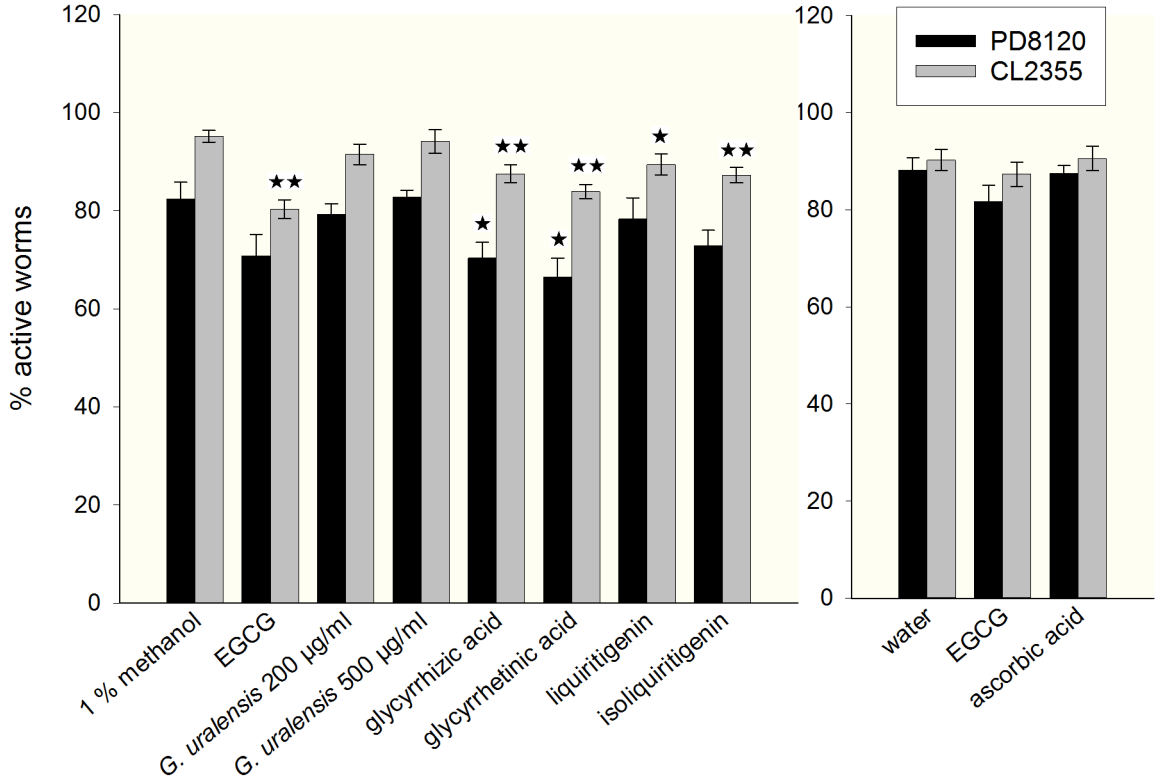

Figure 3.24: Results of the serotonin sensitivity assay on Aβ expressing CL2355 worms and the control strain PD8120. A) The used concentrations for the pure compounds were 100 µg/ml for EGCG and 50 µg/ml for other substances; B) 200 µg/ml ascorbic acid and 100 µg/ml EGCG were used. * $p < 0.05$; ** $p < 0.01$ as compared to controls.

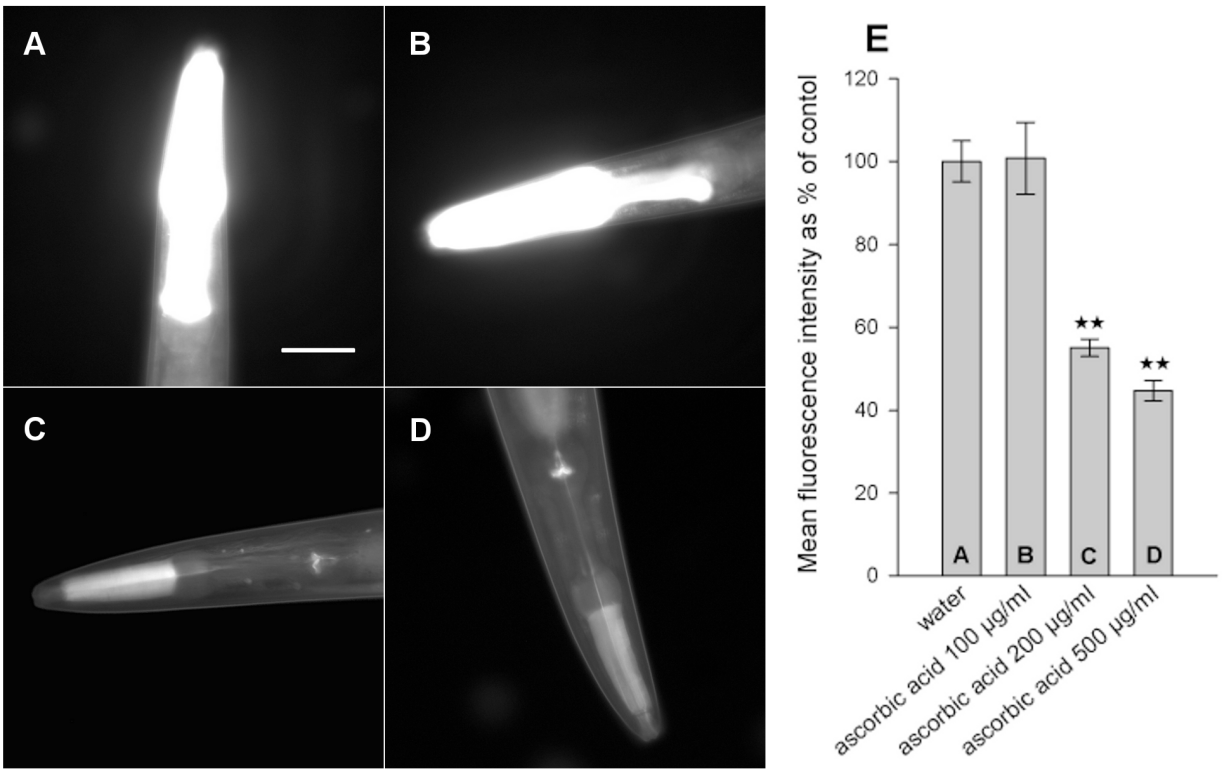

Figure 3.25: Heat shock protein expression levels after treatment with A) water, the scale bar corresponds to 20 µm; B) 100 µg/ml ascorbic acid; C) 200 µg/ml ascorbic acid; D) 500 µg/ml ascorbic acid; E) quantification of the fluorescence intensity. * $p < 0.05$; ** $p < 0.01$ as compared to control.

ment of 100 µg/ml EGCG (15 % decrease, $p < 0.01$), 50 µg/ml GA (8 % decrease, $p < 0.01$), GRA (11 % decrease, $p < 0.01$), LG (6 % decrease, $p = 0.03$), and ILG (8 % decrease, $p < 0.01$) solved in methanol. Treatments with GA and GRA also significantly decreased the activity of the control strain PD8120 (12 %, $p = 0.04$ and 16 %, $p = 0.02$, respectively). The relative number of active worms between both worm strains was significantly different for all treatments except for 50 µg/ml LG and the substances solved in water.

3.4.8 Antioxidant properties

Heat shock protein expression

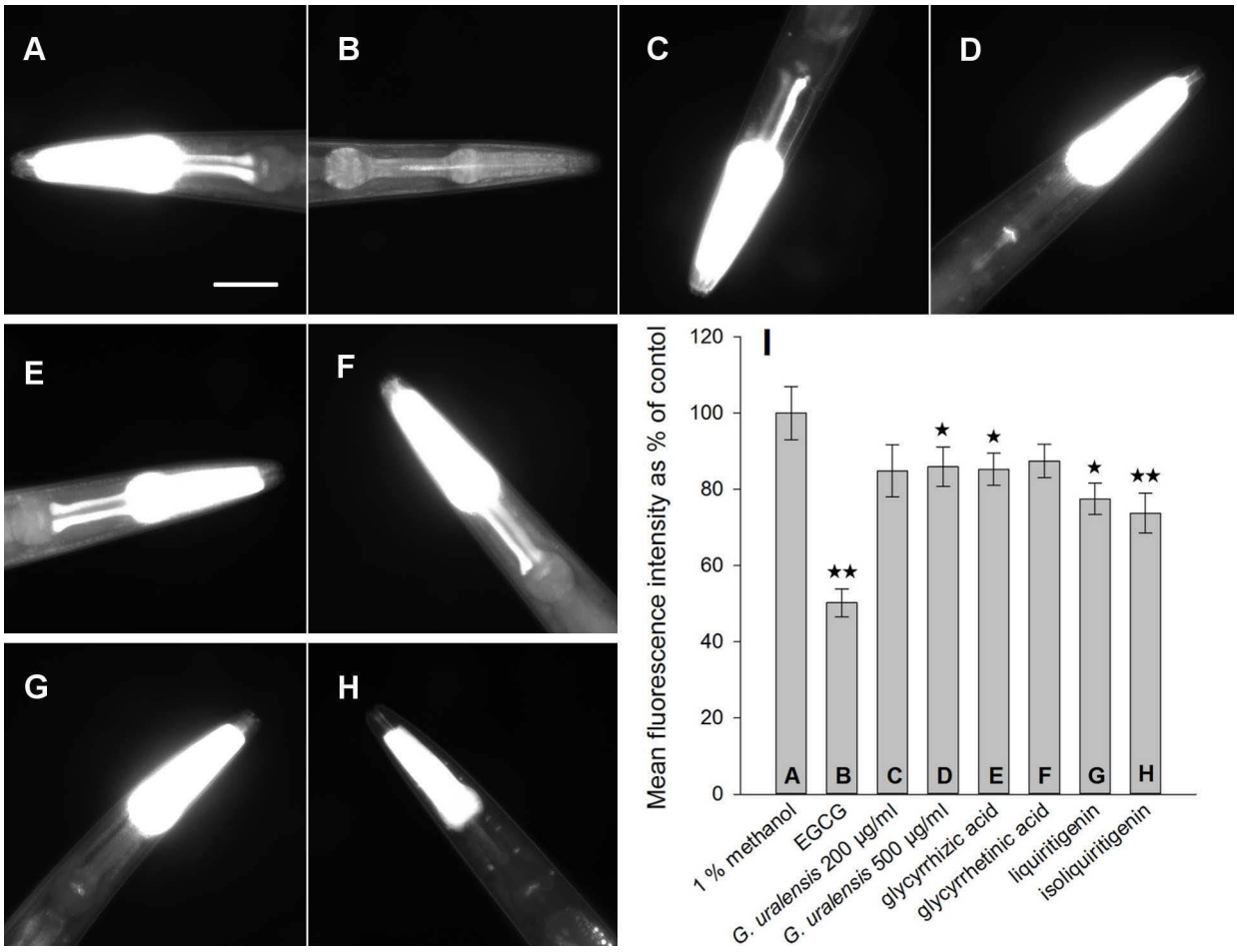

Figure 3.26: Heat shock protein expression levels after treatment with A) 1 % methanol, the scale bar corresponds to 20 µm; B) 100 µg/ml EGCG; C) 200 µg/ml methanolic *G. uralensis* extract; D) 500 µg/ml methanolic *G. uralensis* extract; E) 50 µg/ml of GA; F) 50 µg/ml of GRA; G) 50 µg/ml of LG; H) 50 µg/ml of ILG; I) quantification of the fluorescence intensity. * $p < 0.05$; ** $p < 0.01$ as compared to control.

The expression of *hsp-16.2::GFP* construct in TJ375 worms was triggered by the pro-oxidant juglone, a naphtochinone from *Juglans regia*. The antioxidant activity of the compounds was assessed as the reduction of fluorescence intensity compared to a solvent control (see section 2.2.14). The well known antioxidant ascorbic acid showed a strong dose-dependent decrease of the fluorescence intensity (Fig. 3.25) with no effect for 100 µg/ml treatment, 45 % reduction for 200 µg/ml and 55 % for 500 µg/ml ($p < 0.01$ for both). The positive control EGCG had the strongest effect among the other compounds, lowering the

mean fluorescence intensity by 50 %, $p < 0.01$ (Fig. 3.26). ILG decreased the fluorescence intensity by 26 % ($p < 0.01$), the treatments with 500 µg/ml *G. uralensis* extract, GA, and LG had smaller effects (14 %, $p = 0.04$, 15 %, $p = 0.03$, and 23 %, $p = 0.01$, respectively).

Survival assay

Survival assay measures the antioxidant activity by determining the increase in survival rate under lethal oxidative stress (see section 2.2.15). The effects are summarised in Figure 3.27. Both controls ascorbic acid and EGCG increased the number of living worms markedly (by 72 % for ascorbic acid, by 67 % for EGCG solved in water, and by 74 % for EGCG solved in methanol, $p < 0.01$ for all). The *G. uralensis* extract could not protect the worms from oxidative stress, but the pure compound ILG had an even stronger effect than the controls, increasing the survival by 82 % ($p < 0.01$). Its isomer LG had compared to that only a small effect (19 % increase, $p = 0.03$).

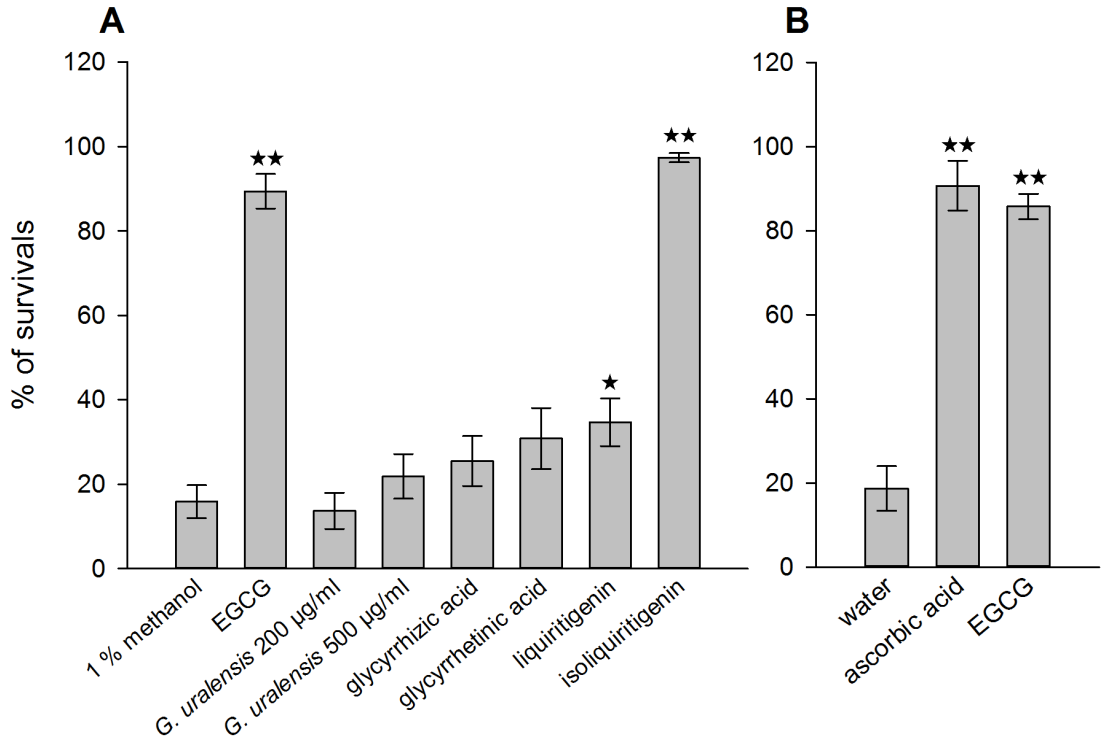

Figure 3.27: Survival under lethal oxidative stress after treatment with *G. uralensis* and pure compounds. A) 50 µg/ml of the pure compounds solved in methanol were used, 100 µg/ml EGCG as positive control, as negative control 1 % methanol was used; B) control treatments with 200 µg/ml ascorbic acid and 100 µg/ml EGCG solved in water. * $p < 0.05$; ** $p < 0.01$ as compared to controls.

3.4.9 DAF-16 delocalisation

Delocalisation of the transcription factor DAF-16 into the nucleus is necessary to start the expression of several stress resistance genes (see section 2.2.16). Under normal conditions the fluorescence in *daf-16::GFP* worms is spread throughout the body (Fig. 3.28 A), under oxidative or heat stress the fluorescence concentrates in the cell nuclei (Fig. 3.28 B, C). A treatment with methanol delocated some of the protein into the nucleus, but around 50 % of the treated worms were still negative. Because of this solvent effect only samples with

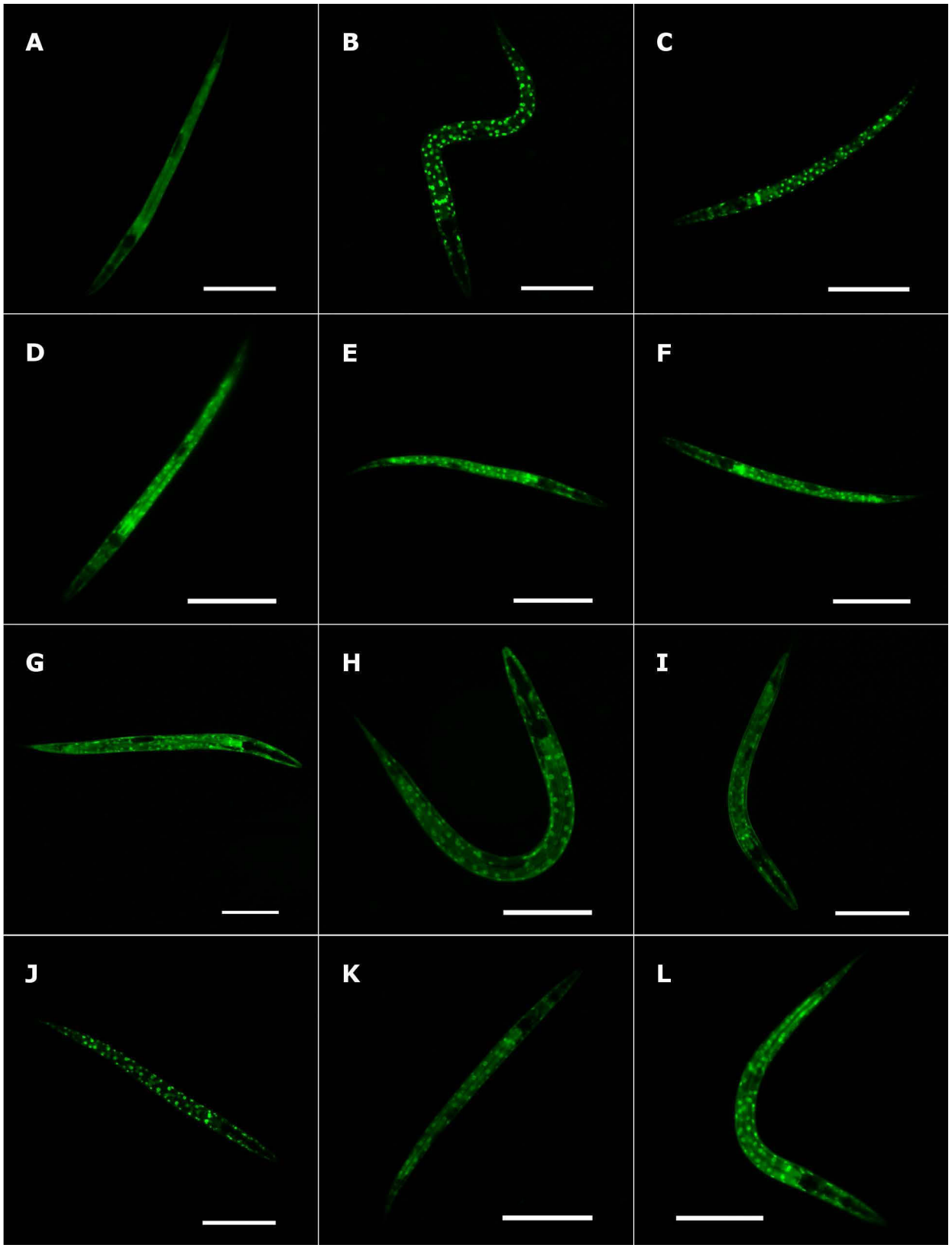

Figure 3.28: Representative pictures of TJ356 worms in DAF-16 delocalisation assay. Worms treated with A) water; B) 20 µM juglone; C) 15 min at 37 °C; D) 1 % methanol; E) 200 µg/ml methanol *G. uralensis* extract; F) 500 µg/ml methanol *G. uralensis* extract; G) 50 µg/ml glycyrrhizic acid; H) 50 µg/ml glycyrrhetinic acid; I) 50 µg/ml liquiritigenin; J) 50 µg/ml isoliquiritigenin; K) 100 µg/ml EGCG solved in methanol; L) 100 µg/ml EGCG solved in water. The scale bars indicate 100 µm. Pictures were taken with BZ9000 from Keyence.

> 70 % worms with nuclear DAF-16 were considered significantly positive. The treatment with GA showed no increase in the nuclear localisation compared with the methanol treatment. Treatments with *G. uralensis* extract, GRA, and LG showed a small increase that did not reach the significance level of 70 %. Also EGCG, that has been reported to be active in this assay before (Abbas & Wink, 2010), showed no significant effect in the current experiment. However, the EGCG solved in water had a stronger effect (15 % more positives than water control) than the substance solved in methanol (no difference to methanol treatment). The only significantly active substance from the ones tested here was ILG that delocated DAF-16 in about 85 % of the worms.

3.4.10 Lifespan assay

The ability of *G. uralensis* extracts and the pure substances found in this extract to extend the lifespan of *C. elegans* was tested in this assay (see section 2.2.17). The results shown in Figures 3.30 and 3.29 indicate, that neither the extract nor the pure substances had a positive effect on the lifespan. The extract rather shortened the half-time of life by 9.6, 5.4, and 1.9 days in the wildtype worms (N2) and by 7.9, 2.9, and 3.1 days in CL2006 for 500, 200, and 50 µg/ml, respectively. This effect was dose-dependent as can be clearly seen on the survival curves and half-time values for the wildtype worms. The effect was less pronounced for the Aβ expressing strain CL2006. From the pure substances GA had no effect, whereas ILG shortened the half-time by 7.3 and 5.8 days in N2 and CL2006, respectively. LG had a stronger toxic effect in CL2006 (3.5 days decrease in half-time) than in the wildtype (2.7 days), GRA had almost no effect in CL2006, but was more toxic than LG in the wildtype (3.5 days decrease in half-time).

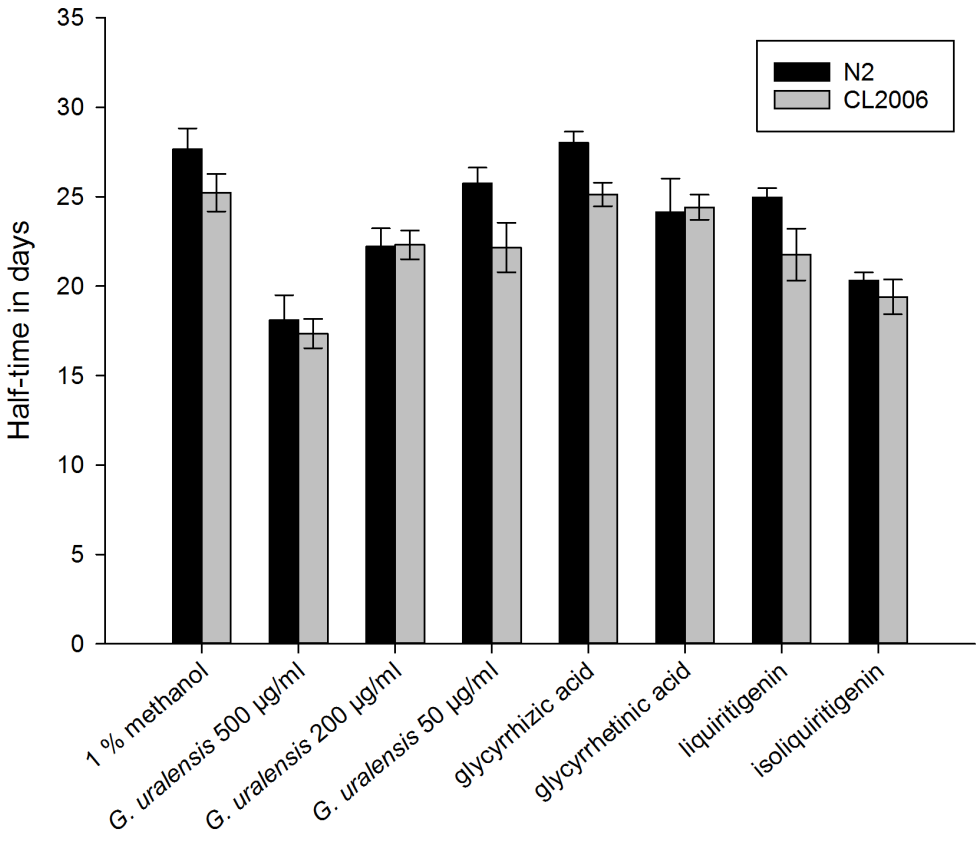

Figure 3.29: Half-times of the lifespan assay. 50 µg/ml of the pure compounds solved in methanol were used, 1 % methanol was used as control.

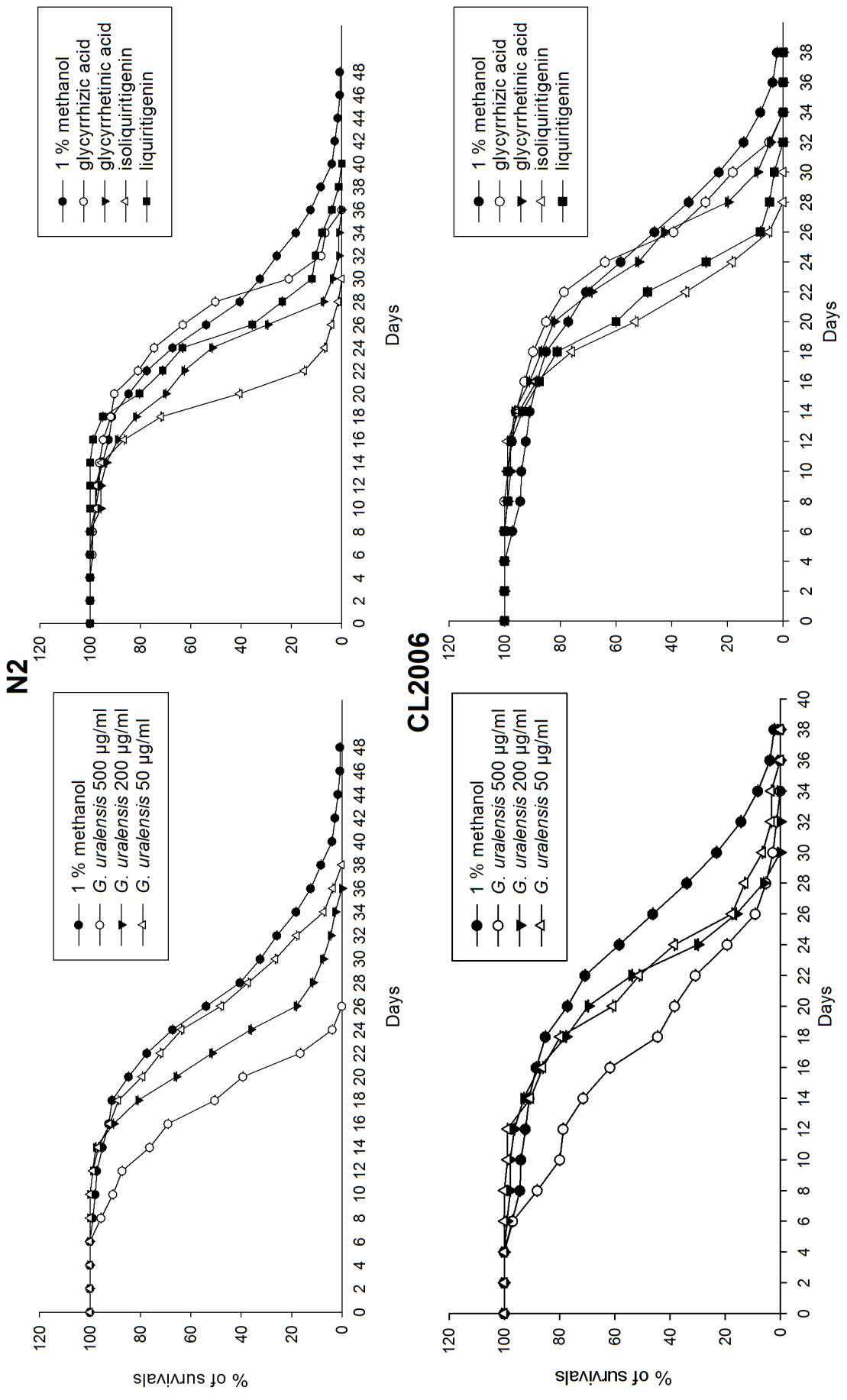

Figure 3.30: Results of the lifespan assay for *G. uralensis*. 50 µg/ml of the pure compounds solved in methanol were used, 1 % methanol was used as control.

3.5 *Carlina acaulis*

3.5.1 GLC-MS analysis of the extract

This analysis was carried out by Frank Sporer, a scientific assistant at Heidelberg University, IPMB, Department of Biology (see section 2.2.5).

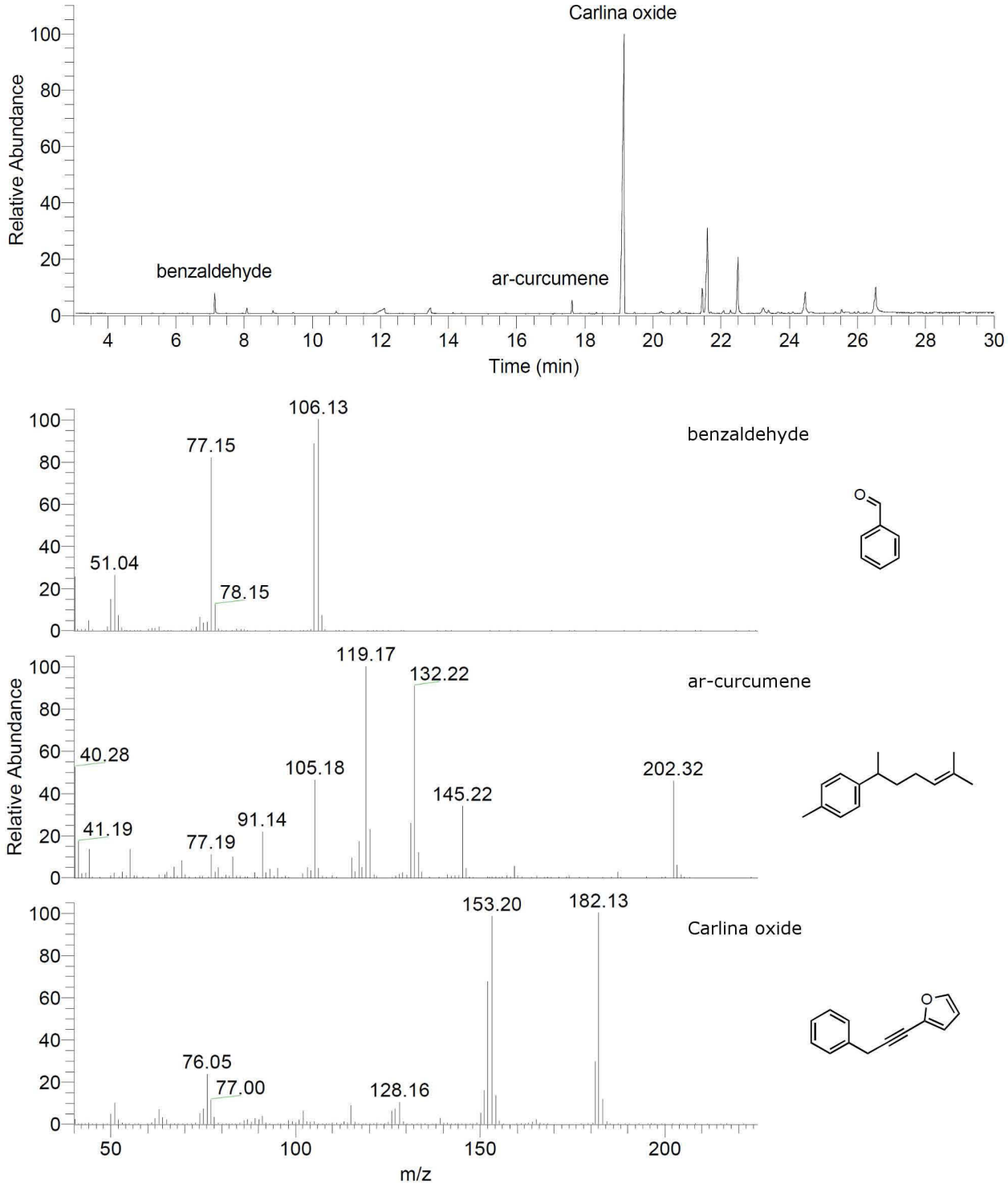

Figure 3.31: GLC-MS total ion current of dichloromethane extract of *C. acaulis* and corresponding MS spectra.

The extract contains mainly Carlina oxide and small amounts of benzaldehyde and ar-

curcumene (Fig. 3.31) that have been reported as constituents of *C. acaulis* volatile oil before (Chalchat *et al.*, 1996; Stojanović-Radić *et al.*, 2012). These compounds could be identified by comparison of their MS-spectra to published data (Đorđević *et al.*, 2005; Qin *et al.*, 2007) and the Chemical Abstract Service database. The MS data for the other peaks in the chromatogram do not match any previously reported substance from *C. acaulis* and could not be identified.

Figure 3.32 shows the chromatograms for isolated Carlina oxide fraction isolated from the dichlormethane extract of *Carlina acaulis* (CarOx) and the residual extract of *Carlina acaulis* after isolation of Carlina oxide (Car0). CarOx (Fig. 3.32 A) contains most of the recovered Carlina oxide (99 % of total recovered substance calculated by peak area) but also curcumene and some of the unidentified substances. Carlina oxide comprises 51 % of all substances in this fraction. Car0 (Fig. 3.32 B) contains only 1.5 % Carlina oxide relative to CarOx, and most of the other substances from the extract. Due to the low concentration of Carlina oxide, it can be considered free of this substance.

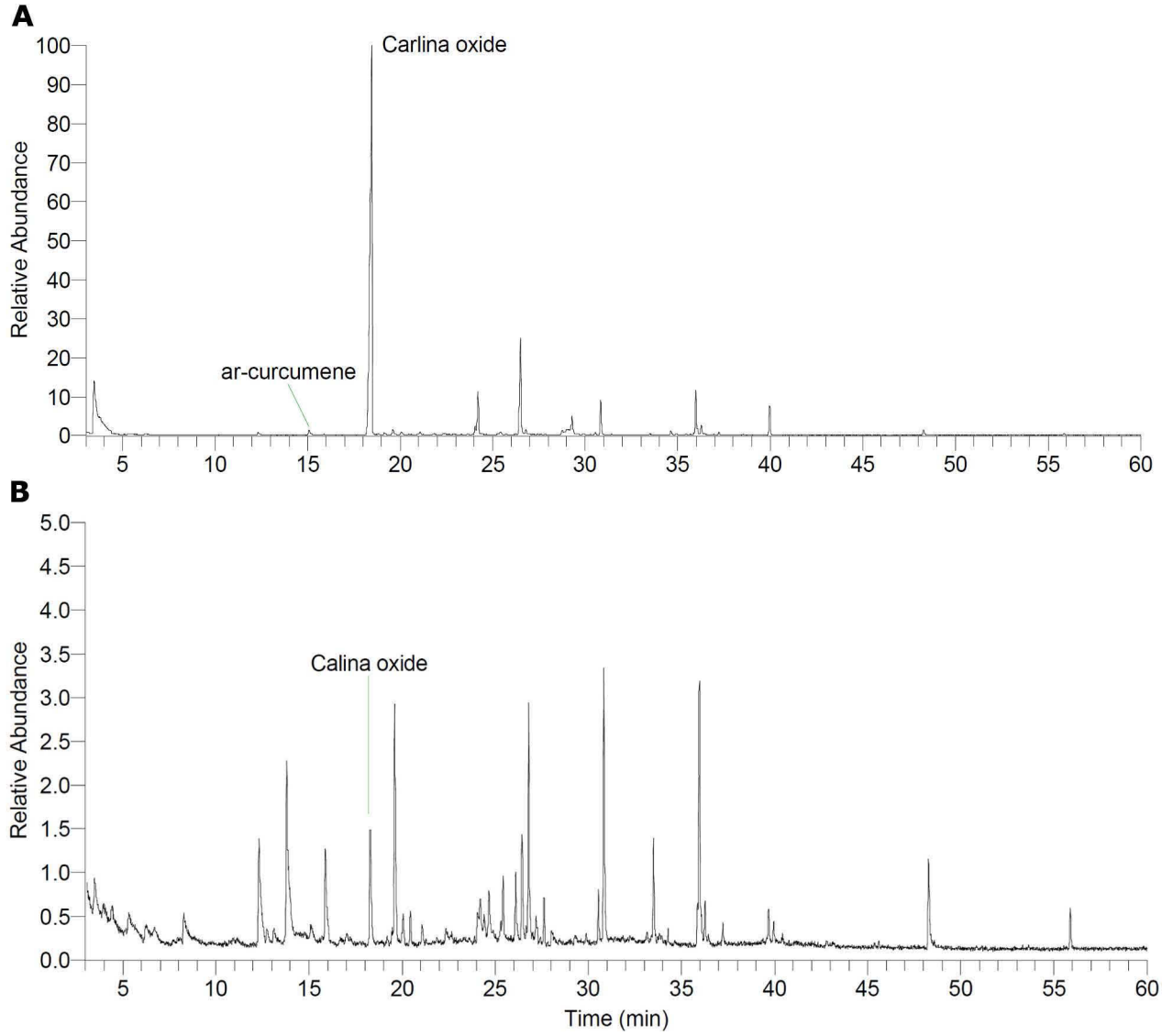

Figure 3.32: GLC-MS total ion current of isolated Carlina oxide fraction (A) and residual extract (B). Scale relative to Carlina oxide in A.

3.5.2 Paralysis assay

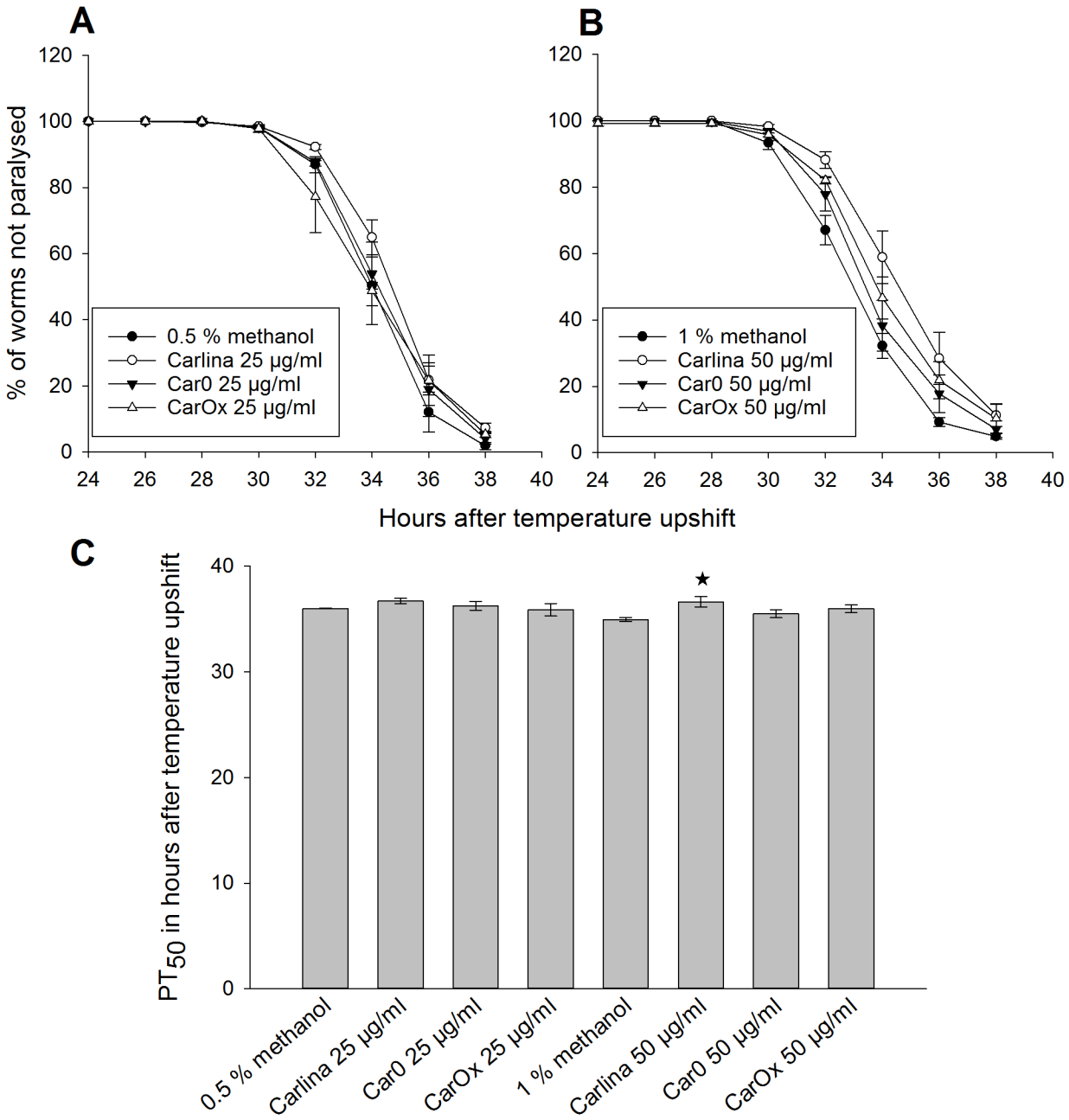

Figure 3.33: Results of the paralysis assay for *C. acaulis* extract (Carlina), the extract without Carlina oxide (Car0) and Carlina oxide (CarOx) in the concentrations of 25 µg/ml (A) and 50 µg/ml (B). C) PT_{50} values for all treatments. * $p < 0.05$ as compared to control.

The paralysis assay was first conducted with 25 µg/ml of *C. acaulis* extract, Car0 and CarOx (see section 2.2.11). No significant differences were observed (Fig. 3.33 A). The extract was most effective with a PT_{50} value of 36.7 h compared to 36 h in a control treated with 0.5 % methanol ($p = 0.05$). Therefore a higher concentration of 50 µg/ml for all treatments was tested (Fig. 3.33 B). Here the extract could increase the PT_{50} from 35 h (control treated with 1 % methanol) to 36.6 h ($p = 0.04$). CarOx (PT_{50} = 36 h) had a slightly stronger effect than Car0 (PT_{50} = 35.5 h), but both were not significant compared to control. Inter-

estingly, the control treatments with 0.5 and 1 % methanol were also significantly different ($p < 0.01$), differing by 1 h.

3.5.3 Chemotaxis assay

The chemotaxis assay (see section 2.2.12) produced no evaluable results. The extract and especially CarOx seem to be toxic for the worms. Since the CL2355 strain is more sensitive than other worms, there were mostly not enough individuals to evaluate. The observed worms showed no increased chemotaxis.

3.5.4 Serotonin sensitivity assay

The serotonin sensitivity assay (see section 2.2.13) showed a significant difference to the solvent control only for 10 µg/ml *C. acaulis* extract ($p = 0.03$) (Fig. 3.34). No dose-dependent effect was observed. In case of 10 µg/ml CarOx there was a significant difference to methanol treatment in the control strain PD8120 ($p = 0.02$), suggesting an action mechanism independent of Aβ expression.

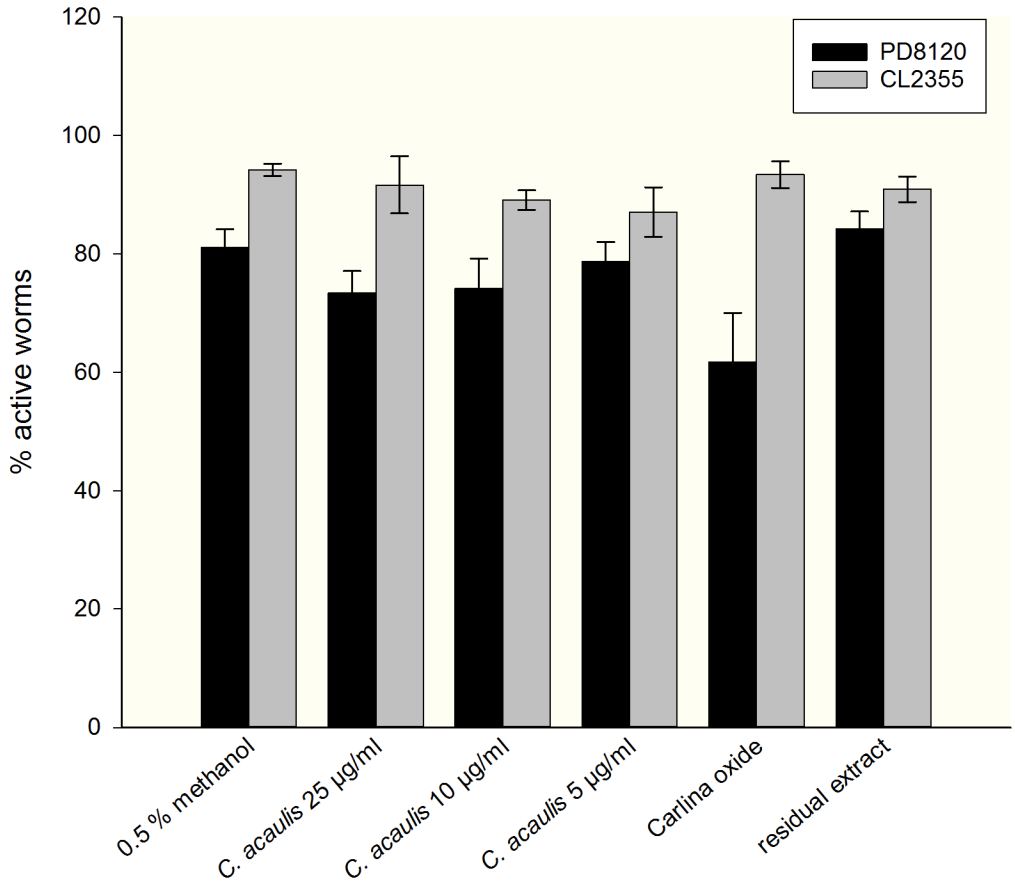

Figure 3.34: Results of the serotonin sensitivity assay for *C. acaulis* on Aβ expressing CL2355 worms and the control strain PD8120. The used concentration for Carlina oxide was 10 µg/ml and for the residual extract 25 µg/ml. * $p < 0.05$ as compared to control.

3.5.5 Antioxidant properties

The antioxidant properties of *C. acaulis* were studied by Kevin Roth, a Master of Science student of Molecular Biotechnology at Heidelberg University, during his laboratory practical at IPMB, Department of Biology.

DPPH$^{\bullet}$ scavenging

The *in vitro* antioxidant activity was tested via DPPH$^{\bullet}$ scavenging assay (see section 2.2.7). The results showed a typical sigmoidal curve, although only the highest tested concentration reached the upper plateau (Fig. 3.35). Relative EC_{50} in this assay was 122 µg/ml.

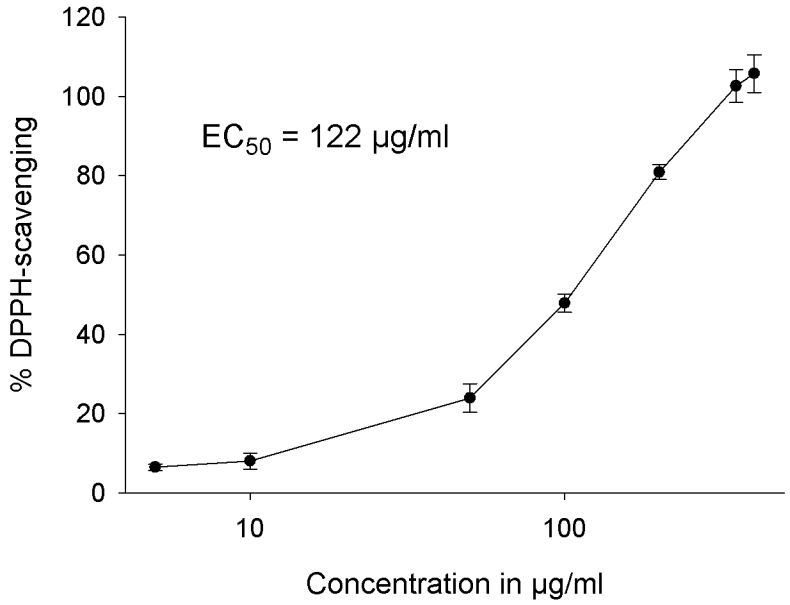

Figure 3.35: DPPH$^{\bullet}$ scavenging activity of *C. acaulis*

Heat shock protein expression

The antioxidant activity *in vivo* was tested in *C. elegans* as effect on the HSP expression (see section 2.2.14). EGCG and ascorbic acid were used as positive controls and both showed a significant decrease in fluorescence intensity, that corresponds to HSP expression levels (Fig. 3.36 E and F, respectively). The extract of *C. acaulis* showed an dose-dependent effect, where only the highest tested concentration (50 µg/ml) resulted in a significant decrease by 47 % (Fig. 3.36 B, $p < 0.01$). This value is similar to the effect of 200 µg/ml ascorbic acid (41 % decrease, $p < 0.01$). Car0 showed no effect for all tested concentrations (Fig. 3.36 C), whereas a strong dose-dependent effect was observed for CarOx. The highest tested concentration, 25 µg/ml, reduced the fluorescence intensity significantly by 64 % (Fig. 3.36 D, $p < 0.01$). This value is only slightly smaller than the strongest effect, observed for the control 100 µg/ml EGCG: 75 % decrease in the fluorescence intensity ($p < 0.01$).

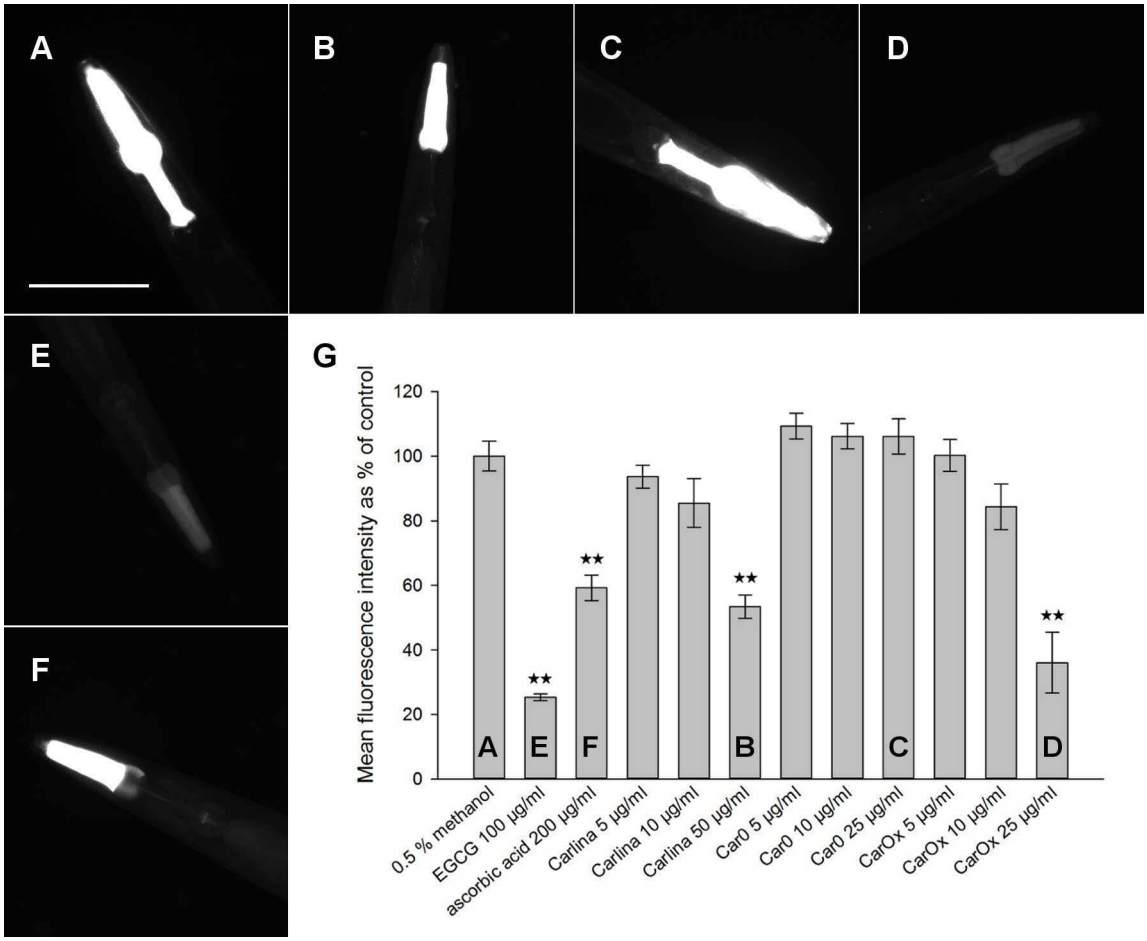

Figure 3.36: Heat shock protein expression levels after treatment with A) 0.5 % methanol; B) 50 µg/ml *C. acaulis* extract (Carlina); C) 25 µg/ml *C. acaulis* extract without Carlina oxide (Car0); D) 25 µg/ml Carlina oxide (CarOx); E) 100 µg/ml EGCG; F) 200 µg/ml ascorbic acid; G) quantification of the fluorescence intensity. The scale bar corresponds to 100 µm. Pictures were taken with BZ9000 from Keyence. ** $p < 0.01$ as compared to control.

3.5.6 DAF-16 delocalisation

The data for this experiment was gathered by Kevin Roth, a Master of Science student of Molecular Biotechnology at Heidelberg University, during his laboratory practical at IPMB, Department of Biology, as described in section 2.2.16. DAF-16 delocalisation into the nuclei was observed after the treatment with *C. acaulis* extract and with CarOx but not for Car0 (Fig. 3.37). The effect was dose-dependent for both treatments and stronger for CarOx, where already 5 µg/ml caused significant DAF-16 delocalisation (63 % of the worms positive, $p < 0.01$; 69 % and 85 % positives for 10 and 25 µg/ml, respectively). The extract showed a significant activation of the DAF-pathway for the concentrations of 10 and 25 µg/ml (60 % ($p = 0.05$) and 77 % ($p < 0.01$) positives, respectively). The positive controls juglone and a heat shock at 37 °C also activated the pathway (84 and 100 % positives, respectively, $p < 0.01$), the untreated worms showed significantly less DAF-16 delocalisation (7 %, $p < 0.01$) than the control treated with methanol (48 % positives).

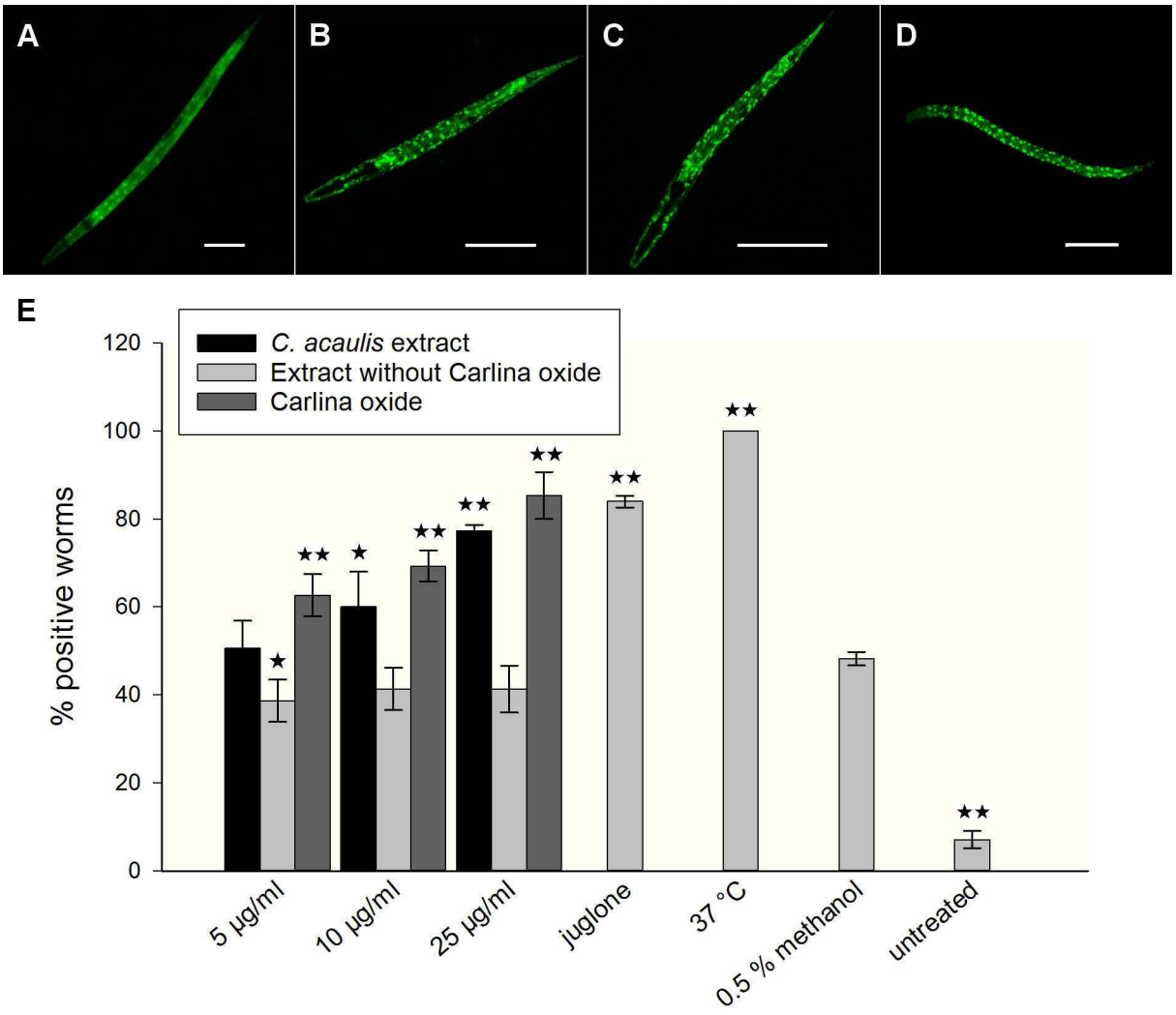

Figure 3.37: DAF-16 localisation in an untreated worm (A) and after treatment with 25 µg/ml *C. acaulis* extract (B), 25 µg/ml extract without Carlina oxide (C) or 25 µg/ml Carlina oxide (D). The scale bars indicate 100 µm, the pictures were taken with BZ9000 from Keyence; E) quantification and dose-dependence of the treatments in this assay. * $p < 0.05$; ** $p < 0.01$ as compared to control.

4 Discussion

4.1 Screening of TCM drug extracts

For this screen 25 plants were chosen based on earlier reports of neuroprotective activity. As a control EGCG was used because of its proven activity in the used *C. elegans* model (Abbas & Wink, 2010) and known mechanism of action (Bieschke *et al.*, 2010; Ehrnhoefer *et al.*, 2008; Hyung *et al.*, 2013). In the study by Abbas & Wink (2010) 220 µM EGCG lowered the number of Aβ aggregates by 33 %, which is in good accordance to the 35 % decrease for 100 µg/ml (≈ 220 µM) EGCG observed in the present study (Fig. 3.2). Thioflavin T showed an even stronger effect than EGCG in the present study. This substance was reported to decrease amyloid pathology in *C. elegans* strain CL4176 (Alavez *et al.*, 2011). These worms develop only a few small plaques before they paralyse, which makes it difficult to image them, and therefore this strain is usually not used for quantification of Aβ aggregates. Due to the different methods these results are not directly comparable to the present work, but in both cases thioflavin T treatment led to decrease in the number of Aβ aggregates.

Of the 55 tested extracts 12 methanol and 9 dichloromethane extracts showed significant activities. The effects of methanol extracts reached a maximum effect of 32 % decrease in Aβ deposits for *A. catechu*. Two other methanol extracts reduced the number of Aβ aggregates by at least 25 %, namely *G. uralensis* (30 %) and *A. oxyphylla* (25 %). These three extracts were pursued further and are discussed in the following sections. The effect of other extracts was smaller but still noteworthy. *S. officinalis* and *R. laevigata* have not had much attention in relation to AD, but both of them decreased Aβ deposition in the *C. elegans* model. Gallic acid from *S. officinalis* has shown neuroprotective effects against Aβ toxicity (Ban *et al.*, 2008). Although the substances underlying the effect on Aβ aggregation were not further elucidated, the dichloromethane extract of this plant was not effective, suggesting that the active compounds are rather hydrophilic like gallic acid. In case of *R. laevigata* the one compound reported to be neuroprotective (1,2-benzenedicarboxylic acid dinonyl ester; Choi *et al.*, 2009) is more lipophilic and would be expected to be contained in dichloromethane extracts. The methanol extract was more effective though, indicating other active substances in this plant. This result is in accordance with the study of Choi *et al.* (2006), who showed protective effects against Aβ toxicity for the methanol extract of *R. laevigata* in cell culture and mice.

Four of the seven extracts tested for *G. biloba* were significantly effective. The seed extracts were more effective than leaves with 22 % decrease in the number of Aβ aggregates for the methanol extract of the TCM drug and 20 % for both extracts of raw seeds, although only half the concentration was used for the dichloromethane extract. This indicates that there are several active ingredients. Some of them are rather lipophilic and are lost either when the seeds ripen, when the sacrotesta is removed, or when the nuts are baked to prepare bái guǒ. Other compounds remain in the nuts or are produced during the preparation of bái guǒ. To our knowledge there are to date no studies of the effects of *G. biloba* seeds or bái guǒ on AD published in international journals. The results presented here, however, suggest that the seeds, either raw or prepared, are possibly more potent than the leaves and deserve further study.

The methanol extract of *G. biloba* leaves showed moderate activity, decreasing the number of Aβ aggregates by 13 %. The dichloromethane extract as well as the special extract EGb761 had no significant effect. The result for EGb761 was unexpected, since many studies have shown its effectiveness in different models (see Tab. 1.1). Two studies have also used the *C. elegans* model CL2006. Smith & Luo (2003) examined the ROS content in this model and found that single compounds found in EGb761 can lower the ROS concentration, but the results for the extract itself were not significant. Wu *et al.* (2006) performed the same Aβ aggregation assay, that was also used in the present work, although with some modifications in treatment protocol and data analysis. The treatment was started at day 4 in contrast to day 2 in the present study, the worms were evaluated on day 6 in both studies. Therefore it is possible that EGb761 has some immediate effects that can be seen after two days, but have vanished after four days. To check this possibility, the experiment should be repeated with different treatment times to compare the effects. Due to limited availability of EGb761 this was omitted in the present study.

Two of the most studied plants besides *G. biloba* in context of cognitive enhancement are *P. ginseng* and *P. notoginseng*. While *P. ginseng* had no effect on Aβ aggregation in the present study, both extracts of *P. notoginseng* had moderate activity. In previous studies the compound quercetin 3-O-β-D-xylopyranosyl-β-D-galactopyranoside has been reported to inhibit Aβ aggregation (Choi *et al.*, 2010). Other substances like notoginsenoside R1 and the polyacetylenes panaxynol and panaxydol could counteract Aβ toxicity in cell culture and mouse models (Ma *et al.*, 2014; Nie *et al.*, 2008; Yan *et al.*, 2014). These reports are in accordance with the present study, indicating that *P. notoginseng* contains several substances that are able to ameliorate Aβ pathology. Since the activity of the extracts was not so high, it would be advisable to identify these active compounds and use them in higher concentrations. Further studies should reveal the suitability of this plant for patients with AD.

E. senticosus, the Siberian ginseng, showed positive results for both methanol and dichloromethane extracts. The decrease in Aβ deposits was 15 % for both extracts, but a ten times lower concentration was used for the dichloromethane extract, so mostly lipophilic compounds seem to be the active ingredients. The methanol extract has been tested before on primary neuron culture, where it could protect the cells from Aβ-induced neurotoxicity (Bai *et al.*, 2011; Tohda *et al.*, 2008). Bai *et al.* (2011) noted activity against axonal and neuritic atrophy mostly in more hydrophilic fractions, whereas hexane fraction of the extract had no effect. They isolated some compounds that were responsible for the protective effect like eleutherosides E and B, isofraxidine, and stigmasterol and sitosterol glycosides. Although aglycons of these compounds might be contained in a dichloromethane extract, it seems more likely that some other compounds are responsible for the effect on Aβ aggregation observed in the present study. This underlines the benefits of using plant extracts in a complex disease like AD, where several compounds can lead to synergistic effects by influencing different targets.

Significant, although low activity was also observed for the methanol extracts of *Phellodendron* spp. (huáng bǎi), *A. galanga*, and *Coptis* spp. (huáng lían). *P. amurense* and *P. chinense*, both species used in the TCM drug huáng bǎi, have been reported to counteract Aβ toxicity in cell culture (Xian *et al.*, 2013). It is possible that this protection is at least partly conferred by inhibiting Aβ aggregation. Same can be said for *A. galanga* that has shown memory enhancing and antioxidant activities in mice treated with $A\beta_{25–35}$. Berberine, the active ingredient of *Coptis* spp., has been shown to lower Aβ levels in a mouse model of AD. This activity was replicated in the present study in the *C. elegans* model with methanol extract of huáng lían. The *Phellodendron* species also contain berberine, although

in smaller amounts than *Coptis* spp. (Van Wyk *et al.*, 2015). Therefore, the effect of huáng bǎi extract might be due to this compound, too. Berberine has several effects besides lowering Aβ levels, including AChE inhibition and should therefore be considered as a possible lead drug for AD therapy (Ji & Shen, 2011).

C. monnieri yielded the most effective dichloromethane extract that decreased the number of Aβ aggregates by 35 %. The methanol extract of this plant had lower but still significant activity with 18 % decrease. This activity might be due to osthole, a rather lipophilic coumarine derivative, that would be expected to be contained mostly in dichloromethane extract. This compound can improve memory in a mouse model of AD via enhancing neurogenesis (Liu *et al.*, 2015b). However, in the study of Liu *et al.* (2015b) no decrease in Aβ deposition was detected. Therefore the present study is the first to report this effect for *C. monnieri* extracts and further research is needed to identify the active compounds. Two other dichloromethane extracts, namely *P. multiflorum* and *M. officinalis*, showed a significant effect. For both only the dichloromethane but not methanol extract was effective, indicating lipophilic substances as active compounds. In *M. officinalis* these compounds could be magnolol and honkiol, that have been reported to counteract Aβ-induced toxicity before (Hoi *et al.*, 2010). For *P. multiflorum* water and ethanol extracts have been studied earlier in context of Aβ toxicity (Liu *et al.*, 2015c; Um *et al.*, 2006). The water extract had a memory enhancing effect in mice and inhibited AChE (Um *et al.*, 2006). The ethanol extract and its active compound 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside were tested on a *Drosophila* model, where they could counteract Aβ toxicity, but no effect on Aβ levels was observed (Liu *et al.*, 2015c). The lipophilic compounds, shown here to act against Aβ aggregation, complement the actions of hydrophilic compounds, making *P. multiflorum* an interesting plant for AD research.

The most effective dichloromethane extract (*C. monnieri*) had stronger activity than the methanol extracts with only tenth of the amount used for the treatment. This might indicate that lipophilic compounds are more active then the hydrophilic substances. They are able to penetrate biomembranes and could therefore have higher bioavailability. However, since the culture medium for *C. elegans* is water based, these substances are poorly soluble and difficult to test. This might account for the fact that there are more methanol extracts showing significant activity. On the other hand, several dichloromethane extracts also lead to an increase in the number of Aβ aggregates. It has to be considered that the solvent DMSO, in which these extracts were solved, has itself a plaque decreasing effect (Fig. 3.3). This observation is in accord with a study showing that 0.8 % DMSO can increase the lifespan of *C. elegans* and even lower concentrations reduce paralysis in CL4176 worms (Frankowski *et al.*, 2013). To account for additive effects, all extracts were compared with the respective concentration of DMSO as control, but a possible synergistic interaction can not be excluded. Interactions counteracting the effect of DMSO could also explain the apparent increase in number of Aβ aggregates for extracts that have no anti-aggregation activity. Due to the complications with dichloromethane extracts and the fact that there were more highly active methanol extracts, further work concentrated on the latter.

Some of the plants tested here were reported to have effects on Aβ levels or toxicity in earlier studies (see Tab. 1.1) but showed no activity in the *C. elegans* model. Many of the other studies tested pure compounds rather than extracts, which might explain the different results. The content of active compounds in the extract is often low, so their effects can be missed. Most studies working with extracts used water as a solvent, hence these extracts also have different composition compared to methanol and dichloromethane extracts used in the present study, making them not directly comparable. Another difference is also the model used. Compared to cell culture *C. elegans* is a more complex model. Some substances

might be active on cells, but fail in the *C. elegans* model due to low bioavailability, intestinal metabolism, or other factors that are not present in cell culture. On the other hand, mice and rats are much more complex animals than a nematode like *C. elegans*. They have developed organs, cell types, and signalling cascades that are not present in nematodes. Therefore, the *C. elegans* model is only suitable for testing direct interactions with Aβ or effects on basic, evolutionary conserved signalling pathways. To detect interactions with specific targets that are only found in vertebrates, other models are needed. These differences in the methodology have to be considered when comparing different studies and are probably responsible for the differing results.

4.2 *Areca catechu*

A. catechu is known for its antiparasitic and cholinomimetic activities, but to date there are no studies about its effects on amyloid aggregation. In the present study both methanol and dichloromethane extracts showed high reduction in number of Aβ aggregates in the *C. elegans* model. The methanol extract, that was studied further, showed a dose-dependent effect (Fig. 3.5), but concentrations higher than 100 µg/ml could not be tested due to toxicity for the worms. *A. catechu* contains mainly alkaloids and polyphenols. To clarify, which of these groups is responsible for the effect, arecoline as the main alkaloid was also tested in the Aβ aggregation assay. It showed no significant activity for concentrations up to 125 µg/ml (data not shown), suggesting that the effect of the extract is due to the polyphenols or some other minor compound.

To examine whether *A. catechu* can also counteract Aβ toxicity, the paralysis assay with the *C. elegans* strain CL4176 was conducted. 100 µg/ml of the extract were used, since this concentration was effective in the first experiment and showed no toxic effects. However, in the paralysis assay this treatment significantly shortened the time until paralysis (Fig. 3.6) and also some worms of the control strain CL802, that do not express Aβ, appeared paralysed after the treatment. The CL4176 and CL802 strains seem to be more sensitive to the toxicity of *A. catechu*, possibly because the worms were treated earlier in this assay. The extract might interact with important processes during development of the worms, making them more vulnerable to the Aβ toxicity or toxic substances in general. Another possibility is that the extract and Aβ both interact with the same target in CL4176, leading to an additive or even synergistic toxicity. A direct interaction with Aβ is also possible and would lead to higher toxicity, if the toxic oligomers were stabilised and further aggregation to less toxic plaques prevented. In this case, however, the toxicity should have also been observed in the CL2006 worms.

The organoselenium compound diphenylselenide, that has shown several positive effects in different AD models, also increased the paralysis rate in *C. elegans* (Zamberlan *et al.*, 2014). Zamberlan *et al.* (2014) showed, that the effect on paralysis is due to muscle inhibition via increased AChE activity. *A. catechu* has cholinomimetic activity and might inhibit AChE (Gilani *et al.*, 2004). Diphenylselenide has shown AChE-inhibitory activity as well in mammalian models, but activated the enzyme in *C. elegans* (Zamberlan *et al.*, 2014). Whether a similar effect could be the cause of increased paralysis after treatment with *A. catechu* needs further testing. Until then other causes of toxicity cannot be excluded.

The toxicity could have been prevented by using lower concentrations of the drug. In the Aβ aggregation assay the concentration 75 µg/ml also showed a significant decrease in the number of deposits, but the effect size was only a half of the higher concentration. Therefore, lowering the concentration would lead to loss of therapeutic effect. If the extract has an effect on larval development, treating the worms later, when they are fully developed,

would also solve the problem. This would be a mechanism specific for *C. elegans* that is not relevant for AD therapy. In the case of an effect on acetylcholinergic neurotransmission, the effect might also be specific for *C. elegans* and further studies in mammals are required to evaluate it. In the present study the work with *A. catechu* was discontinued in favour of the less toxic extract of *G. uralensis*.

For possible usage of *A. catechu* in AD therapy it would be important to further characterise the toxicity of this plant. The effects seen in *C. elegans* might be specific for nematodes and not affect humans or they might be more general. Arecoline has been shown to cause neurotoxicity (Shih *et al.*, 2010), to be cytotoxic, and chewing the seeds can cause oral cancer (Peng *et al.*, 2015). On the other hand, a small study on patients with mild to moderate AD reported no adverse effects for low doses of arecoline that improved the memory of responders (Soncrant *et al.*, 1993). Arecoline acts as a cholinomimetic but had no effect on Aβ aggregation. Therefore, the role of polyphenols and other constituents of *Areca* nuts in the beneficial and toxic activity of the extract should be further clarified. If the toxic compounds can be separated from the beneficial ones, the development of a modified extract or using pure compounds would be advisable.

4.3 *Alpinia oxyphylla*

The methanol extract of *A. oxyphylla* decreased the number of Aβ aggregates by 25 %, being the third most effective methanol extract in the present study. The chloroform fraction of an ethanol extract has been reported before to decrease Aβ levels in mice (Shi *et al.*, 2014). This extract was rich in sesquiterpenes like nootkatone, had antioxidant effects, and inhibited AChE. The decrease in Aβ aggregates shown in the present study is in accordance with these results. The methanol extract probably contains at least some of the sesquiterpenes but also other constituents like protocatechuic acid and 5-hydroxymethylfurfural. Both latter substances have been shown to decrease Aβ levels and have other neuroprotective properties (Liu *et al.*, 2014; Song *et al.*, 2014; Zhang *et al.*, 2015). Based on this initial positive activity the extract of *A. oxyphylla* was studied further.

The paralysis assay, however, did not show a significant difference between treatment with the methanol extract of *A. oxyphylla* and control (Fig. 3.7). There was also no significant difference between control and treatment with EGCG (solved in methanol), in contrast to the results obtained with EGCG solved in water (Fig. 3.22). The effect of EGCG on the CL4176 strain has only been investigated in one study before (Brown *et al.*, 2006). There the compound was solved in ethanol and a small, not significant effect in the paralysis assay was observed. A tendency to longer time until paralysis was also observed in the present study for EGCG solved in methanol. The EGCG solved in water, on the other hand, had a strong, significant effect. In the light of this data it seems possible that the used solvent modulates the activity of EGCG. The main mode of action for EGCG as a polyphenol is building unspecific hydrogen bonds with proteins. The possible interaction with solvent molecules and its impact on the hydrogen bond building ability of EGCG would be an interesting topic for further studies.

Assuming, that the lack of activity for the positive control EGCG was because of a specific solvent effect that does not affect other substances, then the results indicate that the methanol extract of *A. oxyphylla* has no activity against Aβ toxicity. It is possible, that the compounds in this extract only interact with Aβ fibrils, but not the oligomers that cause toxicity in CL4176. However, other studies have shown that this plant can ameliorate cognitive deficits in mice treated with $A\beta_{1–42}$ (Liu *et al.*, 2014; Shi *et al.*, 2014). The lack of a corresponding effect in the present study might be due to a different model organism. It is

also possible, that a higher dose of the extract or a pure active compound is needed to see an effect. Given the promising activity of *A. oxyphylla* in the Aβ aggregation experiment and its low toxicity compared to some other plants, e.g. *A. catechu*, the possible beneficial effects of this plant on AD pathology should be studied more intensively in the future.

4.4 *Glycyrrhiza uralensis*

4.4.1 Determination of the used *Glycyrrhiza* species

The TCM drug gān cǎo can contain three different species of *Glycyrrhiza*: *G. uralensis*, *G. glabra*, and *G. inflata*. Therefore, DNA barcoding was used to determine the exact species. The comparison of ITS sequences from the drug and sequences from known species from database showed the highest similarity with *G. uralensis* (Tab. 3.1). The *ITS* sequence does not differ between *G. glabra* and *G. inflata*, but compared to *G. uralensis* there are two variable sites: one single nucleotide polymorphism in *ITS1* and a substitution of three nucleotides in *ITS2* (Kondo *et al.*, 2007b). Hence, in this case the analysis of the *ITS* sequence was sufficient for the identification.

DNA of the same plant material has been used before to analyse the chloroplast gene for ribulose bisphosphate carboxylase large chain (rbcL) (Herrmann *et al.*, 2011b). According to Kondo *et al.* (2007b), there are two genotypes for *rbcL* in *Glycyrrhiza* species. The first one is found in all three species (*G. glabra*, *G. inflata*, *G. uralensis*), the second one (GenBank accession number AB012126) was only found in *G. uralensis* in the study of Kondo *et al.* (2007b). In contrast to that, Hayashi *et al.* (2000) reported that the *rbcL* sequence is identical in *G. uralensis* and *G. inflata* (GenBank accession numbers AB012126 and AB012127, respectively). Herrmann *et al.* (2011b) reported the *Glycyrrhiza* species used to be *G. inflata* (GenBank accession number JF950025). The two *rbcL* genotypes differ by two nucleotide substitutions at the bases 706 and 736 (based on the sequence AB012126). By comparing the sequences from Kondo and Hayashi to the sequence reported by Herrmann, it occurs that the latter sequence only contains 698 base pairs and is lacking the essential bases. Hence, this sequence cannot be used for identification.

The identity of the plant material as *G. uralensis* is also supported by the phytochemical analysis. Glycycoumarin, a compound specific for the species *G. uralensis* (Kondo *et al.*, 2007a), was found in the methanol extract of the plant (peak 12 in Fig. 3.9, Fig. 3.16) whereas no masses corresponding to glabridin (324 g/mol) or licochalcone A (338 g/mol) were observed (Tab. 3.3). Also the ratio of flavanones to chalchones seems to match most closely the conditions reported for *G. uralensis* (Kondo *et al.*, 2007a; Simmler *et al.*, 2015), although this ratio was not quantitatively determined in the present study. Altogether, both the genetic and phytochemical analysis point to *G. uralensis*, and this result is also consistent with the fact that the drug was purchased in China, where *G. uralensis* is the most common species.

4.4.2 *Glycyrrhiza uralensis* and its major compounds decrease Aβ aggregation

The methanol extract of *G. uralensis* decreased the number of Aβ aggregates in the initial screening by 30 % (Fig. 3.2) and was therefore chosen for further study as one of the most potent extracts. The dichloromethane extract had no significant activity, possibly because due to poor solubility only 25 µg/ml of this extract could be used or because of the different composition of the extracts. Water and ethanol extracts have been studied before on

mice and primary neuron culture (Ahn *et al.*, 2006; Lee *et al.*, 2012b). Memory enhancing and neuroprotective activities were reported, but the effects on Aβ aggregation were not investigated in these studies. The compound LG has been shown to reduce Aβ secretion from neurons and ILG can inhibit Aβ aggregation by binding to this peptide (Chen *et al.*, 2013; Liu *et al.*, 2009). The phytochemical analysis of the extract showed that the methanol extract—in contrast to the dichloromethane extract (Fig. 3.8)—contains glycosylated forms of these compounds (peaks 1–4 in Fig. 3.9). Therefore, these substances could be responsible for the effect seen for the extract.

Quantification of the compounds in the different extracts showed, that 54–64 µg/ml GA is present in water and methanol extracts, 9 µg/ml of LG were found in methanol extract, the other studied compounds were present only in traces (Tab. 3.2). The dichloromethane extract contained only trace amounts of all four compounds in correlation with the lack of significant activity in the aggregation assay. In the LC-MS/MS analysis the aglycons could not be recovered, but liquiritin, liquiritin apioside, isoliquiritin, isoliquiritin apioside, and GA were present in high amounts (Fig. 3.9, Fig. 3.10). Glycosides often function as prodrugs that have to be activated by bacteria in the gut. It is known that the active form of GA in humans is GRA that is produced form GA by intestinal bacteria (Hattori *et al.*, 1983; Krähenbühl *et al.*, 1994; Monder *et al.*, 1989). The *C. elegans* model system used here contains living *E. coli* as a food source for the worms. These bacteria probably also metabolise the extracts, so it can be expected that the worms are exposed to more aglycons than originally present in the extract. Therefore, although only trace amounts were found in the extract, the high amount of glycosides can lead to higher concentrations of LG, ILG, and GRA, and these compounds can significantly contribute to the activity of the extract.

Most of the saponins found in the methanol extract are derivatives of GRA. Assuming that the sugar residues are removed by the bacteria, the aglycon GRA should be representative for possible activity of these compounds. Besides the already mentioned substances, isoprenylated flavonoids and coumarins were found. Glycycoumarin has shown protective activity against Aβ toxicity before (Kanno *et al.*, 2015), but since only a small amount of coumarins was found in the active extract, it is unlikely that these are the main active constituents. Of the isoprenylated flavonoids only isoangustone A could be identified (Tab. 3.3, Fig. 3.18) and there are to date no reports about this substance interacting with Aβ. Therefore, GA as the major compound (peak 8 in Fig. 3.10) and the aglycons LG, ILG, and GRA were chosen for further study.

In the Aβ aggregation assay in CL2006 GA showed a significant effect only for the highest tested dose (100 µg/ml), the other substances were already active at 50 µg/ml, decreasing the number of Aβ aggregates by up to 26 % (Fig. 3.20). LG and ILG had slightly higher activity than the other compounds. These results are in accordance with earlier reports (Chen *et al.*, 2013; Liu *et al.*, 2009), but they also suggest that LG and ILG are not the only active compounds in *G. uralensis* in respect of Aβ aggregation. For further experiments all four compounds were tested in addition to the methanol extract.

4.4.3 *Glycyrrhiza uralensis* and isoliquiritigenin counteract Aβ toxicity

Protective effects against Aβ-induced neurotoxicity have been reported before for different *G. uralensis* extracts, ILG, and LG (Ahn *et al.*, 2006; Lee *et al.*, 2012b; Liu *et al.*, 2009). Here the effects of methanol and water extract as well as the pure compounds GA, GRA, LG, and ILG were tested in *C. elegans* models expressing Aβ in muscles and neurons. The paralysis assay with the strain CL4176 produced the most significant results. Both *G. uralensis*

extracts and ILG delayed the time until paralysis (Fig. 3.22). The control substance EGCG was also active in this assay and had the highest activity of all tested compounds with 2.7 h delay in PT_{50}. This activity is much stronger pronounced than the moderate delay reported by Brown *et al.* (2006). As discussed in section 4.3, there might be an effect of solvent modifying the action of EGCG.

Regarding solvent effects, it was also observed that the treatment with 1 % methanol alone had a significantly different PT_{50} compared to the water-treated control. This effect was dose-dependent, since there was also a significant difference between 0.5 and 1 % methanol (Fig. 3.33; $p < 0.01$). That confirms the observation made by the treatment with *A. catechu* extracts (see section 4.2) that the CL4176 strain is more sensitive than CL2006. Methanol had no effect on the latter strain in concentrations up to 1 % (data not shown). In contrast to *A. catechu*, the effect observed for methanol was not toxic but beneficial. The solvent alone could delay paralysis to the same extent as EGCG. Possibly this activity was mediated by a hormetic effect of early life exposure to a low concentration of a toxin, since methanol has shown hormetic effects at concentrations below 1 % before (Qin *et al.*, 2010). To account for this effect, all treatments were compared to the respective solvent control.

From the two *G. uralensis* extracts tested, the water extract showed higher activity. At a concentration of 200 µg/ml it led to the same delay in paralysis as 500 µg/ml of the methanol extract. The water extract contains slightly more saponins than the methanol extract (compare peaks 5, 6, and 7 in Fig. 3.10), so this could be the cause of higher activity. However, the only saponin tested as a pure compound—GA—showed no difference to the control. Saponins have an amphiphilic structure which allows them to interact with biomembranes and increase their permeability. Therefore, if saponins are not the active ingredients themselves, they can still lead to higher activity of an extract by increasing the bioavailability of the active compounds. Same can be said for the aglycon GRA which showed only minimal, non-significant activity in this assay. Although this molecule has no hydrophilic sugars, the lipophilic triterpene structure can still interact with the lipids and cholesterol molecules in the biomembrane.

ILG was the only pure compound with significant activity in this assay. This result is in accordance with an earlier report of a protective effect against Aβ-induced neuronal death (Lee *et al.*, 2012b). Similar activity has been reported for its isomer LG (Liu *et al.*, 2009). In the paralysis assay, however, LG rather shortened the time until paralysis, although this effect was not significant. The worms of the control strain CL802 did not react to the treatment with LG, suggesting that this compound is not toxic in general. ILG has been reported to interact directly with Aβ (Chen *et al.*, 2013) and similar interactions have been observed for other flavonoids (Bu *et al.*, 2015). ILG and LG are polyphenols like EGCG, so they share a possible mechanism of action. ILG has one more hydroxy group compared to LG, allowing it to build more hydrogen bonds. Hence, if this is the mechanism of action, ILG would be expected to be more potent. This, however, does not explain the opposite activity of LG. Possibly, the unspecific binding via hydrogen bonds is not the only mechanism of action for these flavonoids in the paralysis assay.

The common properties these flavonoids share with EGCG also suggest, that if the solvent affects the action of EGCG (section 4.3), this would also be the case with ILG and LG. The effects of methanol and EGCG are clearly not additive, but rather lead to an abolishment of any visible effect (Fig. 3.7). If the same happens to ILG and LG, then the small negative effect of LG could be interpreted as an abolishment of the methanol effect. It was suggested, that the latter arises from a hormetic response. In this case the antioxidant and neuroprotective effects of LG could protect the worms from methanol toxicity and prevent the induction of hormesis. The same should happen with ILG, but apparently ILG has a

higher protective capacity, so that the delay in paralysis still reaches significance above the methanol control.

Aβ is degraded via autophagy in the CL4176 worms and this process is regulated by the transcription factors HSF-1 and DAF-16 (Cohen *et al.*, 2006; Florez-McClure *et al.*, 2007). Therefore, ILG or other compounds in the *G. uralensis* extract can lower its toxicity also by interacting with proteins involved in autophagy or its regulation. Fluoxetine, a selective serotonin reuptake inhibitor, has been shown to delay paralysis in this model dependent on DAF-16 activation and serotonin (Keowkase *et al.*, 2010). ILG induced DAF-16 delocalisation in the present study (Fig. 3.28), indicating that this transcription factor may be involved in the mechanism of action for this compound as well. However, *G. uralensis* extract failed to activate DAF-16 significantly, although it could delay paralysis. Therefore, there must be other mechanisms underlying its activity.

ILG and LG can modulate serotonin and norepinephrine concentrations (Wang *et al.*, 2008) and several compounds in *G. uralensis* have antioxidant activity. These properties, besides the direct interaction with Aβ peptide, could counteract the downstream toxic effects triggered by Aβ. To further elucidate the mechanisms behind the effects seen in the paralysis assay, the antioxidant activity in *C. elegans* (discussed in section 4.4.4) and the effects on behaviour in the strain CL2355, that expresses Aβ in neurons, were investigated.

Neuronal toxicity in CL2355 leads to impaired chemotaxis behaviour. As can be seen in Figure 3.23, the control strain PD8120 has a CI of about 0.4, whereas CL2355 worms show no chemotaxis at all. This defect is rescued to some extent by the treatments, most significantly by GRA. GRA also showed a small protective effect in the paralysis assay, although it was not statistically significant. Still, these results suggest, that GRA could have protective properties against Aβ toxicity, maybe at higher concentrations. It is also evident, that the chemotaxis behaviour of the control strain is affected as well. This indicates, that the substances have unspecific effects on the behaviour that are not related to Aβ expression. This assay is very sensitive to all environmental factors, leading to high standard deviations. Therefore the effects of *G. uralensis* extract, ILG, or EGCG are not significant, although there is a tendency for higher CI in CL2355 worms after these treatments ($p \leqslant 0.1$).

In the serotonin sensitivity assay an unexpected hyposensitivity in CL2355 worms treated with methanol was observed (Fig. 3.24). By control treatment with water there was no difference between CL2355 and the control strain. It has been reported before that the CL2355 strain is hypersensitive to serotonin (Wu *et al.*, 2006). In later experiments this effect was also observed in our lab (Felix Heiner, PhD student at Heidelberg University, IPMB, Department of Biology, personal communication). To date there is no logical explanation, why the worms behaved differently during the present study. However, there is a difference in sensitivity to serotonin between CL2355 and the control strain, when the worms are treated with methanol. Therefore, the results were evaluated as positive, if a treatment could reduce this difference.

Significant activity was observed for EGCG, LG, and ILG, suggesting that these substances can normalise the serotonergic neurotransmission in this model. ILG and LG have been reported before to modulate serotonin concentrations in mice, resulting in an antidepressant-like effect (Wang *et al.*, 2008), and the antidepressant fluoxetine delays Aβ-induced paralysis in *C. elegans* dependent on serotonin (Keowkase *et al.*, 2010). Similarly, green tea extract containing EGCG has been shown to elevate serotonin concentration in human plasma (Hodgson *et al.*, 2014). Therefore, the effect on serotonergic transmission reported here could also be involved in the mechanism of action of the active compounds in counteracting Aβ toxicity in the paralysis assay.

The compounds GA and GRA can significantly suppress the activity of both the Aβ expressing strain and the control strain. This indicates, that the effect is not specifically related to Aβ. It also suggest that these substances have a general effect on neurotransmission or the enhanced slowing response in *C. elegans*. The inhibition of gap junctions by GRA (Davidson & Baumgarten, 1988) may play a role in this effect. The muscle cells of *C. elegans* are connected by gap junctions (White *et al.*, 1986), therefore GRA could disturb signalling between these cells, leading to reduced mobility. However, such an effect was not observed in other assays following treatment with GRA. On the contrary, in paralysis assay this compound slightly delayed paralysis. Therefore, effects related to serotonin cannot be ruled out and may play a role in the small protective effect of GRA observed in the other assays.

In summary, it can be said that ILG was the most potent substance in counteracting Aβ toxicity. It had a highly significant activity in paralysis and serotonin sensitivity assays and a positive tendency in enhancing chemotaxis. Serotonergic neurotransmission may play a role in this protective effect. The extracts of *G. uralensis* as well as the substances LG and GRA also showed protective effects, although smaller than the effect seen for ILG. *G. uralensis* extracts probably contain several protective compounds that may have additive or synergistic activitiy. ILG as one of the more potent protectors deserves further investigation, weather as pure compound or in combination with other active substances found in *G. uralensis*.

4.4.4 Isoliquiritigenin has antioxidant activity via activating DAF-16

Oxidative stress is one of the important characteristics of AD and ageing in general. Aβ increases the production of ROS (Huang *et al.*, 1999; Opazo *et al.*, 2002). Therefore, one way to counteract Aβ toxicity downstream of the peptide aggregation is antioxidant therapy. The antioxidant capacity of *G. uralensis* extract and its active compounds in *C. elegans* was investigated using two assays. In the HSP expression assay the worms are exposed to low doses of the prooxidant juglone that induces mild oxidative stress. The survival assay is a more rigorous test of antioxidant activity, where the worms are subjected to lethal oxidative stress by high concentrations of juglone.

In the HSP expression assay all substances except the lower concentration of *G. uralensis* extract and GRA showed a moderate activity (Fig. 3.26). As a positive control this experiment was also conducted with ascorbic acid, a well known antioxidant, that exhibited dose-dependent antioxidant activity with up to 55 % reduction in fluorescence intensity (Fig. 3.25). The control substance EGCG reached similar values in accordance with earlier studies in *C. elegans* (Abbas & Wink, 2009, 2010). The effects of other substances were much lower in this assay. In the survival assay only ascorbic acid, EGCG, and the flavonoids LG and ILG showed a significant effect.

From all test substances ILG had the strongest effect in both assays. This is the first report of antioxidant activity of ILG in *C. elegans*, although such effects have been reported before in cell culture systems (Gong *et al.*, 2015; Lee *et al.*, 2012b). In HSP expression assay the fluorescence was decreased only by 26 %, whereas in the survival assay ILG increased the survival rate by 82 %, being even more effective than EGCG. EGCG showed a strong antioxidant effect in both assays. This illustrates, that both compounds act via different mechanisms. EGCG is a radical scavenger, it can directly react with the ROS generated by juglone and thereby prevent damage to the cells. ILG has shown radical scavenging activity in *in vitro* assays as well (Chin *et al.*, 2007; Vaya *et al.*, 1997), but due to the differences in structure it is a less potent radical scavenger than EGCG. Instead it can activate the

cells own protective mechanisms via the transcription factor NRF2 (Gong *et al.*, 2015). This pathway may be combined with the activation of other protective mechanisms like HSP expression, so that the effect is not so strongly seen in the HSP-expression assay, but is highly effective under severe oxidative stress as in survival assay. Since the activity of HSF-1, that controls the expression of HSPs, is necessary to counteract Aβ toxicity (Cohen *et al.*, 2006), the rather small effect of ILG in suppressing HSP expression may be related to the effective delay of paralysis in CL4176.

LG had only a small effect in the antioxidant assays. The antioxidant activity of LG and ILG has been attributed to the activation of NRF2. Wang *et al.* (2015) have shown, that ILG has a much stronger effect on the expression of genes that are under the control of NRF2 than LG. This correlates well with the results of antioxidant activity in *C. elegans* and also with the results in Aβ toxicity assays. NRF2 and its *C. elegans* homologue SKN-1 regulate the expression of stress related genes that could also be involved in detoxification of the Aβ oligomers. In AD patients the function of NRF2 has been shown to be impaired (Mota *et al.*, 2015). Since ILG can activate NRF2 and also influences the expression of some antioxidant genes independent of NRF2 activation (Wang *et al.*, 2015), this compound could be useful for counteracting oxidative stress in early stages of AD.

In *C. elegans* SKN-1 activation is regulated by the same kinases that also control DAF-16 and both transcription factors share some of their target genes (Tullet *et al.*, 2008). The effect of *G. uralensis* and its compounds on DAF-16 nuclear translocation was studied using the *C. elegans* strain TJ356. As shown in Fig. 3.28, the treatment with ILG led to activation of DAF-16, other *Glycyrrhiza* compounds and the extract had no significant effect. EGCG showed also no significant activity in both tested solvents, although it has been reported to be active before. Methanol alone had an effect in this assay, therefore low activities of some compounds like LG might have gone unnoticed.

Treatment with GRA or GA did not lead to nuclear localisation of DAF-16 although they are reported to modulate the activity of PI3K, the human homologue of AGE-1 (Kao *et al.*, 2009, 2013; Wang *et al.*, 2011). In *C. elegans* inhibition of AGE-1 leads to longer lifespan of the worms which is dependent on DAF-16 (Lin *et al.*, 1997; Morris *et al.*, 1996). Kao *et al.* (2009, 2013) have reported activation of PI3K by GA and GRA. This is in accordance with the result that no DAF-16 activation was observed for these treatments in *C. elegans*. Wang *et al.* (2011) reported inhibition of PI3K in active macrophages, contributing for the anti-inflammatory activity of GA and GRA. This shows, that this effect is dependent on the inflammatory state of the organism. Since in the present study no inflammation was induced in the worms, the treatment did not lead to DAF-16 activation. This result is also in line with the lack of antioxidant activity for GA and GRA.

The controls juglone and heat treatment in this test are active because they cause stress to the worms. This rises the question, weather the activity of ILG indicates a protective or a toxic effect? DAF-16, when active, prolongs the lifespan of *C. elegans* (Kenyon *et al.*, 1993) and activates the expression of stress resistance genes (Lee *et al.*, 2003; McElwee *et al.*, 2003; Murphy *et al.*, 2003). This activity is triggered by environmental stimuli that suggest the need of such protection—stressors and toxins (Henderson & Johnson, 2001). Therefore, it is a matter of concentration weather a toxin overpowers the protective effects and kills or damages the worm or is warded off effectively. In the latter case it comes to a hormetic response—a low concentration of the toxic substance leads to higher fitness of the organism, making it more resistant to stress and, at least in *C. elegans*, prolonging its life. Both heat and mild oxidative stress induced by juglone have been shown to trigger a hormetic response (Cypser & Johnson, 2002).

ILG was shown to be protective against the proteotoxicity of Aβ aggregates and against oxidative stress in the previously discussed experiments. Hormesis is a frequent mechanism of action for phytochemicals that is especially important for healthy ageing (Murugaiyah & Mattson, 2015; Son *et al.*, 2008). Hormetic pathways include transcription factors like NRF2, that has been shown to be activated by ILG before, or FOXO. Therefore, it can be assumed that at the used concentration ILG evokes a hormetic response by activating the *C. elegans* homologue of FOXO, DAF-16. Furthermore, this may be the mechanism of action also in other organisms, since NRF2 activation has been reported in mice and human cell cultures (Gong *et al.*, 2015; Wang *et al.*, 2015). To test this hypothesis, a dose-response analysis with a wide range of doses should be conducted.

Prolonged activity of FOXO can lead to AD-like symptoms (Manolopoulos *et al.*, 2010). This is in contrast to the positive effects seen for ILG via activation of DAF-16. In the experiments conducted for the present work, the worms were treated with the substances once, so the activation of DAF-16 was only transitional. It is possible that in an organism with constitutive stress and FOXO/DAF-16 activation an additional induction of this transcription factor has no beneficial effect (see section 4.4.5). If the activity of ILG is mediated by the suggested hormetic effect, it is probably better suited for prevention than treatment of fully developed dementia. However, the role of insulin signalling and FOXO in AD is not entirely established yet. Therefore, further studies with ILG in mammalian models are needed, to test the effect on FOXO, probable interactions with other signalling pathways, and the resulting consequences on AD.

4.4.5 Toxicity of long-term treatment with *Glycyrrhiza uralensis*

DAF-16 activity leads to longer lifespan in *C. elegans*. To test, if *G. uralensis* and its compounds are able to prolong life, lifespan assays were conducted with wildtype worms and CL2006. The experiment was discontinued after the observation that the compounds rather shortened the lifespan in the first run (Fig. 3.30). This effect was dose dependent for the *G. uralensis* extract and more pronounced in wildtype than in CL2006 worms. Possibly the protective effect of this extract against Aβ aggregation and toxicity has some benefits in the CL2006 worms that can counterbalance the toxicity resulting in shorter lifespan.

It is also notable that the lifespan of control worms was about twice as long than the three weeks reported before for worms growing at 20 °C (Klass, 1977). Several factors like temperature or food restriction can influence the lifespan of the worms but could be excluded as a cause for lifespan extension here. One possible explanation that could be determined was a measurable electromagnetic field in the incubator that led to DAF-16 activation in TJ356 worms (data not shown). *C. elegans* contains biogenic magnetite and static magnetic fields have an effect on gene expression of proteins related to development and ageing (Cranfield *et al.*, 2004; Hung *et al.*, 2010). Weather the long lifespan observed was related to the electromagnetic field was not further investigated. Another possibility is, that the treatment with 1 % methanol prolonged the lifespan. To our knowledge the effects of this solvent on *C. elegans* lifespan have not been studied before, but as discussed above, low methanol concentrations have an hormetic effect (Qin *et al.*, 2010). Therefore this treatment could affect stress resistance and lifespan.

From the pure compounds ILG shortened the half-time of life by 7 days and the maximum lifespan by 18 days in the wildtype worms. Its effect in CL2006 was smaller, but still stronger than for other treatments. LG had a similar effect in CL2006 but was closer to the control in the wildtype worms. GA and GRA were rather neutral in CL2006, although GRA shortened the lifespan in wildtype worms by 3.5 days. In wildtype this pattern resem-

bles the activity in DAF-16 delocalisation assay: ILG had the highest activity, followed by LG and GRA, GA had no effect. Hence, the toxicity might be related to prolonged activation of DAF-16, similar to the effects observed for FOXO (Manolopoulos *et al.*, 2010). ILG and LG had an effect against Aβ toxicity and showed antioxidant activity upon a single treatment, whereas GA and GRA did not. ILG and probably also LG act at least in part via hormetic pathways as discussed in section 4.4.4. Therefore, long-term treatment with these compounds would lead to constant activation of stress-related transcription factors like DAF-16/FOXO. That could explain why these compounds are more toxic, especially in the CL2006 strain that is already under stress because of Aβ toxicity, compared to the other compounds.

The results of the lifespan assay demonstrate, that long term treatment with high doses of *G. uralensis* or ILG might be toxic. This might be related to prolonged activation of DAF-16, but it could also have other causes. Therefore this possible toxicity needs further investigation to determine the concentration and time range in which these treatments are non-toxic. It would be also important to establish, weather this toxicity is specific to *C. elegans* or is also found in other species. Liquorice has been used in confectionery and as a food additive for a long time without adverse effects in humans. However, a medicinal preparation with high concentration of the plant extract or a pure compound that is used regularly over a long period of time might have different toxicological effects.

4.4.6 Is isoliquiritin a viable drug candidate?

The results for ILG in the present study prove efficacy against Aβ aggregation, Aβ toxicity, and oxidative stress that are prominent factors in the pathogenesis of AD. These results are in accordance with earlier studies *in vitro* as well as on cell cultures and mice (Chen *et al.*, 2013; Gong *et al.*, 2015; Lee *et al.*, 2012b; Vaya *et al.*, 1997). Additionally, ILG has anti-inflammatory (Kim *et al.*, 2008b; Park & Youn, 2010; Takahashi *et al.*, 2004; Wang *et al.*, 2015) and anti-diabetic properties (Gaur *et al.*, 2014; Ma *et al.*, 2015b) that would help against inflammation and insulin resistance in AD. The antidepressant-like activity reported for ILG (Wang *et al.*, 2008) could also be beneficial, since depression is one of the most frequent co-morbidities in patients with AD. Taken together, these evidence show reasonable efficacy and suggest ILG for further drug development.

Next to the efficacy bioavailability is an important factor in developing a drug. ILG has shown good permeability in a human colon carcinoma cell line (Caco-2) and a blood-brain-barrier model (Yang *et al.*, 2014). The permeability resulted mostly from passive diffusion. Studies in rats and humans with formulations containing ILG have reported low plasma concentrations (Sadakane *et al.*, 2015; Wu *et al.*, 2013). The absorption of ILG is rapid, and 92 % of the drug are absorbed after per oral administration (Choi *et al.*, 2015; Lee *et al.*, 2013; Qiao *et al.*, 2014; Sadakane *et al.*, 2015). However, the bioavailability is low due to high rate of metabolism in gastrointestinal tract and liver, and high protein binding was reported (Choi *et al.*, 2015; Lee *et al.*, 2013). In contrast to the results of Yang *et al.* (2014), only small amounts of ILG were found in the brain of rats and mice (Choi *et al.*, 2015; Lee *et al.*, 2013; Qiao *et al.*, 2014), suggesting a low permeability over the blood-brain-barrier *in vivo*.

In the current study the bioavailability in *C. elegans* was not investigated, but it has been reported before that the drug concentration in worms is similar to that in mice (Zheng *et al.*, 2013). The usage of live *E. coli* could have also mimicked some of the metabolic effects found in intestine of mammals. Therefore, it can be assumed that about 20 % of the substance reached its targets. This may explain, why only relatively small effects were seen in some

assays. On the other hand, a treatment of about 100 worms with 50 µg/ml still yielded significant improvement, suggesting that at least in *C. elegans* only small amounts of the drug are necessary for the desired activity.

The low bioavailability might be overcome by higher doses or alternative administration methods. However, a treatment with high concentrations of a substance needs careful consideration of drug safety. *Glycyrrhiza* spp. have been used for a long time in medicine and food industry, they are generally considered to be safe, and there are little toxicological studies available. Still, using high concentrations of a single compound can have side effects, especially considering the much higher tissue concentrations of ILG in gastrointestinal tract, liver, and kidney than in brain as target organ (Choi *et al.*, 2015; Lee *et al.*, 2013; Qiao *et al.*, 2014). Yang *et al.* (2014) found ILG to be cytotoxic to rat brain microvessel endothelial cells in concentrations higher than 20 µM. In the present study long term treatment of *C. elegans* shortened its lifespan. Therefore, further studies to determine safe concentrations in mammals are needed.

ILG is a promising drug candidate, but before it can be used for human treatment the issues of bioavailability and drug safety need to be solved. Derivatives of the substance might be useful and could also lead to better efficacy (Chen *et al.*, 2013). Also a combination with other substances that can alter the metabolism of ILG or add to its efficacy should be considered. Anyhow, this substance deserves further investigation in other model systems for AD.

4.5 *Carlina acaulis*

4.5.1 Isolation of Carlina oxide

Carlina oxide is the major compound in the dichloromethane extract, as can be seen in Figure 3.31. Small amounts of benzaldehyde and ar-curcumene were also detected, consistent with previous analysis of the essential oil of *C. acaulis* (Chalchat *et al.*, 1996; Stojanović-Radić *et al.*, 2012). The extract also contains some other substances that could not be identified. Based on their masses, these might be oxidation products of Carlina oxide. Since there is no published data about such substances, further analytical characterisation would be necessary to confirm this assumption. Chalchat *et al.* (1996) have reported the presence of small amounts of farnesene and sesquiphellandrene in the essential oil of *C. acaulis*. These highly lipophilic compounds were not found in the dichloromethane extract.

The purification via TLC yielded two fractions. Figure 3.32 shows that the first fraction contains most of the Carlina oxide, ar-curcumene and some of the unknown substances. The second fraction contains negligible amounts of Carlina oxide, but mostly other unidentified substances. Since the small amount of ar-curcumene in the first fraction is not likely to have any notable effect over Carlina oxide, and the unknown compounds are probably related to Carlina oxide, this fraction was handled as a pure substance. The second fraction containing more other substances than Carlina oxide was tested as a control to see, if a possible effect is attributable to Carlina oxide alone.

4.5.2 Dichlormethane extract of *Carlina acaulis* but not Carlina oxide has an effect against Aβ toxicity

C. acaulis has not been studied in context of AD before. It was chosen for the present study because of unexpected activity in the paralysis assay in a preliminary experiment. The effect against Aβ toxicity could be reproduced for 50 µg/ml. The lower concentration of the extract

and both fractions had no activity (Fig. 3.33). This was unexpected, because the *C. acaulis* extract comprises of over 80 % Carlina oxide. Therefore, this compound would be expected to have some activity, or else the extract without Carlina oxide should be active. Instead both fractions showed a small, not significant delay in paralysis. Apparently Carlina oxide and other compounds in the extract have an additive or even synergistic effect. Further research should identify all compounds responsible for the effect and elucidate their interactions.

The effect of *C. acaulis* was further studied in the *C. elegans* strain CL2355 with neuronal Aβ expression. Unfortunately, Carlina oxide seemed to be toxic in the chemotaxis assay and no meaningful results could be produced. In serotonin sensitivity assay lower concentrations were used, reducing the toxic effects. However, there seems to be no specific effect on serotonergic neurotransmission. *C. acaulis* extract showed a small significant effect for 10 µg/ml, but both a higher and a lower concentration had no activity. Carlina oxide had no effect on the CL2355 worms but affected the control stain. This suggests that the observed effects were unspecific and not related neither to Aβ toxicity nor to serotonin signalling.

The extract and Carlina oxide could activate DAF-16. This suggests, that Carlina oxide could have hormetic effects at low concentrations, similar to ILG. There are however no reports that would support or refute this assumption. It would also not explain the small effect of the extract without Carlina oxide in the paralysis assay, since this fraction could not activate DAF-16. *C. acaulis* is known to have antioxidant activity that could play a role in delaying paralysis and is discussed in the next section. Further research is needed to elucidate other possible mechanisms of action.

4.5.3 Carlina oxide exhibits *in vivo* antioxidant activity

Antioxidant activity of *Carlina* extracts and the essential oil has been observed before (Đorđević *et al.*, 2007, 2012), but only *in vitro* tests have been deployed. In the present work we showed the antioxidant activity of the dichloromethane extract of *C. acaulis* and proved that Carlina oxide is the active compound.

The extract has an EC_{50} of 122 µg/ml in the $DPPH^{\bullet}$ assay (Fig. 3.35). Đorđević *et al.* have reported an EC_{50} of 13.6 µl/ml for the essential oil of *C. acanthifolia* (Đorđević *et al.*, 2007) and 208 and 155 µg/ml for the methanol extracts of *C. acaulis* and *C. acanthifolia* roots, respectively (Đorđević *et al.*, 2012). Our result lies between the reported values for essential oil and methanol extracts. Since the essential oil only contains lipophilic compounds in high concentration, whereas a methanol extract can also include more hydrophilic compounds like flavonoids, these values are not directly comparable with the dichloromethane extract studied here. Nevertheless, the results for the dichloromethane extract are in the same range as earlier reported values. This data suggest, that the active antioxidant compound is rather lipophilic.

The main constituent of the extract is Carlina oxide (Fig. 3.31), leading to the assumption that it is also the active ingredient. Đorđević *et al.* (2007) showed via TLC that Carlina oxide has radical scavenging activity. Its antioxidant activity was confirmed in this work by the experiments with the isolated Carlina oxide and the extract without Carlina oxide. Both the full extract and Carlina oxide showed dose-dependent activity in HSP-16.2 expression and DAF-16 delocalisation assays. DAF-16 activation possibly contributes to the antioxidant activity by triggering the expression of SOD, glutathione S-transferase, catalase, metallothioneine, and other protective proteins, including HSP-16.2. Carlina oxide is slightly more active in both assays, whereas the extract without Carlina oxide has no effect. This would be expected when Carlina oxide is the active compound.

Carlina oxide was identified as the responsible compound for antioxidant activity, but was not significantly active in the paralysis assay. This suggests, that antioxidant activity and activation of DAF-16 alone are not sufficient to counteract the Aβ toxicity in CL4176 worms. Drake *et al.* (2003) have proposed that the Aβ-induced toxicity in CL4176 is related to build-up of free radicals and the resulting oxidative stress. The results presented here argue that this is not the only mechanism. The transcription factors DAF-16 and HSF-1, that regulates the expression of HSPs, are both needed to achieve the detoxification of Aβ and protect ageing worms (Cohen *et al.*, 2006; Hsu *et al.*, 2003). Carlina oxide activated DAF-16, but could also effectively prevent HSP expression, indicating that its antioxidant action is not related to HSF-1 activity. In contrast to that, ILG, that could counteract Aβ toxicity, had only a small effect on the HSP expression (see section 4.4.4).

4.5.4 Toxicity of *Carlina acaulis* and Carlina oxide

C. acaulis is a known antimicrobial drug with Carlina oxide as the main active compound (Schmidt-Thomé, 1950), it is also toxic to *Trypanosoma burcei burcei* (Herrmann *et al.*, 2011a) and 0.1 ml of the oil can be deadly to rats (Schmidt-Thomé, 1950). This poses the questions, if there might be toxic effects against *C. elegans*, if the extract is toxic to *E. coli* used as a food source in experiments with *C. elegans*, and if there might be problems considering usage of the drug in humans.

Đorđević *et al.* (2007, 2012) report good antimicrobial effects against several species including *E. coli*, but in general the activity is stronger against gram positive bacteria. This was also observed by Schmidt-Thomé (1950), who reported an initial bacteriostasis but normal growth after 24 h incubation for the gram negative bacteria. Since the bacteria used as food source for *C. elegans* are not supposed to grow during the experiments, the treatment with *C. acaulis* and Carlina oxide is not problematic.

Carlina oxide has been reported to be toxic for small organisms like trypanosomes (Herrmann *et al.*, 2011a). The authors suggest, that it inhibits trypanothione reductase, an enzyme found only in trypanosomes. Still, there might be other not so specific mechanisms. Carlina oxide in higher concentrations (> 50 µg/ml) was also toxic to *C. elegans*, therefore lower concentrations had to be used. In case of paralysis assay, where the worms are exposed to the drug from hatching, and in chemotaxis assay, which is in general more sensitive, a delay in development was observed. Otherwise the worms seemed to tolerate the treatment well, so there is no toxicity to *C. elegans* at tested concentrations. Interestingly, less toxicity was observed in a dichloromethane extract, that had been stored at –20 °C for several months. This extract was still effective in paralysis assay (data not shown), indicating that the active compounds are not necessarily the toxic ones.

Schmidt-Thomé (1950) planned to test the antimicrobial activity of *C. acaulis* essential oil on infected rats but failed because of the toxic effects of intraperitoneal and subcutaneous injections. The rats died in 10 hours. Đorđević *et al.* (2007, 2012) administered the oil or extracts to rats orally and reported no adverse effects. Herrmann *et al.* (2011a) tested extracts of the drug and Carlina oxide against HeLa cells and found no notable toxicity. In traditional medicine the drug is usually applied topically. Therefore it can be said, that the topical and oral administration of *C. acaulis* poses no threat to mammals, but injections should be avoided.

4.6 Conclusion

In the present study *G. uralensis* and ILG were identified as promising drug candidates for the prevention or treatment of AD. ILG exhibited protective properties against Aβ toxicity and oxidative stress possibly via hormetic pathways and by modifying serotonergic neurotransmission. It activated DAF-16, the *C. elegans* homologue of FOXO, that is responsible for several anti-stress reactions and extends lifespan in *C. elegans*. However, the treatments with *G. uralensis* and ILG shortened the life of worms, pointing to a possible long-term toxicity of these treatments. Further research should elucidate the efficacy and therapeutic dosage in mammals, and eventually in humans, allowing for metabolism and bioavailability of ILG and other active substances from *G. uralensis*.

C. acaulis was studied in the context of AD for the first time. A dichlormethane extract of this plant could counteract Aβ toxicity, but the main constituent Carlina oxide was not alone responsible for this effect. This compound, however, was the active constituent in antioxidant assays. The *in vivo* antioxidant activity for *C. acaulis* and Carlina oxide was reported for the first time. Further research is needed to clarify the active constituents and mechanism of action against Aβ toxicity and to elucidate possible toxicological issues in mammals.

The screening identified several other plants that modified Aβ aggregation and may therefore have beneficial effects for people with AD. ILG as well as other secondary metabolites from the effective plants may serve as useful lead structures for the development of new drugs. The plant extracts containing several active compounds can also be effective multitarget therapeutics, especially since there might be still unknown targets in AD. Since finding a cure for AD has proven to be difficult, maybe even impossible for the advanced stages of the disease, prevention is gaining more and more importance. Adding effective plants like *G. uralensis* to the regular diet might be a practical option for prevention.

Some secondary metabolites have specific targets, but more often the mechanism of action is more general. For example, ILG can activate transcription factors related to stress resistance and was suggested to act via hormetic pathways in the present work. Since protein aggregation, oxidative stress, and inflammation are not limited to AD but rather a common theme in many age related neurodegenerative diseases, the plants identified here might also be helpful in Parkinson's disease, dementia with Lewy bodies, or other similar conditions. Therefore, the identified plants and active secondary metabolites should be studied further in other models. Their usage for prevention and treatment of diseases as well as for healthy ageing in general should be considered.

References

Abbas S, Wink M (2009) Epigallocatechin gallate from green tea (*Camellia sinensis*) increases lifespan and stress resistance in *Caenorhabditis elegans*. *Planta Medica* **75**: 216–221.

Abbas S, Wink M (2010) Epigallocatechin gallate inhibits β amyloid oligomerization in *Caenorhabditis elegans* and affects the *daf-2*/insulin-like signaling pathway. *Phytomedicine* **17**: 902–909.

Abe T, Tohgi H, Isobe C, Murata T, Sato C (2002) Remarkable increase in the concentration of 8-hydroxyguanosine in cerebrospinal fluid from patients with Alzheimer's disease. *Journal of Neuroscience Research* **70**: 447–450.

Abisambra JF, Blair LJ, Hill SE, Jones JR, Kraft C, Rogers J, Koren J, Jinwal UK, Lawson L, Johnson AG, Wilcock D, O'Leary JC, Jansen-West K, Muschol M, Golde TE, Weeber EJ, Banko J, Dickey CA (2010) Phosphorylation dynamics regulate Hsp27-mediated rescue of neuronal plasticity deficits in tau transgenic mice. *The Journal of Neuroscience* **30**: 15374–15382.

Ahmed RM, Paterson RW, Warren JD, Zetterberg H, O'Brien JT, Fox NC, Halliday GM, Schott JM (2014) Biomarkers in dementia: clinical utility and new directions. *Journal of Neurology, Neurosurgery and Psychiatry* **85**: 1426–1434.

Ahn J, Um M, Choi W, Kim S, Ha T (2006) Protective effects of *Glycyrrhiza uralensis* Fisch. on the cognitive deficits caused by β-amyloid peptide 25–35 in young mice. *Biogerontology* **7**: 239–247.

Ahuja I, Kissen R, Bones AM (2012) Phytoalexins in defense against pathogens. *Trends in Plant Science* **17**: 73–90.

Alavez S, Vantipalli MC, Zucker DJS, Klang IM, Lithgow GJ (2011) Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan. *Nature* **472**: 226–229.

Albertson DG, Thomson JN (1976) The pharynx of *Caenorhabditis elegans*. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences* **275**: 299–325.

Alexander AG, Marfil V, Li C (2014) Use of *Caenorhabditis elegans* as a model to study Alzheimer's disease and other neurodegenerative diseases. *Frontiers in Genetics* **5**: 279–299.

Alkema MJ, Hunter-Ensor M, Ringstad N, Horvitz HR (2005) Tyramine functions independently of octopamine in the *Caenorhabditis elegans* nervous system. *Neuron* **46**: 247–260.

Alzheimer A (1907) Über eine eigenartige Erkrankung der Hirnrinde. *Allgemeine Zeitschrift für Psychiatrie und Psychisch-gerichtliche Medizin* **64**: 146–148.

Alzheimer A (1911) Über eigenartige Krankheitsfälle des späteren Alters. *Zeitschrift für die gesamte Neurologie und Psychiatrie* **4**: 356–385.

Alzheimer's Association (2015) 2015 Alzheimer's disease facts and figures. *Alzheimer's & Dementia* **11**: 332–384.

Amadoro G, Corsetti V, Atlante A, Florenzano F, Capsoni S, Bussani R, Mercanti D, Calissano P (2012) Interaction between NH_2-tau fragment and Aβ in Alzheimer's disease mitochondria contributes to the synaptic deterioration. *Neurobiology of Aging* **33**: 833.e1–833.e25.

An JH, Blackwell TK (2003) SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. *Genes and Development* **17**: 1882–1893.

Ancolio K, Dumanchin C, Barelli H, Warter JM, Brice A, Campion D, Frébourg T, Checler F (1999) Unusual phenotypic alteration of β amyloid precursor protein (βAPP) maturation by a new Val-715 → Met βAPP-770 mutation responsible for probable early-onset Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 4119–4124.

Apfeld J, Kenyon C (1999) Regulation of lifespan by sensory perception in *Caenorhabditis elegans*. *Nature* **402**: 804–809.

Arena JP, Liu KK, Paress PS, Schaeffer JM, Cully DF (1992) Expression of a glutamate-activated chloride current in *Xenopus* oocytes injected with *Caenorhabditis elegans* RNA: evidence for modulation by avermectin. *Molecular Brain Research* **15**: 339–348.

Arispe N (2004) Architecture of the Alzheimer's AβP ion channel pore. *The Journal of Membrane Biology* **197**: 33–48.

Armstrong RA (2009) The molecular biology of senile plaques and neurofibrillary tangles in Alzheimer's disease. *Folia Neuropathologica* **47**: 289–299.

Armstrong RA (2014) A critical analysis of the 'amyloid cascade hypothesis'. *Folia Neuropathologica* **52**: 211–225.

Arold S, Sullivan P, Bilousova T, Teng E, Miller CA, Poon WW, Vinters HV, Cornwell LB, Saing T, Cole GM, Gylys KH (2012) Apolipoprotein E level and cholesterol are associated with reduced synaptic amyloid β in Alzheimer's disease and apoE TR mouse cortex. *Acta Neuropathologica* **123**: 39–52.

Arriagada PV, Growdon JH, Hedley-Whyte ET, Hyman BT (1992) Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology* **42**: 631–639.

Atwood CS, Obrenovich ME, Liu T, Chan H, Perry G, Smith MA, Martins RN (2003) Amyloid-β: a chameleon walking in two worlds: a review of the trophic and toxic properties of amyloid-β. *Brain Research Brain Research Reviews* **43**: 1–16.

Atwood CS, Scarpa RC, Huang X, Moir RD, Jones WD, Fairlie DP, Tanzi RE, Bush AI (2000) Characterization of copper interactions with alzheimer amyloid β peptides: identification of an attomolar-affinity copper binding site on amyloid β1–42. *Journal of Neurochemistry* **75**: 1219–1233.

Bai Y, Tohda C, Zhu S, Hattori M, Komatsu K (2011) Active components from Siberian ginseng (*Eleutherococcus senticosus*) for protection of amyloid β(25–35)-induced neuritic atrophy in cultured rat cortical neurons. *Journal of Natural Medicines* **65**: 417–423.

Baloyannis SJ (2011) Mitochondria are related to synaptic pathology in Alzheimer's disease. *International Journal of Alzheimer's Disease* **2011**: 305395.

Ban JY, Nguyen HT, Lee HJ, Cho SO, Ju HS, Kim JY, Bae K, Song KS, Seong YH (2008) Neuroprotective properties of gallic acid from Sanguisorbae radix on amyloid β protein (25–35)-induced toxicity in cultured rat cortical neurons. *Biological and Pharmaceutical Bulletin* **31**: 149–153.

Bancher C, Brunner C, Lassmann H, Budka H, Jellinger K, Wiche G, Seitelberger F, Grundke-Iqbal I, Iqbal K, Wisniewski HM (1989) Accumulation of abnormally phosphorylated tau precedes the formation of neurofibrillary tangles in Alzheimer's disease. *Brain Research* **477**: 90–99.

Bargmann CI, Hartwieg E, Horvitz HR (1993) Odorant-selective genes and neurons mediate olfaction in *C. elegans*. *Cell* **74**: 515–527.

Bargmann CI, Horvitz HR (1991) Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in *C. elegans*. *Neuron* **7**: 729–742.

Barrow CJ, Yasuda A, Kenny PTM, Zagorski MG (1992) Solution conformations and aggregational properties of synthetic amyloid β-peptides of Alzheimer's disease: analysis of circular dichroism spectra. *Journal of Molecular Biology* **225**: 1075–1093.

Bastianetto S, Ramassamy C, Doré S, Christen Y, Poirier J, Quirion R (2000) The *Ginkgo biloba* extract (EGb 761) protects hippocampal neurons against cell death induced by β-amyloid. *European Journal of Neuroscience* **12**: 1882–1890.

Benilova I, Karran E, De Strooper B (2012) The toxic Aβ oligomer and Alzheimer's disease: an emperor in need of clothes. *Nature Neuroscience* **15**: 349–357.

Berdichevsky A, Viswanathan M, Horvitz HR, Guarente L (2006) *C. elegans* SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span. *Cell* **125**: 1165–1177.

Berger Z, Roder H, Hanna A, Carlson A, Rangachari V, Yue M, Wszolek Z, Ashe K, Knight J, Dickson D, Andorfer C, Rosenberry TL, Lewis J, Hutton M, Janus C (2007) Accumulation of pathological tau species and memory loss in a conditional model of tauopathy. *Journal of Neuroscience* **27**: 3650–3662.

Bertram L, McQueen MB, Mullin K, Blacker D, Tanzi RE (2007) Systematic meta-analyses of Alzheimer disease genetic association studies: the AlzGene database. *Nature Genetics* **39**: 17–23.

Beyreuther K, Pollwein P, Multhaup G, Mönning U, König G, Dyrks T, Schubert W, Masters CL (1993) Regulation and expression of the Alzheimer's β/A4 amyloid protein precursor in health, disease, and Down's syndrome. *Annals of the New York Academy of Sciences* **695**: 91–102.

Bieschke J, Russ J, Friedrich RP, Ehrnhoefer DE, Wobst H, Neugebauer K, Wanker EE (2010) EGCG remodels mature alpha-synuclein and amyloid-β fibrils and reduces cellular toxicity. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 7710–7715.

Binder LI, Frankfurter A, Rebhun LI (1985) The distribution of tau in the mammalian central nervous system. *Journal of Cell Biology* **101**: 1371–1378.

Birks J (2006) Cholinesterase inhibitors for Alzheimer's disease. *The Cochrane Database of Systematic Reviews* Cd005593.

Bitan G, Fradinger EA, Spring SM, Teplow DB (2005) Neurotoxic protein oligomers—what you see is not always what you get. *Amyloid* **12**: 88–95.

Blackard J W G, Sood GK, Crowe DR, Fallon MB (1998) Tacrine. A cause of fatal hepatotoxicity? *Journal of Clinical Gastroenterology* **26**: 57–59.

Blois MS (1958) Antioxidant determinations by the use of a stable free radical. *Nature* **181**: 1199–1200.

Bohlmann F, Mannhardt HJ (1957) Acetylenverbindungen im Pflanzenreich. *Fortschritte der Chemie Organischer Naturstoffe / Progress in the Chemistry of Organic Natural Products / Progrès dans la Chimie des Substances Organiques Naturelles* **14**: 1–70.

Bohlmann F, Rode KM (1967) Polyacetylenverbindungen, CXXVII. Die Polyine der Gattung *Carlina* L. *Chemische Berichte* **100**: 1507–1514.

Boyd-Kimball D, Poon HF, Lynn BC, Cai J, Pierce WM Jr, Klein JB, Ferguson J, Link CD, Butterfield DA (2006) Proteomic identification of proteins specifically oxidized in *Caenorhabditis elegans* expressing human Aβ(1–42): Implications for Alzheimer's disease. *Neurobiology of Aging* **27**: 1239–1249.

Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathologica* **82**: 239–259.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248–254.

Bré MH, Karsenti E (1990) Effects of brain microtubule-associated proteins on microtubule dynamics and the nucleating activity of centrosomes. *Cell Motility and the Cytoskeleton* **15**: 88–98.

Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71–94.

Bright J, Hussain S, Dang V, Wright S, Cooper B, Byun T, Ramos C, Singh A, Parry G, Stagliano N, Griswold-Prenner I (2015) Human secreted tau increases amyloid-β production. *Neurobiology of Aging* **36**: 693–709.

Brown MK, Evans JL, Luo Y (2006) Beneficial effects of natural antioxidants EGCG and α-lipoic acid on life span and age-dependent behavioral declines in *Caenorhabditis elegans*. *Pharmacology, Biochemistry and Behavior* **85**: 620–628.

Bu XL, Rao PP, Wang YJ (2015) Anti-amyloid aggregation activity of natural compounds: implications for Alzheimer's drug discovery. *Molecular Neurobiology* in press.

Burdick D, Kosmoski J, Knauer MF, Glabe CG (1997) Preferential adsorption, internalization and resistance to degradation of the major isoform of the Alzheimer's amyloid peptide, A β 1–42, in differentiated PC12 cells. *Brain Research* **746**: 275–284.

Burdick D, Soreghan B, Kwon M, Kosmoski J, Knauer M, Henschen A, Yates J, Cotman C, Glabe C (1992) Assembly and aggregation properties of synthetic Alzheimer's A4/β amyloid peptide analogs. *Journal of Biological Chemistry* **267**: 546–554.

Bürkland R (2014) *Tervise alkeemia*. Pilgrim: Tallinn.

Bush AI, Pettingell WH, Multhaup G, d Paradis M, Vonsattel JP, Gusella JF, Beyreuther K, Masters CL, Tanzi RE (1994) Rapid induction of Alzheimer Aβ amyloid formation by zinc. *Science* **265**: 1464–1467.

Byerly L, Cassada RC, Russell RL (1976) The life cycle of the nematode *Caenorhabditis elegans*: I. Wild-type growth and reproduction. *Developmental Biology* **51**: 23–33.

Calabrese EJ (2015) Hormesis within a mechanistic context. *Homeopathy* **104**: 90–96.

Calkins MJ, Reddy PH (2011) Amyloid β impairs mitochondrial anterograde transport and degenerates synapses in Alzheimer's disease neurons. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1812**: 507–513.

Canevelli M, Adali N, Kelaiditi E, Cantet C, Ousset PJ, Cesari M (2014) Effects of *Gingko biloba* supplementation in Alzheimer's disease patients receiving cholinesterase inhibitors: data from the ICTUS study. *Phytomedicine* **21**: 888–892.

Capetillo-Zarate E, Gracia L, Tampellini D, Gouras GK (2012) Intraneuronal Aβ accumulation, amyloid plaques, and synapse pathology in Alzheimer's disease. *Neurodegenerative Diseases* **10**: 56–59.

Cassada RC, Russell RL (1975) The dauerlarva, a post-embryonic developmental variant of the nematode *Caenorhabditis elegans*. *Developmental Biology* **46**: 326–342.

Castellano JM, Deane R, Gottesdiener AJ, Verghese PB, Stewart FR, West T, Paoletti AC, Kasper TR, Demattos RB, Zlokovic BV, Holtzman DM (2012) Low-density lipoprotein receptor overexpression enhances the rate of brain-to-blood Aβ clearance in a mouse model of β-amyloidosis. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 15502–15507.

Castellano JM, Kim J, Stewart FR, Jiang H, DeMattos RB, Patterson BW, Fagan AM, Morris JC, Mawuenyega KG, Cruchaga C, Goate AM, Bales KR, Paul SM, Bateman RJ, Holtzman DM (2011) Human apoE isoforms differentially regulate brain amyloid-β peptide clearance. *Science Translational Medicine* **3**: 89ra57.

Cha MY, Han SH, Son SM, Hong HS, Choi YJ, Byun J, Mook-Jung I (2012) Mitochondria-specific accumulation of amyloid β induces mitochondrial dysfunction leading to apoptotic cell death. *PLoS One* **7**: e34929.

Chalchat JC, Đorđević S, Gorunović M (1996) Composition of the essential oil from the root of *Carlina acaulis* L. Asteraceae. *Journal of Essential Oil Research* **8**: 577–578.

Chartier-Harlin MC, Crawford F, Houlden H, Warren A, Hughes D, Fidani L, Goate A, Rossor M, Roques P, Hardy J, Mullan M (1991) Early-onset Alzheimer's disease caused by mutations at codon 717 of the β-amyloid precursor protein gene. *Nature* **353**: 844–846.

Chen F, Eckman EA, Eckman CB (2006) Reductions in levels of the Alzheimer's amyloid β peptide after oral administration of ginsenosides. *FASEB Journal* **20**: 1269–1271.

Chen HJ, Kang SP, Lee IJ, Lin YL (2014) Glycyrrhetinic acid suppressed NF-κB activation in TNF-α-induced hepatocytes. *Journal of Agricultural and Food Chemistry* **62**: 618–625.

Chen YP, Zhang ZY, Li YP, Li D, Huang SL, Gu LQ, Xu J, Huang ZS (2013) Syntheses and evaluation of novel isoliquiritigenin derivatives as potential dual inhibitors for amyloid-β aggregation and 5-lipoxygenase. *European Journal of Medicinal Chemistry* **66**: 22–31.

Chiang WC, Ching TT, Lee HC, Mousigian C, Hsu AL (2012) HSF-1 regulators DDL-1/2 link insulin-like signaling to heat-shock responses and modulation of longevity. *Cell* **148**: 322–334.

Chin J, Palop JJ, Yu GQ, Kojima N, Masliah E, Mucke L (2004) Fyn kinase modulates synaptotoxicity, but not aberrant sprouting, in human amyloid precursor protein transgenic mice. *The Journal of Neuroscience* **24**: 4692–4697.

Chin YW, Jung HA, Liu Y, Su BN, Castoro JA, Keller WJ, Pereira MA, Kinghorn AD (2007) Antioxidant constituents of the roots and stolons of licorice (*Glycyrrhiza glabra*). *Journal of Agricultural and Food Chemistry* **55**: 4691–4697.

Choi EM, Suh KS, Lee YS (2014) Liquiritigenin restores osteoblast damage through regulating oxidative stress and mitochondrial dysfunction. *Phytotherapy Research* **28**: 880–886.

Choi RC, Zhu JT, Leung KW, Chu GK, Xie HQ, Chen VP, Zheng KY, Lau DT, Dong TT, Chow PC, Han YF, Wang ZT, Tsim KW (2010) A flavonol glycoside, isolated from roots of *Panax notoginseng*, reduces amyloid-β-induced neurotoxicity in cultured neurons: signaling transduction and drug development for Alzheimer's disease. *Journal of Alzheimer's Disease* **19**: 795–811.

Choi SJ, Kim MJ, Heo HJ, Kim HK, Hong B, Kim CJ, Kim BG, Shin DH (2006) Protective effect of *Rosa laevigata* against amyloid β peptide-induced oxidative stress. *Amyloid* **13**: 6–12.

Choi SJ, Kim MJ, Heo HJ, Kim JK, Jun WJ, Kim HK, Kim EK, Kim MO, Cho HY, Hwang HJ, Kim YJ, Shin DH (2009) Ameliorative effect of 1,2-benzenedicarboxylic acid dinonyl ester against amyloid β peptide-induced neurotoxicity. *Amyloid* **16**: 15–24.

Choi YH, Kim YJ, Chae HS, Chin YW (2015) *In vivo* gastroprotective effect along with pharmacokinetics, tissue distribution and metabolism of isoliquiritigenin in mice. *Planta Medica* **81**: 586–593.

Chyung JH, Selkoe DJ (2003) Inhibition of receptor-mediated endocytosis demonstrates generation of amyloid β-protein at the cell surface. *Journal of Biological Chemistry* **278**: 51035–51043.

Cirrito JR, Deane R, Fagan AM, Spinner ML, Parsadanian M, Finn MB, Jiang H, Prior JL, Sagare A, Bales KR, Paul SM, Zlokovic BV, Piwnica-Worms D, Holtzman DM (2005) P-glycoprotein deficiency at the blood-brain barrier increases amyloid-β deposition in an Alzheimer disease mouse model. *Journal of Clinical Investigation* **115**: 3285–3290.

Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ (1992) Mutation of the β-amyloid precursor protein in familial Alzheimer's disease increases β-protein production. *Nature* **360**: 672–674.

Clarke JR, Lyra ESNM, Figueiredo CP, Frozza RL, Ledo JH, Beckman D, Katashima CK, Razolli D, Carvalho BM, Frazão R, Silveira MA, Ribeiro FC, Bomfim TR, Neves FS, Klein WL, Medeiros R, LaFerla FM, Carvalheira JB, Saad MJ, Munoz DP, Velloso LA, Ferreira ST, De Felice FG (2015) Alzheimer-associated Aβ oligomers impact the central nervous system to induce peripheral metabolic deregulation. *EMBO Molecular Medicine* **7**: 190–210.

Cohen E, Bieschke J, Perciavalle RM, Kelly JW, Dillin A (2006) Opposing activities protect against age-onset proteotoxicity. *Science* **313**: 1604–1610.

Cohen E, Du D, Joyce D, Kapernick EA, Volovik Y, Kelly JW, Dillin A (2010) Temporal requirements of insulin/IGF-1 signaling for proteotoxicity protection. *Aging Cell* **9**: 126–134.

Colton CA, Chernyshev ON, Gilbert DL, Vitek MP (2000) Microglial contribution to oxidative stress in Alzheimer's disease. *Annals of the New York Academy of Sciences* **899**: 292–307.

Cook DG, Forman MS, Sung JC, Leight S, Kolson DL, Iwatsubo T, Lee VM, Doms RW (1997) Alzheimer's Aβ(1–42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells. *Nature Medicine* **3**: 1021–1023.

Corder EH, Saunders AM, Risch NJ, Strittmatter WJ, Schmechel DE, Gaskell J P C, Rimmler JB, Locke PA, Conneally PM, Schmader KE, Small GW, Roses AD, Haines JL, Pericak-Vance MA (1994) Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. *Nature Genetics* **7**: 180–184.

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261**: 921–923.

Couchie D, Mavilia C, Georgieff IS, Liem RK, Shelanski ML, Nunez J (1992) Primary structure of high molecular weight tau present in the peripheral nervous system. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 4378–4381.

Cowan CM, Quraishe S, Mudher A (2012) What is the pathological significance of tau oligomers? *Biochemical Society Transactions* **40**: 693–697.

Craddock TJ, Tuszynski JA, Chopra D, Casey N, Goldstein LE, Hameroff SR, Tanzi RE (2012) The zinc dyshomeostasis hypothesis of Alzheimer's disease. *PLoS One* **7**: e33552.

Cranfield CG, Dawe A, Karloukovski V, Dunin-Borkowski RE, de Pomerai D, Dobson J (2004) Biogenic magnetite in the nematode *Caenorhabditis elegans*. *Proceedings Biological sciences / The Royal Society* **271**: S436–439.

Crespo-Biel N, Theunis C, Van Leuven F (2012) Protein tau: prime cause of synaptic and neuronal degeneration in Alzheimer's disease. *International Journal of Alzheimer's Disease* **2012**: 251426–251438.

Cruts M, Theuns J, Van Broeckhoven C (2012) Locus-specific mutation databases for neurodegenerative brain diseases. *Human Mutation* **33**: 1340–1344.

Cuajungco MP, Goldstein LE, Nunomura A, Smith MA, Lim JT, Atwood CS, Huang X, Farrag YW, Perry G, Bush AI (2000) Evidence that the β-amyloid plaques of Alzheimer's disease represent the redox-silencing and entombment of Aβ by zinc. *Journal of Biological Chemistry* **275**: 19439–19442.

Cypser JR, Johnson TE (2002) Multiple stressors in *Caenorhabditis elegans* induce stress hormesis and extended longevity. *Journals of Gerontology Series A, Biological Sciences and Medical Sciences* **57**: B109–114.

Daigle I, Li C (1993) *apl-1*, a *Caenorhabditis elegans* gene encoding a protein related to the human β-amyloid protein precursor. *Proceedings of the National Academy of Sciences of the United States of America* **90**: 12045–12049.

D'Avanzo C, Sliwinski C, Wagner SL, Tanzi RE, Kim DY, Kovacs DM (2015) γ-Secretase modulators reduce endogenous amyloid β_{42} levels in human neural progenitor cells without altering neuronal differentiation. *FASEB Journal* **29**: 3335–3341.

Davidson JS, Baumgarten IM (1988) Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. Structure-activity relationships. *Journal of Pharmacology and Experimental Therapeutics* **246**: 1104–1107.

Davies P, Maloney AJ (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet* **2**: 1403.

De Jonghe C, Esselens C, Kumar-Singh S, Craessaerts K, Serneels S, Checler F, Annaert W, Van Broeckhoven C, De Strooper B (2001) Pathogenic APP mutations near the γ-secretase cleavage site differentially affect Aβ secretion and APP C-terminal fragment stability. *Human Molecular Genetics* **10**: 1665–1671.

Deane R, Du Yan S, Submamaryan RK, LaRue B, Jovanovic S, Hogg E, Welch D, Manness L, Lin C, Yu J, Zhu H, Ghiso J, Frangione B, Stern A, Schmidt AM, Armstrong DL, Arnold B, Liliensiek B, Nawroth P, Hofman F, Kindy M, Stern D, Zlokovic B (2003) RAGE mediates amyloid-β peptide transport across the blood-brain barrier and accumulation in brain. *Nature Medicine* **9**: 907–913.

Deibel MA, Ehmann WD, Markesbery WR (1996) Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress. *Journal of the Neurological Sciences* **143**: 137–142.

Denzer I, Münch G, Pischetsrieder M, Friedland K (2016) S-allyl-l-cysteine and isoliquiritigenin improve mitochondrial function in cellular models of oxidative and nitrosative stress. *Food Chemistry* **194**: 843–848.

Devi L, Prabhu BM, Galati DF, Avadhani NG, Anandatheerthavarada HK (2006) Accumulation of amyloid precursor protein in the mitochondrial import channels of human Alzheimer's disease brain is associated with mitochondrial dysfunction. *Journal of Neuroscience* **26**: 9057–9068.

Dhanasekaran M, Holcomb LA, Hitt AR, Tharakan B, Porter JW, Young KA, Manyam BV (2009) *Centella asiatica* extract selectively decreases amyloid β levels in hippocampus of Alzheimer's disease animal model. *Phytotherapy Research* **23**: 14–19.

Dhingra D, Parle M, Kulkarni SK (2004) Memory enhancing activity of *Glycyrrhiza glabra* in mice. *Journal of Ethnopharmacology* **91**: 361–365.

Dinamarca MC, Ríos JA, Inestrosa NC (2012) Postsynaptic receptors for amyloid-β oligomers as mediators of neuronal damage in Alzheimer's disease. *Frontiers in Physiology* **3**: 464–450.

Ding H, Johnson GV (2008) The last tangle of tau. *Journal of Alzheimer's Disease* **14**: 441–447.

Đorđević S, Petrović S, Dobrić S, Milenković M, Vučićević D, Žižić S, Kukić J (2007) Antimicrobial, anti-inflammatory, anti-ulcer and antioxidant activities of *Carlina acanthifolia* root essential oil. *Journal of Ethnopharmacology* **109**: 458–463.

Đorđević S, Petrović S, Ristić M, Đoković D (2005) Composition of *Carlina acanthifolia* root essential oil. *Chemistry of Natural Compounds* **41**: 410–412.

Đorđević S, Tadić V, Petrović S, Kukić-Marković J, Dobrić S, Milenković M, Hadžifejzović N (2012) Bioactivity assays on *Carlina acaulis* and *C. acanthifolia* root and herb extracts. *Digest Journal of Nanomaterials and Biostructures* **7**: 1213–1222.

Doig AJ, Derreumaux P (2015) Inhibition of protein aggregation and amyloid formation by small molecules. *Current Opinion in Structural Biology* **30c**: 50–56.

Donahue JE, Flaherty SL, Johanson CE, Duncan JAI, Silverberg GD, Miller MC, Tavares R, Yang W, Wu Q, Sabo E, Hovanesian V, Stopa EG (2006) RAGE, LRP-1, and amyloid-β protein in Alzheimer's disease. *Acta Neuropathologica* **112**: 405–415.

Dosanjh LE, Brown MK, Rao G, Link CD, Luo Y (2010) Behavioral phenotyping of a transgenic *Caenorhabditis elegans* expressing neuronal amyloid-β. *Journal of Alzheimers Disease* **19**: 681–690.

Dostal V, Link CD (2010) Assaying β-amyloid toxicity using a transgenic *C. elegans* model. *Journal of Visualized Experiments* **44**: e2252.

Dotti CG, Banker GA, Binder LI (1987) The expression and distribution of the microtubule-associated proteins tau and microtubule-associated protein 2 in hippocampal neurons in the rat in situ and in cell culture. *Neuroscience* **23**: 121–130.

Dou F, Netzer WJ, Tanemura K, Li F, Hartl FU, Takashima A, Gouras GK, Greengard P, Xu H (2003) Chaperones increase association of tau protein with microtubules. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 721–726.

Drake J, Link CD, Butterfield DA (2003) Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid β-peptide (1–42) in a transgenic *Caenorhabditis elegans* model. *Neurobiology of Aging* **24**: 415–420.

Durairajan SS, Liu LF, Lu JH, Chen LL, Yuan Q, Chung SK, Huang L, Li XS, Huang JD, Li M (2012) Berberine ameliorates β-amyloid pathology, gliosis, and cognitive impairment in an Alzheimer's disease transgenic mouse model. *Neurobiology of Aging* **33**: 2903–2919.

Durell SR, Guy HR, Arispe N, Rojas E, Pollard HB (1994) Theoretical models of the ion channel structure of amyloid β-protein. *Biophysical Journal* **67**: 2137–2145.

Dusenbery DB (1974) Analysis of chemotaxis in the nematode *Caenorhabditis elegans* by countercurrent separation. *Journal of Experimental Zoology* **188**: 41–47.

Dyrks T, Weidemann A, Multhaup G, Salbaum JM, Lemaire HG, Kang J, Müller-Hill B, Masters CL, Beyreuther K (1988) Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's disease. *EMBO Journal* **7**: 949–957.

Eckman CB, Mehta ND, Crook R, Perez-tur J, Prihar G, Pfeiffer E, Graff-Radford N, Hinder P, Yager D, Zenk B, Refolo LM, Prada CM, Younkin SG, Hutton M, Hardy J (1997) A new pathogenic mutation in the APP gene (I716V) increases the relative proportion of Aβ 42(43). *Human Molecular Genetics* **6**: 2087–2089.

Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, Haass C (2003) Reconstitution of γ-secretase activity. *Nature Cell Biology* **5**: 486–488.

Ehrnhoefer DE, Bieschke J, Boeddrich A, Herbst M, Masino L, Lurz R, Engemann S, Pastore A, Wanker EE (2008) EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. *Nature Structural and Molecular Biology* **15**: 558–566.

Esch FS, Keim PS, Beattie EC, Blacher RW, Culwell AR, Oltersdorf T, McClure D, Ward PJ (1990) Cleavage of amyloid β peptide during constitutive processing of its precursor. *Science* **248**: 1122–1124.

Ethell DW (2010) An amyloid-notch hypothesis for Alzheimer's disease. *Neuroscientist* **16**: 614–617.

Evin G, Hince C (2013) BACE1 as a therapeutic target in Alzheimer's disease: rationale and current status. *Drugs and Aging* **30**: 755–764.

Ewald CY, Cheng R, Tolen L, Shah V, Gillani A, Nasrin A, Li C (2012a) Pan-neuronal expression of APL-1, an APP-related protein, disrupts olfactory, gustatory, and touch plasticity in *Caenorhabditis elegans*. *Journal of Neuroscience* **32**: 10156–10169.

Ewald CY, Raps DA, Li C (2012b) APL-1, the Alzheimer's amyloid precursor protein in *Caenorhabditis elegans*, modulates multiple metabolic pathways throughout development. *Genetics* **191**: 493–507.

Farag MA, Porzel A, Wessjohann LA (2012) Comparative metabolite profiling and fingerprinting of medicinal licorice roots using a multiplex approach of GC-MS, LC-MS and 1D NMR techniques. *Phytochemistry* **76**: 60–72.

Fay DS, Fluet A, Johnson CJ, Link CD (1998) *In vivo* aggregation of β-amyloid peptide variants. *Journal of Neurochemistry* **71**: 1616–1625.

Fellous A, Francon J, Lennon AM, Nunez J (1977) Microtubule assembly *in vitro*. Purification of assembly-promoting factors. *European Journal of Biochemistry* **78**: 167–174.

Ferreira ST, Klein WL (2011) The Aβ oligomer hypothesis for synapse failure and memory loss in Alzheimer's disease. *Neurobiology of Learning and Memory* **96**: 529–543.

Fiore C, Eisenhut M, Ragazzi E, Zanchin G, Armanini D (2005) A history of the therapeutic use of liquorice in Europe. *Journal of Ethnopharmacology* **99**: 317–324.

Florez-McClure ML, Hohsfield LA, Fonte G, Bealor MT, Link CD (2007) Decreased insulin-receptor signaling promotes the autophagic degradation of β-amyloid peptide in *C. elegans*. *Autophagy* **3**: 569–580.

Fontaine SN, Sabbagh JJ, Baker J, Martinez-Licha CR, Darling A, Dickey CA (2015) Cellular factors modulating the mechanism of tau protein aggregation. *Cellular and Molecular Life Sciences* **72**: 1863–1879.

Fonte V, Kapulkin V, Taft A, Fluet A, Friedman D, Link CD (2002) Interaction of intracellular β amyloid peptide with chaperone proteins. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 9439–9444.

Fonte V, Kipp DR, Yerg J, Merin D, Forrestal M, Wagner E, Roberts CM, Link CD (2008) Suppression of *in vivo* β-amyloid peptide toxicity by overexpression of the HSP-16.2 small chaperone protein. *Journal of Biological Chemistry* **283**: 784–791.

Francis PT, Palmer AM, Snape M, Wilcock GK (1999) The cholinergic hypothesis of Alzheimer's disease: a review of progress. *Journal of Neurology, Neurosurgery & Psychiatry* **66**: 137–147.

Frankowski H, Alavez S, Spilman P, Mark KA, Nelson JD, Mollahan P, Rao RV, Chen SF, Lithgow GJ, Ellerby HM (2013) Dimethyl sulfoxide and dimethyl formamide increase lifespan of *C. elegans* in liquid. *Mechanisms of Ageing and Development* **134**: 69–78.

Garai K, Verghese PB, Baban B, Holtzman DM, Frieden C (2014) The binding of apolipoprotein E to oligomers and fibrils of amyloid-β alters the kinetics of amyloid aggregation. *Biochemistry* **53**: 6323–6331.

Gaur R, Yadav KS, Verma RK, Yadav NP, Bhakuni RS (2014) *In vivo* anti-diabetic activity of derivatives of isoliquiritigenin and liquiritigenin. *Phytomedicine* **21**: 415–422.

Gauthier S, Schlaefke S (2014) Efficacy and tolerability of *Ginkgo biloba* extract EGb 761® in dementia: a systematic review and meta-analysis of randomized placebo-controlled trials. *Clinical Interventions in Aging* **9**: 2065–2077.

Geldenhuys WJ, Darvesh AS (2014) Pharmacotherapy of Alzheimer's disease: current and future trends. *Expert Review of Neurotherapeutics* 1–3.

Gervais FG, Xu D, Robertson GS, Vaillancourt JP, Zhu Y, Huang J, LeBlanc A, Smith D, Rigby M, Shearman MS, Clarke EE, Zheng H, Van Der Ploeg LH, Ruffolo SC, Thornberry NA, Xanthoudakis S, Zamboni RJ, Roy S, Nicholson DW (1999) Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-β precursor protein and amyloidogenic Aβ peptide formation. *Cell* **97**: 395–406.

Ghayur MN, Kazim SF, Rasheed H, Khalid A, Jumani MI, Choudhary MI, Gilani AH (2011) Identification of antiplatelet and acetylcholinesterase inhibitory constituents in betel nut. *Zhong Xi Yi Jie He Xue Bao (Journal of Chinese Integrative Medicine)* **9**: 619–625.

Ghetti B, Oblak AL, Boeve BF, Johnson KA, Dickerson BC, Goedert M (2015) Frontotemporal dementia caused by *microtubule-associated protein tau* gene (*MAPT*) mutations: a chameleon for neuropathology and neuroimaging. *Neuropathology and Applied Neurobiology* **41**: 24–46.

Giaccone G, Rossi G, Morbin M, Tagliavini F, Bugiani O (2002) A713T mutation of the *APP* gene in an Italian family with Alzheimer disease and severe congophilic angiopathy. *Neurobiology of Aging* **23**: 320.

Gilani AH, Ghayur MN, Saify ZS, Ahmed SP, Choudhary MI, Khalid A (2004) Presence of cholinomimetic and acetylcholinesterase inhibitory constituents in betel nut. *Life Sciences* **75**: 2377–2389.

Gilman H, Van Ess PR, Burtner RR (1933) The constitution of Carlina-oxide. *Journal of the American Chemical Society* **55**: 3461–3463.

Glenner GG, Wong CW (1984a) Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. *Biochemical and Biophysical Research Communications* **122**: 1131–1135.

Glenner GG, Wong CW (1984b) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochemical and Biophysical Research Communications* **120**: 885–890.

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, Mant R, Newton P, Rooke K, Roques P, Talbot C, Pericak-Vance M, Roses A, Williamson R, Rossor M, Owen M, Hardy J (1991) Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* **349**: 704–706.

Goedert M, Spillantini MG, Jakes R, Rutherford D, Crowther RA (1989a) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron* **3**: 519–526.

Goedert M, Spillantini MG, Potier MC, Ulrich J, Crowther RA (1989b) Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. *EMBO Journal* **8**: 393–399.

Goldgaber D, Lerman MI, McBride OW, Saffiotti U, Gajdusek DC (1987) Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease. *Science* **235**: 877–880.

Gómez-Isla T, Hollister R, West H, Mui S, Growdon JH, Petersen RC, Parisi JE, Hyman BT (1997) Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. *Annals of Neurology* **41**: 17–24.

Gong H, Zhang B, Yan M, Fang P, Li H, Hu C, Yang Y, Cao P, Jiang P, Fan X (2015) A protective mechanism of licorice (*Glycyrrhiza uralensis*): isoliquiritigenin stimulates detoxification system via Nrf2 activation. *Journal of Ethnopharmacology* **162**: 134–139.

Gottlieb S, Ruvkun G (1994) *daf-2*, *daf-16* and *daf-23*: genetically interacting genes controlling dauer formation in *Caenorhabditis elegans*. *Genetics* **137**: 107–120.

Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR (2000) Intraneuronal Aβ42 accumulation in human brain. *American Journal of Pathology* **156**: 15–20.

Gowing E, Roher AE, Woods AS, Cotter RJ, Chaney M, Little SP, Ball MJ (1994) Chemical characterization of Aβ 17–42 peptide, a component of diffuse amyloid deposits of Alzheimer disease. *Journal of Biological Chemistry* **269**: 10987–10990.

Grabowski TJ, Cho HS, Vonsattel JP, Rebeck GW, Greenberg SM (2001) Novel amyloid precursor protein mutation in an Iowa family with dementia and severe cerebral amyloid angiopathy. *Annals of Neurology* **49**: 697–705.

Gravina SA, Ho L, Eckman CB, Long KE, Otvos L Jr, Younkin LH, Suzuki N, Younkin SG (1995) Amyloid β protein (Aβ) in Alzheimer's disease brain. Biochemical and immunocytochemical analysis with antibodies specific for forms ending at Aβ40 or Aβ42(43). *Journal of Biological Chemistry* **270**: 7013–7016.

Gray NE, Sampath H, Zweig JA, Quinn JF, Soumyanath A (2015) *Centella asiatica* attenuates amyloid-β-induced oxidative stress and mitochondrial dysfunction. *Journal of Alzheimer's Disease* **45**: 933–946.

Greenberg SG, Davies P, Schein JD, Binder LI (1992) Hydrofluoric acid-treated τ_{PHF} proteins display the same biochemical properties as normal τ. *Journal of Biological Chemistry* **267**: 564–569.

Grimm MO, Mett J, Stahlmann CP, Haupenthal VJ, Zimmer VC, Hartmann T (2013) Neprilysin and Aβ clearance: impact of the APP intracellular domain in NEP regulation and implications in Alzheimer's disease. *Frontiers in Aging Neuroscience* **5**: 98–124.

Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM (1986a) Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. *Journal of Biological Chemistry* **261**: 6084–6089.

Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI (1986b) Abnormal phosphorylation of the microtubule-associated protein τ(tau) in Alzheimer cytoskeletal pathology. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 4913–4917.

Guardia-Laguarta C, Pera M, Clarimón J, Molinuevo JL, Sánchez-Valle R, Lladó A, Coma M, Gómez-Isla T, Blesa R, Ferrer I, Lleó A (2010) Clinical, neuropathologic, and biochemical profile of the amyloid precursor protein I716F mutation. *Journal of Neuropathology and Experimental Neurology* **69**: 53–59.

Guo J, Chang L, Zhang X, Pei S, Yu M, Gao J (2014a) Ginsenoside compound K promotes β-amyloid peptide clearance in primary astrocytes via autophagy enhancement. *Experimental and Therapeutic Medicine* **8**: 1271–1274.

Guo J, Shang E, Zhao J, Fan X, Duan J, Qian D, Tao W, Tang Y (2014b) Data mining and frequency analysis for licorice as a "Two-Face" herb in Chinese Formulae based on Chinese Formulae Database. *Phytomedicine* **21**: 1281–1286.

Gustke N, Trinczek B, Biernat J, Mandelkow EM, Mandelkow E (1994) Domains of τ protein and interactions with microtubules. *Biochemistry* **33**: 9511–9522.

Haass C, Hung AY, Selkoe DJ, Teplow DB (1994) Mutations associated with a locus for familial Alzheimer's disease result in alternative processing of amyloid β-protein precursor. *Journal of Biological Chemistry* **269**: 17741–17748.

Haass C, Koo EH, Mellon A, Hung AY, Selkoe DJ (1992) Targeting of cell-surface β-amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. *Nature* **357**: 500–503.

Halliwell B, Gutteridge JM (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochemical Journal* **219**: 1–14.

Hanish Singh JC, Alagarsamy V, Diwan PV, Sathesh Kumar S, Nisha JC, Narsimha Reddy Y (2011) Neuroprotective effect of *Alpinia galanga* (L.) fractions on A$\beta_{(25–35)}$ induced amnesia in mice. *Journal of Ethnopharmacology* **138**: 85–91.

Hansson Petersen CA, Alikhani N, Behbahani H, Wiehager B, Pavlov PF, Alafuzoff I, Leinonen V, Ito A, Winblad B, Glaser E, Ankarcrona M (2008) The amyloid β-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 13145–13150.

Hardy J, Allsop D (1991) Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends in Pharmacological Sciences* **12**: 383–388.

Hardy J, Houlden H, Collinge J, Kennedy A, Newman S, Rossor M, Lannfelt L, Lilius L, Wmblad B, Crook R, Duff K (1993) Apolipoprotein E genotype and Alzheimer's disease. *The Lancet* **342**: 737–738.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**: 353–356.

Hardy JA, Higgins GA (1992) Alzheimer's disease: the amyloid cascade hypothesis. *Science* **256**: 184–185.

Harman D (1956) Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology* **11**: 298–300.

Hartman RE, Shah A, Fagan AM, Schwetye KE, Parsadanian M, Schulman RN, Finn MB, Holtzman DM (2006) Pomegranate juice decreases amyloid load and improves behavior in a mouse model of Alzheimer's disease. *Neurobiology of Disease* **24**: 506–515.

Hassan WM, Merin DA, Fonte V, Link CD (2009) AIP-1 ameliorates β-amyloid peptide toxicity in a *Caenorhabditis elegans* Alzheimer's disease model. *Human Molecular Genetics* **18**: 2739–2747.

Hattori M, Sakamoto T, Kobashi K, Namba T (1983) Metabolism of glycyrrhizin by human intestinal flora. *Planta Medica* **48**: 38–42.

Hawkes CA, McLaurin J (2009) Selective targeting of perivascular macrophages for clearance of β-amyloid in cerebral amyloid angiopathy. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 1261–1266.

Hayashi H, Hosono N, Kondo M, Hiraoka N, Ikeshiro Y, Shibano M, Kusano G, Yamamoto H, Tanaka T, Inoue K (2000) Phylogenetic relationship of six *Glycyrrhiza* species based on *rbcL* sequences and chemical constituents. *Biological and Pharmaceutical Bulletin* **23**: 602–606.

He J, Petrovic AG, Dzyuba SV, Berova N, Nakanishi K, Polavarapu PL (2008) Spectroscopic investigation of *Ginkgo biloba* terpene trilactones and their interaction with amyloid peptide Aβ(25–35). *Spectrochimica Acta Part A, Molecular and Biomolecular Spectroscopy* **69**: 1213–1222.

Heinrich M, Teoh HL (2004) Galanthamine from snowdrop—the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. *Journal of Ethnopharmacology* **92**: 147–162.

Henderson ST, Johnson TE (2001) *daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. *Current Biology* **11**: 1975–1980.

Hendriks L, van Duijn CM, Cras P, Cruts M, Van Hul W, van Harskamp F, Warren A, McInnis MG, Antonarakis SE, Martin JJ, Hofman A, Van Broeckhoven C (1992) Presenile dementia and cerebral haemorrhage linked to a mutation at codon 692 of the β-amyloid precursor protein gene. *Nature Genetics* **1**: 218–221.

Heo HJ, Kim DO, Choi SJ, Shin DH, Lee CY (2004) Potent inhibitory effect of flavonoids in *Scutellaria baicalensis* on amyloid β protein-induced neurotoxicity. *Journal of Agricultural and Food Chemistry* **52**: 4128–4132.

Hernandez-Zimbron LF, Luna-Muñoz J, Mena R, Vazquez-Ramirez R, Kubli-Garfias C, Cribbs DH, Manoutcharian K, Gevorkian G (2012) Amyloid-β peptide binds to cytochrome c oxidase subunit 1. *PLoS One* **7**: e42344.

Herrmann F, Hamoud R, Sporer F, Tahrani A, Wink M (2011a) Carlina oxide—a natural polyacetylene from *Carlina acaulis* (Asteraceae) with potent antitrypanosomal and antimicrobial properties. *Planta Medica* **77**: 1905–1911.

Herrmann F, Romero MR, Blazquez AG, Kaufmann D, Ashour ML, Kahl S, Marin JJ, Efferth T, Wink M (2011b) Diversity of pharmacological properties in Chinese and European medicinal plants: cytotoxicity, antiviral and antitrypanosomal screening of 82 herbal drugs. *Diversity* **3**: 547–580.

Herrup K (2015) The case for rejecting the amyloid cascade hypothesis. *Nature Neuroscience* **18**: 794–799.

Hertweck M, Göbel C, Baumeister R (2004) *C. elegans* SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span. *Developmental Cell* **6**: 577–588.

Hickman SE, Allison EK, El Khoury J (2008) Microglial dysfunction and defective β-amyloid clearance pathways in aging Alzheimer's disease mice. *Journal of Neuroscience* **28**: 8354–8360.

Himmler A, Drechsel D, Kirschner MW, Martin DW Jr (1989) Tau consists of a set of proteins with repeated C-terminal microtubule-binding domains and variable N-terminal domains. *Molecular and Cellular Biology* **9**: 1381–1388.

Hodgson AB, Randell RK, Mahabir-Jagessar TK, Lotito S, Mulder T, Mela DJ, Jeukendrup AE, Jacobs DM (2014) Acute effects of green tea extract intake on exogenous and endogenous metabolites in human plasma. *Journal of Agricultural and Food Chemistry* **62**: 1198–1208.

Hoi CP, Ho YP, Baum L, Chow AH (2010) Neuroprotective effect of honokiol and magnolol, compounds from *Magnolia officinalis*, on β-amyloid-induced toxicity in PC12 cells. *Phytotherapy Research* **24**: 1538–1542.

Horvitz HR, Chalfie M, Trent C, Sulston JE, Evans PD (1982) Serotonin and octopamine in the nematode *Caenorhabditis elegans*. *Science* **216**: 1012–1014.

Hosseinzadeh H, Nassiri-Asl M (2015) Pharmacological effects of *Glycyrrhiza* spp. and its bioactive constituents: update and review. *Phytotherapy Research* n/a–n/a.

Hoyer S (1988) Glucose and related brain metabolism in dementia of Alzheimer type and its morphological significance. *AGE* **11**: 158–166.

Hoyer S, Nitsch R, Oesterreich K (1991) Predominant abnormality in cerebral glucose utilization in late-onset dementia of the Alzheimer type: a cross-sectional comparison against advanced late-onset and incipient early-onset cases. *Journal of Neural Transmission Parkinsons Disease and Dementia Section* **3**: 1–14.

Hsin H, Kenyon C (1999) Signals from the reproductive system regulate the lifespan of *C. elegans*. *Nature* **399**: 362–366.

Hsu AL, Murphy CT, Kenyon C (2003) Regulation of aging and age-related disease by DAF-16 and heat-shock factor. *Science* **300**: 1142–1145.

Huang X, Cuajungco MP, Atwood CS, Hartshorn MA, Tyndall JD, Hanson GR, Stokes KC, Leopold M, Multhaup G, Goldstein LE, Scarpa RC, Saunders AJ, Lim J, Moir RD, Glabe C, Bowden EF, Masters CL, Fairlie DP, Tanzi RE, Bush AI (1999) Cu(II) potentiation of alzheimer Aβ neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction. *Journal of Biological Chemistry* **274**: 37111–37116.

Hung YC, Lee JH, Chen HM, Huang GS (2010) Effects of static magnetic fields on the development and aging of *Caenorhabditis elegans*. *Journal of Experimental Biology* **213**: 2079–2085.

Hussain I, Powell D, Howlett DR, Tew DG, Meek TD, Chapman C, Gloger IS, Murphy KE, Southan CD, Ryan DM, Smith TS, Simmons DL, Walsh FS, Dingwall C, Christie G (1999) Identification of a novel aspartic protease (Asp 2) as β-secretase. *Molecular and Cellular Neurosciences* **14**: 419–427.

Hyung SJ, DeToma AS, Brender JR, Lee S, Vivekanandan S, Kochi A, Choi JS, Ramamoorthy A, Ruotolo BT, Lim MH (2013) Insights into antiamyloidogenic properties of the green tea extract (–)-epigallocatechin-3-gallate toward metal-associated amyloid-β species. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 3743–3748.

Ihara Y, Nukina N, Miura R, Ogawara M (1986) Phosphorylated tau protein is integrated into paired helical filaments in Alzheimer's disease. *Journal of Biochemistry* **99**: 1807–1810.

Ittner LM, Ke YD, Delerue F, Bi M, Gladbach A, van Eersel J, Wölfing H, Chieng BC, Christie MJ, Napier IA, Eckert A, Staufenbiel M, Hardeman E, Götz J (2010) Dendritic function of tau mediates amyloid-β toxicity in Alzheimer's disease mouse models. *Cell* **142**: 387–397.

Iwata N, Tsubuki S, Takaki Y, Watanabe K, Sekiguchi M, Hosoki E, Kawashima-Morishima M, Lee HJ, Hama E, Sekine-Aizawa Y, Saido TC (2000) Identification of the major $A\beta_{1–42}$-degrading catabolic pathway in brain parenchyma: suppression leads to biochemical and pathological deposition. *Nature Medicine* **6**: 143–150.

Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y (1994) Visualization of Aβ42(43) and Aβ40 in senile plaques with end-specific Aβ monoclonals: evidence that an initially deposited species is Aβ42(43). *Neuron* **13**: 45–53.

Jaiswal R, Deshpande S, Kuhnert N (2011) Profiling the chlorogenic acids of *Rudbeckia hirta*, *Helianthus tuberosus*, *Carlina acaulis* and *Symphyotrichum novae-angliae* leaves by LC-MS^n. *Phytochemical Analysis* **22**: 432–441.

Ji HF, Shen L (2011) Berberine: a potential multipotent natural product to combat Alzheimer's disease. *Molecules* **16**: 6732–6740.

Jiang Q, Lee CYD, Mandrekar S, Wilkinson B, Cramer P, Zelcer N, Mann K, Lamb B, Willson TM, Collins JL, Richardson JC, Smith JD, Comery TA, Riddell D, Holtzman DM, Tontonoz P, Landreth GE (2008) ApoE promotes the proteolytic degradation of Aβ. *Neuron* **58**: 681–693.

Jonsson T, Atwal JK, Steinberg S, Snaedal J, Jonsson PV, Bjornsson S, Stefansson H, Sulem P, Gudbjartsson D, Maloney J, Hoyte K, Gustafson A, Liu Y, Lu Y, Bhangale T, Graham RR, Huttenlocher J, Bjornsdottir G, Andreassen OA, Jönsson EG, Palotie A, Behrens TW, Magnusson OT, Kong A, Thorsteinsdottir U, Watts RJ, Stefansson K (2012) A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. *Nature* **488**: 96–99.

Jović J, Mihajilov-Krstev T, Žabar A, Stojanović-Radić Z (2012) Influence of solvent on antimicrobial activity of Carlinae radix essential oil and decoct. *Biologica Nyssana* **3**: 61–67.

Junghans T (2003) Die Eberwurz—Wetterbote in Silber und Gold. *Pharmazie in unserer Zeit* **32**: 167–167.

Kaether C, Schmitt S, Willem M, Haass C (2006) Amyloid precursor protein and Notch intracellular domains are generated after transport of their precursors to the cell surface. *Traffic* **7**: 408–415.

Kamino K, Orr HT, Payami H, Wijsman EM, Alonso ME, Pulst SM, Anderson L, O'Dahl S, Nemens E, White JA, Sadovnick A, Ball M, Kaye J, Warren A, McInnis M, Antonarakis SE, Korenberg JR, Sharma V, Kukull W, Larson E, Heston LL, Martin GM, Bird TD, Schellenberg GD (1992) Linkage and mutational analysis of familial Alzheimer disease kindreds for the APP gene region. *American Journal of Human Genetics* **51**: 998–1014.

Kanaan NM, Morfini GA, LaPointe NE, Pigino GF, Patterson KR, Song Y, Andreadis A, Fu Y, Brady ST, Binder LI (2011) Pathogenic forms of tau inhibit kinesin-dependent axonal transport through a mechanism involving activation of axonal phosphotransferases. *Journal of Neuroscience* **31**: 9858–9868.

Kang J, Lemaire HG, Unterbeck A, Salbaum JM, Masters CL, Grzeschik KH, Multhaup G, Beyreuther K, Müller-Hill B (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**: 733–736.

Kang J, Müller-Hill B (1990) Differential splicing of Alzheimer's disease amyloid A4 precursor RNA in rat tissues: PreA4695 mRNA is predominantly produced in rat and human brain. *Biochemical and Biophysical Research Communications* **166**: 1192–1200.

Kanno H, Kawakami Z, Iizuka S, Tabuchi M, Mizoguchi K, Ikarashi Y, Kase Y (2013) *Glycyrrhiza* and *Uncaria* Hook contribute to protective effect of traditional Japanese medicine yokukansan against amyloid β oligomer-induced neuronal death. *Journal of Ethnopharmacology* **149**: 360–370.

Kanno H, Kawakami Z, Tabuchi M, Mizoguchi K, Ikarashi Y, Kase Y (2015) Protective effects of glycycoumarin and procyanidin B1, active components of traditional Japanese medicine yokukansan, on amyloid β oligomer-induced neuronal death. *Journal of Ethnopharmacology* **159**: 122–128.

Kao TC, Shyu MH, Yen GC (2009) Neuroprotective effects of glycyrrhizic acid and 18β-glycyrrhetinic acid in PC12 cells via modulation of the PI3K/Akt pathway. *Journal of Agricultural and Food Chemistry* **57**: 754–761.

Kao TC, Wu CH, Yen GC (2013) Glycyrrhizic acid and 18β-glycyrrhetinic acid recover glucocorticoid resistance via PI3K-induced AP1, CRE and NFAT activation. *Phytomedicine* **20**: 295–302.

Kao TC, Wu CH, Yen GC (2014) Bioactivity and potential health benefits of licorice. *Journal of Agricultural and Food Chemistry* **62**: 542–553.

Karran E, Mercken M, De Strooper B (2011) The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. *Nature Reviews Drug Discovery* **10**: 698–712.

Kawakami Z, Ikarashi Y, Kase Y (2011) Isoliquiritigenin is a novel NMDA receptor antagonist in Kampo medicine yokukansan. *Cellular and Molecular Neurobiology* **31**: 1203–1212.

Kayed R, Sokolov Y, Edmonds B, McIntire TM, Milton SC, Hall JE, Glabe CG (2004) Permeabilization of lipid bilayers is a common conformation-dependent activity of soluble amyloid oligomers in protein misfolding diseases. *Journal of Biological Chemistry* **279**: 46363–46366.

Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A *C. elegans* mutant that lives twice as long as wild type. *Nature* **366**: 461–464.

Keowkase R, Aboukhatwa M, Luo Y (2010) Fluoxetine protects against amyloid-beta toxicity, in part via *daf-16* mediated cell signaling pathway, in *Caenorhabditis elegans*. *Neuropharmacology* **59**: 358–365.

Kidd M (1963) Paired helical filaments in electron microscopy of Alzheimer's disease. *Nature* **197**: 192–193.

Kim DH, Kim S, Jeon SJ, Son KH, Lee S, Yoon BH, Cheong JH, Ko KH, Ryu JH (2008a) The effects of acute and repeated oroxylin A treatments on Aβ(25–35)-induced memory impairment in mice. *Neuropharmacology* **55**: 639–647.

Kim J, Kim SH, Lee DS, Lee DJ, Kim SH, Chung S, Yang HO (2013) Effects of fermented ginseng on memory impairment and β-amyloid reduction in Alzheimer's disease experimental models. *Journal of Ginseng Research* **37**: 100–107.

Kim JY, Park SJ, Yun KJ, Cho YW, Park HJ, Lee KT (2008b) Isoliquiritigenin isolated from the roots of *Glycyrrhiza uralensis* inhibits LPS-induced iNOS and COX-2 expression via the attenuation of NF-κB in RAW 264.7 macrophages. *European Journal of Pharmacology* **584**: 175–184.

Kimberly WT, LaVoie MJ, Ostaszewski BL, Ye W, Wolfe MS, Selkoe DJ (2003) γ-Secretase is a membrane protein complex comprised of presenilin, nicastrin, aph-1, and pen-2. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 6382–6387.

Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G (1997) *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. *Science* **277**: 942–946.

Kinoshita A, Fukumoto H, Shah T, Whelan CM, Irizarry MC, Hyman BT (2003) Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. *Journal of Cell Science* **116**: 3339–3346.

Kitazawa M, Cheng D, Tsukamoto MR, Koike MA, Wes PD, Vasilevko V, Cribbs DH, LaFerla FM (2011) Blocking IL-1 signaling rescues cognition, attenuates tau pathology, and restores neuronal β-catenin pathway function in an Alzheimer's disease model. *Journal of Immunology* **187**: 6539–6549.

Klass MR (1977) Aging in the nematode *Caenorhabditis elegans*: major biological and environmental factors influencing life span. *Mechanisms of Ageing and Development* **6**: 413–429.

Knops J, Suomensaari S, Lee M, McConlogue L, Seubert P, Sinha S (1995) Cell-type and amyloid precursor protein-type specific inhibition of Aβ release by bafilomycin A1, a selective inhibitor of vacuolar ATPases. *Journal of Biological Chemistry* **270**: 2419–2422.

Ko BS, Jang JS, Hong SM, Sung SR, Lee JE, Lee MY, Jeon WK, Park S (2007) Changes in components, glycyrrhizin and glycyrrhetinic acid, in raw *Glycyrrhiza uralensis* Fisch, modify insulin sensitizing and insulinotropic actions. *Bioscience, Biotechnology, and Biochemistry* **71**: 1452–1461.

Kondo K, Shiba M, Nakamura R, Morota T, Shoyama Y (2007a) Constituent properties of licorices derived from *Glycyrrhiza uralensis*, *G. glabra*, or *G. inflata* identified by genetic information. *Biological and Pharmaceutical Bulletin* **30**: 1271–1277.

Kondo K, Shiba M, Yamaji H, Morota T, Zhengmin C, Huixia P, Shoyama Y (2007b) Species identification of licorice using nrDNA and cpDNA genetic markers. *Biological and Pharmaceutical Bulletin* **30**: 1497–1502.

Koo EH, Squazzo SL (1994) Evidence that production and release of amyloid β-protein involves the endocytic pathway. *Journal of Biological Chemistry* **269**: 17386–17389.

Kopeikina KJ, Carlson GA, Pitstick R, Ludvigson AE, Peters A, Luebke JI, Koffie RM, Frosch MP, Hyman BT, Spires-Jones TL (2011) Tau accumulation causes mitochondrial distribution deficits in neurons in a mouse model of tauopathy and in human Alzheimer's disease brain. *American Journal of Pathology* **179**: 2071–2082.

Krabbe G, Halle A, Matyash V, Rinnenthal JL, Eom GD, Bernhardt U, Miller KR, Prokop S, Kettenmann H, Heppner FL (2013) Functional impairment of microglia coincides with β-amyloid deposition in mice with Alzheimer-like pathology. *PLoS One* **8**: e60921.

Krähenbühl S, Hasler F, Frey BM, Frey FJ, Brenneisen R, Krapf R (1994) Kinetics and dynamics of orally administered 18β-glycyrrhetinic acid in humans. *Journal of Clinical Endocrinology and Metabolism* **78**: 581–585.

Krishnan KJ, Ratnaike TE, Gruyter HL, Jaros E, Turnbull DM (2011) Mitochondrial DNA deletions cause the biochemical defect observed in Alzheimer's disease. *Neurobiology of Aging* **33**: 2210–2214.

Krstic D, Madhusudan A, Doehner J, Vogel P, Notter T, Imhof C, Manalastas A, Hilfiker M, Pfister S, Schwerdel C, Riether C, Meyer U, Knuesel I (2012) Systemic immune challenges trigger and drive Alzheimer-like neuropathology in mice. *Journal of Neuroinflammation* **9**: 151.

Kumar-Singh S, De Jonghe C, Cruts M, Kleinert R, Wang R, Mercken M, De Strooper B, Vanderstichele H, Löfgren A, Vanderhoeven I, Backhovens H, Vanmechelen E, Kroisel PM, Van Broeckhoven C (2000) Nonfibrillar diffuse amyloid deposition due to a γ_{42}-secretase site mutation points to an essential role for N-truncated Aβ_{42} in Alzheimer's disease. *Human Molecular Genetics* **9**: 2589–2598.

Kwok JB, Li QX, Hallupp M, Whyte S, Ames D, Beyreuther K, Masters CL, Schofield PR (2000) Novel Leu723Pro amyloid precursor protein mutation increases amyloid β42(43) peptide levels and induces apoptosis. *Annals of Neurology* **47**: 249–253.

Kwon ES, Narasimhan SD, Yen K, Tissenbaum HA (2010) A new DAF-16 isoform regulates longevity. *Nature* **466**: 498–502.

Laconi S, Madeddu MA, Pompei R (2014) Autophagy activation and antiviral activity by a licorice triterpene. *Phytotherapy Research* **28**: 1890–1892.

Lacor PN, Buniel MC, Chang L, Fernandez SJ, Gong Y, Viola KL, Lambert MP, Velasco PT, Bigio EH, Finch CE, Krafft GA, Klein WL (2004) Synaptic targeting by Alzheimer's-related amyloid β oligomers. *The Journal of Neuroscience* **24**: 10191–10200.

Lacor PN, Buniel MC, Furlow PW, Sanz Clemente A, Velasco PT, Wood M, Viola KL, Klein WL (2007) Aβ oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. *Journal of Neuroscience* **27**: 796–807.

LaDu MJ, Munson GW, Jungbauer L, Getz GS, Reardon CA, L MT, Yu C (2011) Preferential interactions between ApoE-containing lipoproteins and Aβ revealed by a detection method that combines size exclusion chromatography with non-reducing gel-shift. *Biochimica et Biophysica Acta* **1821**: 295–302.

Lai SW, Yu MS, Yuen WH, Chang RC (2006) Novel neuroprotective effects of the aqueous extracts from *Verbena officinalis* Linn. *Neuropharmacology* **50**: 641–650.

Lambert MP, Barlow AK, Chromy BA, Edwards C, Freed R, Liosatos M, Morgan TE, Rozovsky I, Trommer B, Viola KL, Wals P, Zhang C, Finch CE, Krafft GA, Klein WL (1998) Diffusible, nonfibrillar ligands derived from A$\beta_{1–42}$ are potent central nervous system neurotoxins. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 6448–6453.

Larson M, Sherman MA, Amar F, Nuvolone M, Schneider JA, Bennett DA, Aguzzi A, Lesné SE (2012) The complex PrP^c-Fyn couples human oligomeric Aβ with pathological tau changes in Alzheimer's disease. *Journal of Neuroscience* **32**: 16857–16871.

Lasagna-Reeves CA, Castillo-Carranza DL, Sengupta U, Clos AL, Jackson GR, Kayed R (2011) Tau oligomers impair memory and induce synaptic and mitochondrial dysfunction in wild-type mice. *Molecular Neurodegeneration* **6**: 39–52.

Laurén J, Gimbel DA, Nygaard HB, Gilbert JW, Strittmatter SM (2009) Cellular prion protein mediates impairment of synaptic plasticity by amyloid-β oligomers. *Nature* **457**: 1128–1132.

Lee CY, Tse W, Landreth GE (2012a) ApoE promotes Aβ trafficking and degradation by modulating microglial cholesterol levels. *Journal of Biological Chemistry* **287**: 2032–2044.

Lee HG, Casadesus G, Zhu X, Takeda A, Perry G, Smith MA (2004) Challenging the amyloid cascade hypothesis: senile plaques and amyloid-β as protective adaptations to Alzheimer disease. *Annals of the New York Academy of Sciences* **1019**: 1–4.

Lee HK, Yang EJ, Kim JY, Song KS, Seong YH (2012b) Inhibitory effects of Glycyrrhizae radix and its active component, isoliquiritigenin, on Aβ(25–35)-induced neurotoxicity in cultured rat cortical neurons. *Archives of Pharmacal Research* **35**: 897–904.

Lee JW, Lee YK, Lee BJ, Nam SY, Lee SI, Kim YH, Kim KH, Oh KW, Hong JT (2010a) Inhibitory effect of ethanol extract of *Magnolia officinalis* and 4-O-methylhonokiol on memory impairment and neuronal toxicity induced by β-amyloid. *Pharmacology, Biochemistry and Behavior* **95**: 31–40.

Lee RYN, Hench J, Ruvkun G (2001) Regulation of *C. elegans* DAF-16 and its human ortholog FKHRL1 by the *daf-2* insulin-like signaling pathway. *Current Biology* **11**: 1950–1957.

Lee SS, Kennedy S, Tolonen AC, Ruvkun G (2003) DAF-16 target genes that control *C. elegans* life-span and metabolism. *Science* **300**: 644–647.

Lee YJ, Choi DY, Han SB, Kim YH, Kim KH, Hwang BY, Kang JK, Lee BJ, Oh KW, Hong JT (2012c) Inhibitory effect of ethanol extract of *Magnolia officinalis* on memory impairment and amyloidogenesis in a transgenic mouse model of Alzheimer's disease via regulating β-secretase activity. *Phytotherapy Research* **26**: 1884–1892.

Lee YK, Chin YW, Bae JK, Seo JS, Choi YH (2013) Pharmacokinetics of isoliquiritigenin and its metabolites in rats: low bioavailability is primarily due to the hepatic and intestinal metabolism. *Planta Medica* **79**: 1656–1665.

Lee YS, Kim SH, Kim JK, Shin HK, Kang YH, Park JH, Lim SS (2010b) Rapid identification and preparative isolation of antioxidant components in licorice. *Journal of Separation Science* **33**: 664–671.

Levy E, Carman MD, Fernandez-Madrid IJ, Power MD, Lieberburg I, van Duinen SG, Bots GT, Luyendijk W, Frangione B (1990) Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. *Science* **248**: 1124–1126.

Lewczuk P, Mroczko B, Fagan A, Kornhuber J (2014) Biomarkers of Alzheimer's disease and mild cognitive impairment: a current perspective. *Advances in Medical Sciences* **60**: 76–82.

Lewis JA, Fleming JT, McLafferty S, Murphy H, Wu C (1987) The levamisole receptor, a cholinergic receptor of the nematode *Caenorhabditis elegans*. *Molecular Pharmacology* **31**: 185–193.

Lezi E, Swerdlow RH (2012) Mitochondria in neurodegeneration. *Advances in Experimental Medicine and Biology* **942**: 269–286.

Li F, Dong HX, Gong QH, Wu Q, Jin F, Shi JS (2015) Icariin decreases both APP and Aβ levels and increases neurogenesis in the brain of Tg2576 mice. *Neuroscience* **304**: 29–35.

Li Q, Zhao HF, Zhang ZF, Liu ZG, Pei XR, Wang JB, Li Y (2009) Long-term green tea catechin administration prevents spatial learning and memory impairment in senescence-accelerated mouse prone-8 mice by decreasing $A\beta_{1–42}$ oligomers and upregulating synaptic plasticity-related proteins in the hippocampus. *Neuroscience* **163**: 741–749.

Li W, Kennedy SG, Ruvkun G (2003) *daf-28* encodes a *C. elegans* insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway. *Genes and Development* **17**: 844–858.

Li Y, Zhao H, Wang Y, Zheng H, Yu W, Chai H, Zhang J, Falck JR, Guo AM, Yue J, Peng R, Yang J (2013a) Isoliquiritigenin induces growth inhibition and apoptosis through downregulating arachidonic acid metabolic network and the deactivation of PI3K/Akt in human breast cancer. *Toxicology and Applied Pharmacology* **272**: 37–48.

Li YH, Chen F, Wang JF, Wang Y, Zhang JQ, Guo T (2013b) Analysis of nine compounds from *Alpinia oxyphylla* fruit at different harvest time using UFLC-MS/MS and an extraction method optimized by orthogonal design. *Chemistry Central Journal* **7**: 134–142.

Lichtenthaler SF (2011) α-Secretase in Alzheimer's disease: molecular identity, regulation and therapeutic potential. *Journal of Neurochemistry* **116**: 10–21.

Lin H, Bhatia R, Lal R (2001a) Amyloid β protein forms ion channels: implications for Alzheimer's disease pathophysiology. *The FASEB Journal* **15**: 2433–2444.

Lin K, Dorman JB, Rodan A, Kenyon C (1997) *daf-16*: an HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. *Science* **278**: 1319–1322.

Lin K, Hsin H, Libina N, Kenyon C (2001b) Regulation of the *Caenorhabditis elegans* longevity protein DAF-16 by insulin/IGF-1 and germline signaling. *Nature Genetics* **28**: 139–145.

Lin X, Koelsch G, Wu S, Downs D, Dashti A, Tang J (2000) Human aspartic protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 1456–1460.

Lindwall G, Cole RD (1984a) Phosphorylation affects the ability of tau protein to promote microtubule assembly. *Journal of Biological Chemistry* **259**: 5301–5305.

Lindwall G, Cole RD (1984b) The purification of tau protein and the occurrence of two phosphorylation states of tau in brain. *Journal of Biological Chemistry* **259**: 12241–12245.

Ling D, Magallanes M, Salvaterra PM (2014) Accumulation of amyloid-like $A\beta_{1–42}$ in AEL (autophagy-endosomal-lysosomal) vesicles: potential implications for plaque biogenesis. *ASN Neuro* **6**: e00139.

Link CD (1995) Expression of human β-amyloid peptide in transgenic *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 9368–9372.

Link CD, Johnson CJ, Fonte V, Paupard MC, Hall DH, Styren S, Mathis CA, Klunk WE (2001) Visualization of fibrillar amyloid deposits in living, transgenic *Caenorhabditis elegans* animals using the sensitive amyloid dye, X-34. *Neurobiology of Aging* **22**: 217–226.

Link CD, Taft A, Kapulkin V, Duke K, Kim S, Fei Q, Wood DE, Sahagan BG (2003) Gene expression analysis in a transgenic *Caenorhabditis elegans* Alzheimer's disease model. *Neurobiology of Aging* **24**: 397–413.

Liu A, Zhao X, Li H, Liu Z, Liu B, Mao X, Guo L, Bi K, Jia Y (2014) 5-Hydroxymethylfurfural, an antioxidant agent from *Alpinia oxyphylla* Miq. improves cognitive impairment in Aβ mouse model of Alzheimer's disease. *International Immunopharmacology* **23**: 719–725.

Liu H, Wang J, Wang J, Wang P, Xue Y (2015a) Paeoniflorin attenuates $A\beta_{1–42}$-induced inflammation and chemotaxis of microglia *in vitro* and inhibits NF-κB- and VEGF/Flt-1 signaling pathways. *Brain Research* **1618**: 149–158.

Liu H, Xue X, Shi H, Qi L, Gong D (2015b) Osthole upregulates BDNF to enhance adult hippocampal neurogenesis in APP/PS1 transgenic mice. *Biological and Pharmaceutical Bulletin* **38**: 1439–1449.

Liu QF, Lee JH, Kim YM, Lee S, Hong YK, Hwang S, Oh Y, Lee K, Yun HS, Lee IS, Jeon S, Chin YW, Koo BS, Cho KS (2015c) *In vivo* screening of traditional medicinal plants for neuroprotective activity against Aβ42 cytotoxicity by using *Drosophila* models of Alzheimer's disease. *Biological and Pharmaceutical Bulletin* In press.

Liu RT, Tang JT, Zou LB, Fu JY, Lu QJ (2011) Liquiritigenin attenuates the learning and memory deficits in an amyloid protein precursor transgenic mouse model and the underlying mechanisms. *European Journal of Pharmacology* **669**: 76–83.

Liu RT, Zou LB, Fu JY, Lu QJ (2010) Effects of liquiritigenin treatment on the learning and memory deficits induced by amyloid β-peptide (25–35) in rats. *Behavioural Brain Research* **210**: 24–31.

Liu RT, Zou LB, Lu QJ (2009) Liquiritigenin inhibits $A\beta_{25–35}$-induced neurotoxicity and secretion of $A\beta_{1–40}$ in rat hippocampal neurons. *Acta Pharmacologica Sinica* **30**: 899–906.

Liu X, Hao W, Qin Y, Decker Y, Wang X, Burkart M, Schötz K, Menger MD, Fassbender K, Liu Y (2015d) Long-term treatment with *Ginkgo biloba* extract EGb 761 improves symptoms and pathology in a transgenic mouse model of Alzheimer's disease. *Brain, Behavior and Immunity* **46**: 121–131.

Liu X, Yang MH, Wang XB, Xie SS, Li ZR, Kim DH, Park JS, Kong LY (2015e) Lignans from the root of *Paeonia lactiflora* and their anti-β-amyloid aggregation activities. *Fitoterapia* **103**: 136–142.

Lloret A, Fuchsberger T, Giraldo E, Viña J (2015) Molecular mechanisms linking amyloid β toxicity and Tau hyperphosphorylation in Alzheimer's disease. *Free Radical Biology and Medicine* **83**: 186–191.

Lourenco MV, Ferreira ST, De Felice FG (2015) Neuronal stress signaling and eIF2α phosphorylation as molecular links between Alzheimer's disease and diabetes. *Progress in Neurobiology* **129**: 37–57.

Lovell MA, Ehmann WD, Butler SM, Markesbery WR (1995) Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease. *Neurology* **45**: 1594–1601.

Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR (1998) Copper, iron and zinc in Alzheimer's disease senile plaques. *Journal of the Neurological Sciences* **158**: 47–52.

Lovestone S, Reynolds CH (1997) The phosphorylation of tau: a critical stage in neurodevelopment and neurodegenerative processes. *Neuroscience* **78**: 309–324.

Lublin AL, Link CD (2013) Alzheimer's disease drug discovery: *in vivo* screening using *Caenorhabditis elegans* as a model for β-amyloid peptide-induced toxicity. *Drug Discovery Today: Technologies* **10**: e115–119.

Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y, Sue L, Beach T, Kurth JH, Rydel RE, Rogers J (1999) Soluble amyloid β peptide concentration as a predictor of synaptic change in Alzheimer's disease. *American Journal of Pathology* **155**: 853–862.

Luna-Muñoz J, Chávez-Macías L, García-Sierra F, Mena R (2007) Earliest stages of tau conformational changes are related to the appearance of a sequence of specific phospho-dependent tau epitopes in Alzheimer's disease. *Journal of Alzheimer's Disease* **12**: 365–375.

Luo L, Jin Y, Kim ID, Lee JK (2013) Glycyrrhizin attenuates kainic acid-induced neuronal cell death in the mouse hippocampus. *Experimental Neurobiology* **22**: 107–115.

Luo YF, Zhang J, Liu NQ, Luo Y, Zhao BL (2011) Copper ions influence the toxicity of β-amyloid(1–42) in a concentration-dependent manner in a *Caenorhabditis elegans* model of Alzheimer's disease. *Science China Life Sciences* **54**: 527–534.

Lustbader JW, Cirilli M, Lin C, Xu HW, Takuma K, Wang N, Caspersen C, Chen X, Pollak S, Chaney M, Trinchese F, Liu S, Gunn-Moore F, Lue LF, Walker DG, Kuppusamy P, Zewier ZL, Arancio O, Stern D, Yan SS, Wu H (2004) ABAD directly links Aβ to mitochondrial toxicity in Alzheimer's disease. *Science* **304**: 448–452.

Lutomski J (1983) Chemie und therapeutische Verwendung von Süßholz (*Glycyrrhiza glabra* L.). *Pharmazie in unserer Zeit* **12**: 49–54.

Lyketsos CG, Carrillo MC, Ryan JM, Khachaturian AS, Trzepacz P, Amatniek J, Cedarbaum J, Brashear R, Miller DS (2011) Neuropsychiatric symptoms in Alzheimer's disease. *Alzheimer's & Dementia* **7**: 532–539.

Ma B, Meng X, Wang J, Sun J, Ren X, Qin M, Sun J, Sun G, Sun X (2014) Notoginsenoside R1 attenuates amyloid-β-induced damage in neurons by inhibiting reactive oxygen species and modulating MAPK activation. *International Immunopharmacology* **22**: 151–159.

Ma L, Wang J, Li Y (2015a) Insulin resistance and cognitive dysfunction. *Clinica Chimica Acta* **444**: 18–23.

Ma X, Fang F, Song M, Ma S (2015b) The effect of isoliquirigenin on learning and memory impairments induced by high-fat diet via inhibiting TNF-α/JNK/IRS signaling. *Biochemical and Biophysical Research Communications* **464**: 1090–1095.

Maccioni RB, Vera JC, Dominguez J, Avila J (1989) A discrete repeated sequence defines a tubulin binding domain on microtubule-associated protein tau. *Archives of Biochemistry and Biophysics* **275**: 568–579.

Mahadevan S, Park Y (2008) Multifaceted therapeutic benefits of *Ginkgo biloba* L.: chemistry, efficacy, safety, and uses. *Journal of Food Science* **73**: R14–19.

Manolopoulos KN, Klotz LO, Korsten P, Bornstein SR, Barthel A (2010) Linking Alzheimer's disease to insulin resistance: the FoxO response to oxidative stress. *Molecular Psychiatry* **15**: 1046–1052.

Markesbery WR, Lovell MA (1998) Four-hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease. *Neurobiology of Aging* **19**: 33–36.

Masters CL, Simms G, Weinman NA, Multhaup G, McDonald BL, Beyreuther K (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proceedings of the National Academy of Sciences* **82**: 4245–4249.

Matsunaga S, Kishi T, Iwata N (2015) Memantine monotherapy for Alzheimer's disease: a systematic review and meta-analysis. *PLoS One* **10**: e0123289.

Mattson MP (2008) Hormesis defined. *Ageing Research Reviews* **7**: 1–7.

Maupas E (1900) Modes et formes de reproduction des nématodes. *Archives de zoologie expérimentale et générale* **8**: 463–624.

Maurer I, Zierz S, Möller HJ (2000) A selective defect of cytochrome c oxidase is present in brain of Alzheimer disease patients. *Neurobiology of Aging* **21**: 455–462.

Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, Yarasheski KE, Bateman RJ (2010) Decreased clearance of CNS β-amyloid in Alzheimer's disease. *Science* **330**: 1774.

Mayeux R, Stern Y (2012) Epidemiology of Alzheimer disease. *Cold Spring Harbor Perspectives in Medicine* **2**: a006239.

McColl G, Roberts BR, Gunn AP, Perez KA, Tew DJ, Masters CL, Barnham KJ, Cherny RA, Bush AI (2009) The *Caenorhabditis elegans* $A\beta_{1–42}$ model of Alzheimer disease predominantly expresses $A\beta_{3–42}$. *Journal of Biological Chemistry* **284**: 22697–22702.

McElwee J, Bubb K, Thomas JH (2003) Transcriptional outputs of the *Caenorhabditis elegans* forkhead protein DAF-16. *Aging Cell* **2**: 111–121.

McIntire SL, Jorgensen E, Kaplan J, Horvitz HR (1993) The GABAergic nervous system of *Caenorhabditis elegans*. *Nature* **364**: 337–341.

Mecocci P, MacGarvey U, Beal MF (1994) Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Annals of Neurology* **36**: 747–751.

Mersereau JE, Levy N, Staub RE, Baggett S, Zogovic T, Chow S, Ricke WA, Tagliaferri M, Cohen I, Bjeldanes LF, Leitman DC (2008) Liquiritigenin is a plant-derived highly selective estrogen receptor β agonist. *Molecular and Cellular Endocrinology* **283**: 49–57.

Meusel H, Kästner A (1990) *Lebensgeschichte der Gold- und Silberdisteln: Monographie der mediterran-mitteleuropäischen Compositen-Gattung Carlina*. Volume 1 of *Denkschriften / Österreichische Akademie der Wissenschaften, Mathematisch-Naturwissenschaftliche Klasse*, Springer-Verlag: Wien.

Mikulca JA, Nguyen V, Gajdosik DA, Teklu SG, Giunta EA, Lessa EA, Tran CH, Terak EC, Raffa RB (2014) Potential novel targets for Alzheimer pharmacotherapy: II. Update on secretase inhibitors and related approaches. *Journal of Clinical Pharmacy and Therapeutics* **39**: 25–37.

Mildner A, Schlevogt B, Kierdorf K, Böttcher C, Erny D, Kummer MP, Quinn M, Brück W, Bechmann I, Heneka MT, Priller J, Prinz M (2011) Distinct and non-redundant roles of microglia and myeloid subsets in mouse models of Alzheimer's disease. *Journal of Neuroscience* **31**: 11159–11171.

Miller DL, Papayannopoulos IA, Styles J, Bobin SA, Lin YY, Biemann K, Iqbal K (1993) Peptide compositions of the cerebrovascular and senile plaque core amyloid deposits of Alzheimer's disease. *Archives of Biochemistry and Biophysics* **301**: 41–52.

Miners JS, Barua N, Kehoe PG, Gill S, Love S (2011) Aβ-degrading enzymes: potential for treatment of Alzheimer disease. *Journal of Neuropathology and Experimental Neurology* **70**: 944–959.

Miners JS, Palmer JC, Tayler H, Palmer LE, Ashby E, Kehoe PG, Love S (2014) Aβ degradation or cerebral perfusion? Divergent effects of multifunctional enzymes. *Frontiers in Aging Neuroscience* **6**: 238–250.

Ming LJ, Yin AC (2013) Therapeutic effects of glycyrrhizic acid. *Natural Product Communications* **8**: 415–418.

Monder C, Stewart PM, Lakshmi V, Valentino R, Burt D, Edwards CR (1989) Licorice inhibits corticosteroid 11β-dehydrogenase of rat kidney and liver: *in vivo* and *in vitro* studies. *Endocrinology* **125**: 1046–1053.

Montoro P, Maldini M, Russo M, Postorino S, Piacente S, Pizza C (2011) Metabolic profiling of roots of liquorice (*Glycyrrhiza glabra*) from different geographical areas by ESI/MS/MS and determination of major metabolites by LC-ESI/MS and LC-ESI/MS/MS. *Journal of Pharmaceutical and Biomedical Analysis* **54**: 535–544.

Moreira PI, Santos MS, Moreno A, Rego AC, Oliveira C (2002) Effect of amyloid β-peptide on permeability transition pore: a comparative study. *Journal of Neuroscience Research* **69**: 257–267.

Morgen K, Frölich L (2015) The metabolism hypothesis of Alzheimer's disease: from the concept of central insulin resistance and associated consequences to insulin therapy. *Journal of Neural Transmission* **122**: 499–504.

Mori I, Ohshima Y (1995) Neural regulation of thermotaxis in *Caenorhabditis elegans*. *Nature* **376**: 344–348.

Mori T, Koyama N, Segawa T, Maeda M, Maruyama N, Kinoshita N, Hou H, Tan J, Town T (2014) Methylene blue modulates β-secretase, reverses cerebral amyloidosis, and improves cognition in transgenic mice. *Journal of Biological Chemistry* **289**: 30303–30317.

Morley JF, Morimoto RI (2004) Regulation of longevity in *Caenorhabditis elegans* by heat shock factor and molecular chaperones. *Molecular Biology of the Cell* **15**: 657–664.

Morris JZ, Tissenbaum HA, Ruvkun G (1996) A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*. *Nature* **382**: 536–539.

Mota SI, Costa RO, Ferreira IL, Santana I, Caldeira GL, Padovano C, Fonseca AC, Baldeiras I, Cunha C, Letra L, Oliveira CR, Pereira CM, Rego AC (2015) Oxidative stress involving changes in Nrf2 and ER stress in early stages of Alzheimer's disease. *Biochimica et Biophysica Acta* **1852**: 1428–1441.

Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L (1992) A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of β-amyloid. *Nature Genetics* **1**: 345–347.

Murakami A (2014) Dose-dependent functionality and toxicity of green tea polyphenols in experimental rodents. *Archives of Biochemistry and Biophysics* **557**: 3–10.

Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, Li H, Kenyon C (2003) Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans*. *Nature* **424**: 277–283.

Murrell J, Farlow M, Ghetti B, Benson MD (1991) A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* **254**: 97–99.

Murrell JR, Hake AM, Quaid KA, Farlow MR, Ghetti B (2000) Early-onset Alzheimer disease caused by a new mutation (V717L) in the amyloid precursor protein gene. *Archives of Neurology* **57**: 885–887.

Murugaiyah V, Mattson MP (2015) Neurohormetic phytochemicals: an evolutionary-bioenergetic perspective. *Neurochemistry International* **89**: 271–280.

Musiek ES, Holtzman DM (2015) Three dimensions of the amyloid hypothesis: time, space and 'wingmen'. *Nature Neuroscience* **18**: 800–806.

Mutisya EM, Bowling AC, Beal MF (1994) Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. *Journal of Neurochemistry* **63**: 2179–2184.

Nassiri-Asl M, Hosseinzadeh H (2008) Review of pharmacological effects of *Glycyrrhiza* sp. and its bioactive compounds. *Phytotherapy Research* **22**: 709–724.

Neve RL, Harris P, Kosik KS, Kurnit DM, Donlon TA (1986) Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. *Brain Research* **387**: 271–280.

Nhan HS, Chiang K, Koo EH (2015) The multifaceted nature of amyloid precursor protein and its proteolytic fragments: friends and foes. *Acta Neuropathologica* **129**: 1–19.

Nie BM, Jiang XY, Cai JX, Fu SL, Yang LM, Lin L, Hang Q, Lu PL, Lu Y (2008) Panaxydol and panaxynol protect cultured cortical neurons against Aβ25–35-induced toxicity. *Neuropharmacology* **54**: 845–853.

Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, Stenh C, Luthman J, Teplow DB, Younkin SG, Näslund J, Lannfelt L (2001) Localization and trafficking of amyloid-β protein precursor and pecretases: impact on Alzheimer's disease. *Nature Neuroscience* **4**: 887–893.

Nixon RA, Yang DS (2011) Autophagy failure in Alzheimer's disease—locating the primary defect. *Neurobiology of Disease* **43**: 38–45.

Noda Y, Asada M, Kubota M, Maesako M, Watanabe K, Uemura M, Kihara T, Shimohama S, Takahashi R, Kinoshita A, Uemura K (2013) Copper enhances APP dimerization and promotes Aβ production. *Neuroscience Letters* **547**: 10–15.

Nunomura A, Perry G, Pappolla MA, Wade R, Hirai K, Chiba S, Smith MA (1999) RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. *Journal of Neuroscience* **19**: 1959–1964.

Obici L, Demarchi A, de Rosa G, Bellotti V, Marciano S, Donadei S, Arbustini E, Palladini G, Diegoli M, Genovese E, Ferrari G, Coverlizza S, Merlini G (2005) A novel Aβ PP mutation exclusively associated with cerebral amyloid angiopathy. *Annals of Neurology* **58**: 639–644.

Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, Ruvkun G (1997) The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature* **389**: 994–999.

Ogg S, Ruvkun G (1998) The *C. elegans* PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. *Molecular Cell* **2**: 887–893.

Oh SW, Mukhopadhyay A, Dixit BL, Raha T, Green MR, Tissenbaum HA (2006) Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation. *Nature Genetics* **38**: 251–257.

Oh SW, Mukhopadhyay A, Svrzikapa N, Jiang F, Davis RJ, Tissenbaum HA (2005) JNK regulates lifespan in *Caenorhabditis elegans* by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 4494–4499.

Oikonomou G, Shaham S (2011) The glia of *Caenorhabditis elegans*. *Glia* **59**: 1253–1263.

Okello EJ, McDougall GJ, Kumar S, Seal CJ (2011) *In vitro* protective effects of colon-available extract of *Camellia sinensis* (tea) against hydrogen peroxide and β-amyloid ($A\beta_{1–42}$) induced cytotoxicity in differentiated PC12 cells. *Phytomedicine* **18**: 691–696.

Opazo C, Huang X, Cherny RA, Moir RD, Roher AE, White AR, Cappai R, Masters CL, Tanzi RE, Inestrosa NC, Bush AI (2002) Metalloenzyme-like activity of Alzheimer's disease β-amyloid. Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H_2O_2. *Journal of Biological Chemistry* **277**: 40302–40308.

Panda D, Goode BL, Feinstein SC, Wilson L (1995) Kinetic stabilization of microtubule dynamics at steady state by tau and microtubule-binding domains of tau. *Biochemistry* **34**: 11117–11127.

Panowski SH, Wolff S, Aguilaniu H, Durieux J, Dillin A (2007) PHA-4/Foxa mediates diet-restriction-induced longevity of *C. elegans*. *Nature* **447**: 550–555.

Papatheodorou I, Petrovs R, Thornton JM (2014) Comparison of the mammalian insulin signalling pathway to invertebrates in the context of FOXO-mediated ageing. *Bioinformatics* **30**: 2999–3003.

Paradis S, Ailion M, Toker A, Thomas JH, Ruvkun G (1999) A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in *Caenorhabditis elegans*. *Genes and Development* **13**: 1438–1452.

Paradis S, Ruvkun G (1998) *Caenorhabditis elegans* Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. *Genes and Development* **12**: 2488–2498.

Park M, Lee JH, Choi JK, Hong YD, Bae IH, Lim KM, Park YH, Ha H (2014) 18β-glycyrrhetinic acid attenuates anandamide-induced adiposity and high-fat diet induced obesity. *Molecular Nutrition and Food Research* **58**: 1436–1446.

Park SJ, Youn HS (2010) Suppression of homodimerization of toll-like receptor 4 by isoliquiritigenin. *Phytochemistry* **71**: 1736–1740.

Parle M, Dhingra D, Kulkarni SK (2004) Memory-strengthening activity of *Glycyrrhiza glabra* in exteroceptive and interoceptive behavioral models. *Journal of Medicinal Food* **7**: 462–466.

Parsons CG, Danysz W, Dekundy A, Pulte I (2013) Memantine and cholinesterase inhibitors: complementary mechanisms in the treatment of Alzheimer's disease. *Neurotoxicity Research* **24**: 358–369.

Parvathy S, Hussain I, Karran EH, Turner AJ, Hooper NM (1999) Cleavage of Alzheimer's amyloid precursor protein by α-secretase occurs at the surface of neuronal cells. *Biochemistry* **38**: 9728–9734.

Pasalar P, Najmabadi H, Noorian AR, Moghimi B, Jannati A, Soltanzadeh A, Krefft T, Crook R, Hardy J (2002) An Iranian family with Alzheimer's disease caused by a novel APP mutation (Thr714Ala). *Neurology* **58**: 1574–1575.

Pasternak SH, Bagshaw RD, Guiral M, Zhang S, Ackerley CA, Pak BJ, Callahan JW, Mahuran DJ (2003) Presenilin-1, nicastrin, amyloid precursor protein, and γ-secretase activity are co-localized in the lysosomal membrane. *Journal of Biological Chemistry* **278**: 26687–26694.

Patterson KR, Remmers C, Fu Y, Brooker S, Kanaan NM, Vana L, Ward S, Reyes JF, Philibert K, Glucksman MJ, Binder LI (2011a) Characterization of prefibrillar tau oligomers in vitro and in Alzheimer disease. *Journal of Biological Chemistry* **286**: 23063–23076.

Patterson KR, Ward SM, Combs B, Voss K, Kanaan NM, Morfini G, Brady ST, Gamblin TC, Binder LI (2011b) Heat shock protein 70 prevents both tau aggregation and the inhibitory effects of preexisting tau aggregates on fast axonal transport. *Biochemistry* **50**: 10300–10310.

Peña-Casanova J, Sánchez-Benavides G, de Sola S, Manero-Borrás RM, Casals-Coll M (2012) Neuropsychology of Alzheimer's disease. *Archives of Medical Research* **43**: 686–693.

Peng W, Liu YJ, Wu N, Sun T, He XY, Gao YX, Wu CJ (2015) *Areca catechu* L. (Arecaceae): a review of its traditional uses, botany, phytochemistry, pharmacology and toxicology. *Journal of Ethnopharmacology* **164**: 340–356.

Perkins LA, Hedgecock EM, Thomson JN, Culotti JG (1986) Mutant sensory cilia in the nematode *Caenorhabditis elegans*. *Developmental Biology* **117**: 456–487.

Perry E, Walker M, Grace J, Perry R (1999) Acetylcholine in mind: a neurotransmitter correlate of consciousness? *Trends in Neurosciences* **22**: 273–280.

Perry G, Nunomura A, Raina AK, Smith MA (2000) Amyloid-β junkies. *Lancet* **355**: 757.

Phaniendra A, Jestadi DB, Periyasamy L (2015) Free radicals: properties, sources, targets, and their implication in various diseases. *Indian Journal of Clinical Biochemistry* **30**: 11–26.

Pierce SB, Costa M, Wisotzkey R, Devadhar S, Homburger SA, Buchman AR, Ferguson KC, Heller J, Platt DM, Pasquinelli AA, Liu LX, Doberstein SK, Ruvkun G (2001) Regulation of DAF-2 receptor signaling by human insulin and *ins-1*, a member of the unusually large and diverse *C. elegans* insulin gene family. *Genes and Development* **15**: 672–686.

Pike CJ, Overman MJ, Cotman CW (1995) Amino-terminal deletions enhance aggregation of β-amyloid peptides *in vitro*. *Journal of Biological Chemistry* **270**: 23895–23898.

Pimplikar SW (2009) Reassessing the amyloid cascade hypothesis of Alzheimer's disease. *International Journal of Biochemistry and Cell Biology* **41**: 1261–1268.

Prasad Gabbita S, Lovell MA, Markesbery WR (1998) Increased nuclear DNA oxidation in the brain in Alzheimer's disease. *Journal of Neurochemistry* **71**: 2034–2040.

Prince M, Jackson J (eds.) (2009) *World Alzheimer Report*, Alzheimer's Disease International.

Qi-Takahara Y, Morishima-Kawashima M, Tanimura Y, Dolios G, Hirotani N, Horikoshi Y, Kametani F, Maeda M, Saido TC, Wang R, Ihara Y (2005) Longer forms of amyloid β protein: implications for the mechanism of intramembrane cleavage by γ-secretase. *The Journal of Neuroscience* **25**: 436–445.

Qian XZ (ed.) (2007) *An illustrated atlas of the commonly used Chinese materia medica*. People's Medical Publishing House: Beijing, China.

Qiao H, Zhang X, Wang T, Liang L, Chang W, Xia H (2014) Pharmacokinetics, biodistribution and bioavailability of isoliquiritigenin after intravenous and oral administration. *Pharmaceutical Biology* **52**: 228–236.

Qin LT, Liu SS, Liu HL, Zhang YH (2010) Support vector regression and least squares support vector regression for hormetic dose-response curves fitting. *Chemosphere* **78**: 327–334.

Qin NY, Yang FQ, Wang YT, Li SP (2007) Quantitative determination of eight components in rhizome (*Jianghuang*) and tuberous root (*Yujin*) of *Curcuma longa* using pressurized liquid extraction and gas chromatography-mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **43**: 486–492.

Qing ZJ, Yong W, Hui LY, Yong LW, Long LH, Ao DJ, Xia PL (2012) Two new natural products from the fruits of *Alpinia oxyphylla* with inhibitory effects on nitric oxide production in lipopolysaccharide-activated RAW264.7 macrophage cells. *Archives of Pharmacal Research* **35**: 2143–2146.

Qiu WQ, Walsh DM, Ye Z, Vekrellis K, Zhang J, Podlisny MB, Rosner MR, Safavi A, Hersh LB, Selkoe DJ (1998) Insulin-degrading enzyme regulates extracellular levels of amyloid β-protein by degradation. *Journal of Biological Chemistry* **273**: 32730–32738.

Quintanilla RA, von Bernhardi R, Godoy JA, Inestrosa NC, Johnson GV (2014) Phosphorylated tau potentiates Aβ-induced mitochondrial damage in mature neurons. *Neurobiology of Disease* **71**: 260–269.

Raynaud J, Rasolojaona L (1979) Flavonoïdes des feuilles de *Carlina acaulis*. *Planta Medica* **37**: 168–171.

Rebolledo DL, Aldunate R, Kohn R, Neira I, Minniti AN, Inestrosa NC (2011) Copper reduces Aβ oligomeric species and ameliorates neuromuscular synaptic defects in a *C. elegans* model of inclusion body myositis. *Journal of Neuroscience* **31**: 10149–10158.

Reitz C, Mayeux R (2014) Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers. *Biochemical Pharmacology* **88**: 640–651.

Rhein V, Song X, Wiesner A, Ittner LM, Baysang G, Meier F, Ozmen L, Bluethmann H, Dröse S, Brandt U, Savaskan E, Czech C, Götz J, Eckert A (2009) Amyloid-β and tau synergistically impair the oxidative phosphorylation system in triple transgenic Alzheimer's disease mice. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 20057–20062.

Riddell DR, Christie G, Hussain I, Dingwall C (2001) Compartmentalization of β-secretase (Asp2) into low-buoyant density, noncaveolar lipid rafts. *Current Biology* **11**: 1288–1293.

Riddle DL, Swanson MM, Albert PS (1981) Interacting genes in nematode dauer larva formation. *Nature* **290**: 668–671.

Rijal Upadhaya A, Kosterin I, Kumar S, von Arnim CA, Yamaguchi H, Fändrich M, Walter J, Thal DR (2014) Biochemical stages of amyloid-β peptide aggregation and accumulation in the human brain and their association with symptomatic and pathologically preclinical Alzheimer's disease. *Brain* **137**: 887–903.

Ristow M, Schmeisser S (2011) Extending life span by increasing oxidative stress. *Free Radical Biology and Medicine* **51**: 327–336.

Robakis NK, Ramakrishna N, Wolfe G, Wisniewski HM (1987) Molecular cloning and characterization of a cDNA encoding the cerebrovascular and the neuritic plaque amyloid peptides. *Proceedings of the National Academy of Sciences* **84**: 4190–4194.

Roberson ED, Scearce-Levie K, Palop JJ, Yan F, Cheng IH, Wu T, Gerstein H, Yu GQ, Mucke L (2007) Reducing endogenous tau ameliorates amyloid β-induced deficits in an Alzheimer's disease mouse model. *Science* **316**: 750–754.

Rogers JT, Randall JD, Cahill CM, Eder PS, Huang X, Gunshin H, Leiter L, McPhee J, Sarang SS, Utsuki T, Greig NH, Lahiri DK, Tanzi RE, Bush AI, Giordano T, Gullans SR (2002) An iron-responsive element type II in the 5'-untranslated region of the Alzheimer's amyloid precursor protein transcript. *Journal of Biological Chemistry* **277**: 45518–45528.

Roher AE, Lowenson JD, Clarke S, Woods AS, Cotter RJ, Gowing E, Ball MJ (1993a) β-Amyloid-(1–42) is a major component of cerebrovascular amyloid deposits: implications for the pathology of Alzheimer disease. *Proceedings of the National Academy of Sciences* **90**: 10836–10840.

Roher AE, Palmer KC, Yurewicz EC, Ball MJ, Greenberg BD (1993b) Morphological and biochemical analyses of amyloid plaque core proteins purified from Alzheimer disease brain tissue. *Journal of Neurochemistry* **61**: 1916–1926.

Rossi G, Giaccone G, Maletta R, Morbin M, Capobianco R, Mangieri M, Giovagnoli AR, Bizzi A, Tomaino C, Perri M, Di Natale M, Tagliavini F, Bugiani O, Bruni AC (2004) A family with Alzheimer disease and strokes associated with A713T mutation of the APP gene. *Neurology* **63**: 910–912.

Sadakane C, Watanabe J, Fukutake M, Nisimura H, Maemura K, Kase Y, Kono T (2015) Pharmacokinetic profiles of active components after oral administration of a Kampo medicine, Shakuyakukanzoto, to healthy adult Japanese volunteers. *Journal of Pharmaceutical Sciences* **104**: 3952–3959.

Sawin ER, Ranganathan R, Horvitz HR (2000) *C. elegans* locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. *Neuron* **26**: 619–631.

Schackwitz WS, Inoue T, Thomas JH (1996) Chemosensory neurons function in parallel to mediate a pheromone response in *C. elegans*. *Neuron* **17**: 719–728.

Schaeffer JM, White T, Bergstrom AR, Wilson KE, Turner MJ (1990) Identification of glutamate-binding sites in *Caenorhabditis elegans*. *Pesticide Biochemistry and Physiology* **36**: 220–228.

Schafer WR, Kenyon CJ (1995) A calcium-channel homologue required for adaptation to dopamine and serotonin in *Caenorhabditis elegans*. *Nature* **375**: 73–78.

Scheffler K, Krohn M, Dunkelmann T, Stenzel J, Miroux B, Ibrahim S, von Bohlen und Halbach O, Heinze HJ, Walker LC, Gsponer JA, Pahnke J (2012) Mitochondrial DNA polymorphisms specifically modify cerebral β-amyloid proteostasis. *Acta Neuropathologica* **124**: 199–208.

Scheuner D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, Bird TD, Hardy J, Hutton M, Kukull W, Larson E, Levy-Lahad E, Viitanen M, Peskind E, Poorkaj P, Schellenberg G, Tanzi R, Wasco W, Lannfelt L, Selkoe D, Younkin S (1996) Secreted amyloid β-protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Medicine* **2**: 864–870.

Schiavi A, Ventura N (2014) The interplay between mitochondria and autophagy and its role in the aging process. *Experimental Gerontology* **56**: 147–153.

Schilcher H, Hagels H (1990) Carlinae radix: Verfälschung, Verwechslung oder Ersatzdroge? *Deutsche Apotheker Zeitung* **130**: 2186–2190.

Schmidt-Thomé J (1950) Über die antibakterielle Wirkung der Silberdistelwurzel. *Zeitschrift für Naturforschung* **5b**: 409–412.

Schneider LS (2012) Ginkgo and AD: key negatives and lessons from GuidAge. *Lancet Neurology* **11**: 836–837.

Schubert W, Prior R, Weidemann A, Dircksen H, Multhaup G, Masters CL, Beyreuther K (1991) Localization of Alzheimer β A4 amyloid precursor protein at central and peripheral synaptic sites. *Brain Research* **563**: 184–194.

Sebaugh JL (2011) Guidelines for accurate EC_{50}/IC_{50} estimation. *Pharmaceutical Statistics* **10**: 128–134.

Sebollela A, Mustata GM, Luo K, Velasco PT, Viola KL, Cline EN, Shekhawat GS, Wilcox KC, Dravid VP, Klein WL (2014) Elucidating molecular mass and shape of a neurotoxic Aβ oligomer. *ACS Chemical Neuroscience* **5**: 1238–1245.

Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. *Science* **298**: 789–791.

Selkoe DJ, Abraham CR, Podlisny MB, Duffy LK (1986) Isolation of low-molecular-weight proteins from amyloid plaque fibers in Alzheimer's disease. *Journal of Neurochemistry* **46**: 1820–1834.

Semmler FW (1906) Zusammensetzung des ätherischen Öls der Eberwurzel (*Carlina acaulis* L.). *Berichte der deutschen chemischen Gesellschaft* **39**: 726–731.

Semmler FW, Ascher E (1909) Zur Kenntnis der Bestandteile ätherischer Öle. (Über Carlinaoxyd und über einige synthetische Versuche). *Berichte der deutschen chemischen Gesellschaft* **42**: 2355–2360.

Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT (2011) Neuropathological alterations in Alzheimer disease. *Cold Spring Harbor Perspectives in Medicine* **1**: a006189.

Seward ME, Swanson E, Norambuena A, Reimann A, Cochran JN, Li R, Roberson ED, Bloom GS (2013) Amyloid-β signals through tau to drive ectopic neuronal cell cycle re-entry in Alzheimer's disease. *Journal of Cell Science* **126**: 1278–1286.

Shen B, Truong J, Helliwell R, Govindaraghavan S, Sucher NJ (2013) An *in vitro* study of neuroprotective properties of traditional Chinese herbal medicines thought to promote healthy ageing and longevity. *BMC Complementary and Alternative Medicine* **13**: 373.

Shi GF, An LJ, Jiang B, Guan S, Bao YM (2006) *Alpinia* protocatechuic acid protects against oxidative damage *in vitro* and reduces oxidative stress *in vivo*. *Neuroscience Letters* **403**: 206–210.

Shi SH, Zhao X, Liu B, Li H, Liu AJ, Wu B, Bi KS, Jia Y (2014) The effects of sesquiterpenes-rich extract of *Alpinia oxyphylla* Miq. on amyloid-induced cognitive impairment and neuronal abnormalities in the cortex and hippocampus of mice. *Oxidative Medicine and Cellular Longevity* **2014**: 451802–451813.

Shibata M, Yamada S, Kumar SR, Calero M, Bading J, Frangione B, Holtzman DM, Miller CA, Strickland DK, Ghiso J, Zlokovic BV (2000) Clearance of Alzheimer's amyloid-β_{1-40} peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier. *Journal of Clinical Investigation* **106**: 1489–1499.

Shih YT, Chen PS, Wu CH, Tseng YT, Wu YC, Lo YC (2010) Arecoline, a major alkaloid of the areca nut, causes neurotoxicity through enhancement of oxidative stress and suppression of the antioxidant protective system. *Free Radical Biology and Medicine* **49**: 1471–1479.

Shui G, Bao YM, Bo J, An LJ (2006) Protective effect of protocatechuic acid from *Alpinia oxyphylla* on hydrogen peroxide-induced oxidative PC12 cell death. *European Journal of Pharmacology* **538**: 73–79.

Simard AR, Soulet D, Gowing G, Julien JP, Rivest S (2006) Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. *Neuron* **49**: 489–502.

Simmler C, Anderson JR, Gauthier L, Lankin DC, McAlpine JB, Chen SN, Pauli GF (2015) Metabolite profiling and classification of DNA-authenticated licorice botanicals. *Journal of Natural Products* **78**: 2007–2022.

Sindi S, Mangialasche F, Kivipelto M (2015) Advances in the prevention of Alzheimer's disease. *F1000Prime Reports* **7**: 50–61.

Sinha S, Anderson JP, Barbour R, Basi GS, Caccavello R, Davis D, Doan M, Dovey HF, Frigon N, Hong J, Jacobson-Croak K, Jewett N, Keim P, Knops J, Lieberburg I, Power M, Tan H, Tatsuno G, Tung J, Schenk D, Seubert P, Suomensaari SM, Wang S, Walker D, Zhao J, McConlogue L, John V (1999) Purification and cloning of amyloid precursor protein β-secretase from human brain. *Nature* **402**: 537–540.

Small SA, Duff K (2008) Linking Aβ and tau in late-onset Alzheimer's disease: a dual pathway hypothesis. *Neuron* **60**: 534–542.

Smith CD, Carney JM, Starke-Reed PE, Oliver CN, Stadtman ER, Floyd RA, Markesbery WR (1991) Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 10540–10543.

Smith DG, Cappai R, Barnham KJ (2007) The redox chemistry of the Alzheimer's disease amyloid β peptide. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1768**: 1976–1990.

Smith JV, Luo Y (2003) Elevation of oxidative free radicals in Alzheimer's disease models can be attenuated by *Ginkgo biloba* extract EGb 761. *Journal of Alzheimer's Disease* **5**: 287–300.

Smith JV, Luo Y (2004) Studies on molecular mechanisms of *Ginkgo biloba* extract. *Applied Microbiology and Biotechnology* **64**: 465–472.

Smolarkiewicz M, Skrzypczak T, Wojtaszek P (2013) The very many faces of presenilins and the γ-secretase complex. *Protoplasma* **250**: 997–1011.

Snowdon DA (2003) Healthy aging and dementia: findings from the nun study. *Annals of Internal Medicine* **139**: 450–454.

Son TG, Camandola S, Mattson MP (2008) Hormetic dietary phytochemicals. *Neuromolecular Medicine* **10**: 236–246.

Soncrant TT, Raffaele KC, Asthana S, Berardi A, Morris PP, Haxby JV (1993) Memory improvement without toxicity during chronic, low dose intravenous arecoline in Alzheimer's disease. *Psychopharmacology* **112**: 421–427.

Song Y, Cui T, Xie N, Zhang X, Qian Z, Liu J (2014) Protocatechuic acid improves cognitive deficits and attenuates amyloid deposits, inflammatory response in aged AβPP/PS1 double transgenic mice. *International Immunopharmacology* **20**: 276–281.

Soumyanath A, Zhong YP, Henson E, Wadsworth T, Bishop J, Gold BG, Quinn JF (2012) *Centella asiatica* extract improves behavioral deficits in a mouse model of Alzheimer's disease: investigation of a possible mechanism of action. *International Journal of Alzheimer's Disease* **2012**: 381974–381982.

Spencer B, Masliah E (2014) Immunotherapy for Alzheimer's disease: past, present and future. *Frontiers in Aging Neuroscience* **6**: 114–120.

Spoel SH, Dong X (2012) How do plants achieve immunity? Defence without specialized immune cells. *Nature Reviews Immunology* **12**: 89–100.

Stojanović-Radić Z, Čomić L, Radulović N, Blagojević P, Mihajilov-Krstev T, Rajković J (2012) Commercial *Carlinae radix* herbal drug: botanical identity, chemical composition and antimicrobial properties. *Pharmaceutical Biology* **50**: 933–940.

Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD (1993) Apolipoprotein E: high-avidity binding to β-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America* **90**: 1977–1981.

Su Y, Wang Q, Wang C, Chan K, Sun Y, Kuang H (2014) The treatment of Alzheimer's disease using Chinese medicinal plants: from disease models to potential clinical applications. *Journal of Ethnopharmacology* **152**: 403–423.

Sulston J, Dew M, Brenner S (1975) Dopaminergic neurons in the nematode *Caenorhabditis elegans*. *The Journal of Comparative Neurology* **163**: 215–226.

Sulston J, Hodgkin J (1988) Methods, in: *The nematode Caenorhabditis elegans* (ed. WB Wood). Cold Spring Harbor Laboratory Press: New York.

Sulston JE, Schierenberg E, White JG, Thomson JN (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Developmental Biology* **100**: 64–119.

Summers WK (2006) Tacrine, and Alzheimer's treatments. *Journal of Alzheimer's Disease* **9**: 439–445.

Swerdlow RH, Burns JM, Khan SM (2014) The Alzheimer's disease mitochondrial cascade hypothesis: progress and perspectives. *Biochimica et Biophysica Acta* **1842**: 1219–1231.

Swerdlow RH, Khan SM (2004) A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease. *Medical Hypotheses* **63**: 8–20.

Sydow A, Van der Jeugd A, Zheng F, Ahmed T, Balschun D, Petrova O, Drexler D, Zhou L, Rune G, Mandelkow E, D'Hooge R, Alzheimer C, Mandelkow EM (2011) Tau-induced defects in synaptic plasticity, learning, and memory are reversible in transgenic mice after switching off the toxic tau mutant. *Journal of Neuroscience* **31**: 2511–2525.

Szabados T, Dul C, Majtenyi K, Hargitai J, Penzes Z, Urbanics R (2004) A chronic Alzheimer's model evoked by mitochondrial poison sodium azide for pharmacological investigations. *Behavioural Brain Research* **154**: 31–40.

Takahashi T, Takasuka N, Iigo M, Baba M, Nishino H, Tsuda H, Okuyama T (2004) Isoliquiritigenin, a flavonoid from licorice, reduces prostaglandin E2 and nitric oxide, causes apoptosis, and suppresses aberrant crypt foci development. *Cancer Science* **95**: 448–453.

Tam JH, Pasternak SH (2012) Amyloid and Alzheimer's disease: inside and out. *Canadian Journal of Neurological Sciences* **39**: 286–298.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731–2739.

Tang ZH, Li T, Chang LL, Zhu H, Tong YG, Chen XP, Wang YT, Lu JJ (2014) Glycyrrhetinic acid triggers a protective autophagy by activation of extracellular regulated protein kinases in hepatocellular carcinoma cells. *Journal of Agricultural and Food Chemistry* **62**: 11910–11916.

Teng L, Meng Q, Lu J, Xie J, Wang Z, Liu Y, Wang D (2014) Liquiritin modulates ERK and AKT/GSK-3β-dependent pathways to protect against glutamate-induced cell damage in differentiated PC12 cells. *Molecular Medicine Reports* **10**: 818–824.

Terreni L, Fogliarino S, Franceschi M, Forloni G (2002) Novel pathogenic mutation in an Italian patient with familial Alzheimer's disease detected in APP gene. *Neurobiology of Aging* **23**: S319.

The *Caenorhabditis elegans* Sequencing Consortium (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282**: 2012–2018.

Theuns J, Marjaux E, Vandenbulcke M, Van Laere K, Kumar-Singh S, Bormans G, Brouwers N, Van den Broeck M, Vennekens K, Corsmit E, Cruts M, De Strooper B, Van Broeckhoven C, Vandenberghe R (2006) Alzheimer dementia caused by a novel mutation located in the APP C-terminal intracytosolic fragment. *Human Mutation* **27**: 888–896.

Tissenbaum HA, Guarente L (2001) Increased dosage of a *sir-2* gene extends lifespan in *Caenorhabditis elegans*. *Nature* **410**: 227–230.

Tohda C, Ichimura M, Bai Y, Tanaka K, Zhu S, Komatsu K (2008) Inhibitory effects of *Eleutherococcus senticosus* extracts on amyloid β(25–35)-induced neuritic atrophy and synaptic loss. *Journal of Pharmacological Sciences* **107**: 329–339.

Trinczek B, Biernat J, Baumann K, Mandelkow EM, Mandelkow E (1995) Domains of tau protein, differential phosphorylation, and dynamic instability of microtubules. *Molecular Biology of the Cell* **6**: 1887–1902.

Tsigelny IF, Sharikov Y, Kouznetsova VL, Greenberg JP, Wrasidlo W, Gonzalez T, Desplats P, Michael SE, Trejo-Morales M, Overk CR, Masliah E (2014) Structural diversity of Alzheimer's disease amyloid-β dimers and their role in oligomerization and fibril formation. *Journal of Alzheimer's Disease* **39**: 583–600.

Tullet JMA, Hertweck M, An JH, Baker J, Hwang JY, Liu S, Oliveira RP, Baumeister R, Blackwell TK (2008) Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in *C. elegans*. *Cell* **132**: 1025–1038.

Um JW, Nygaard HB, Heiss JK, Kostylev MA, Stagi M, Vortmeyer A, Wisniewski T, Gunther EC, Strittmatter SM (2012) Alzheimer amyloid-β oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. *Nature Neuroscience* **15**: 1227–1235.

Um MY, Choi WH, Aan JY, Kim SR, Ha TY (2006) Protective effect of *Polygonum multiflorum* Thunb on amyloid β-peptide 25–35 induced cognitive deficits in mice. *Journal of Ethnopharmacology* **104**: 144–148.

Van Assche R, Temmerman L, Dias DA, Boughton B, Boonen K, Braeckman BP, Schoofs L, Roessner U (2015) Metabolic profiling of a transgenic Alzheimer model. *Metabolomics* **11**: 477–486.

Van Wyk BE, Wink C, Wink M (2015) *Handbuch der Arzneipflanzen: ein Bildatlas*. 3 edition, Wissenschaftliche Verlagsgesellschaft: Stuttgart.

Varadarajan S, Yatin S, Kanski J, Jahanshahi F, Butterfield DA (1999) Methionine residue 35 is important in amyloid β-peptide-associated free radical oxidative stress. *Brain Research Bulletin* **50**: 133–141.

Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, Collins F, Treanor J, Rogers G, Citron M (1999) β-Secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**: 735–741.

Vassar R, Kuhn PH, Haass C, Kennedy ME, Rajendran L, Wong PC, Lichtenthaler SF (2014) Function, therapeutic potential and cell biology of BACE proteases: current status and future prospects. *Journal of Neurochemistry* **130**: 4–28.

Vaya J, Belinky PA, Aviram M (1997) Antioxidant constituents from licorice roots: isolation, structure elucidation and antioxidative capacity toward LDL oxidation. *Free Radical Biology and Medicine* **23**: 302–313.

Velliquette RA, O'Connor T, Vassar R (2005) Energy inhibition elevates β-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. *The Journal of Neuroscience* **25**: 10874–10883.

Vetrivel KS, Cheng H, Lin W, Sakurai T, Li T, Nukina N, Wong PC, Xu H, Thinakaran G (2004) Association of γ-secretase with lipid rafts in post-Golgi and endosome membranes. *Journal of Biological Chemistry* **279**: 44945–44954.

Vigo-Pelfrey C, Lee D, Keim P, Lieberburg I, Schenk DB (1993) Rapid communication: characterization of β-amyloid peptide from human cerebrospinal fluid. *Journal of Neurochemistry* **61**: 1965–1968.

Viola K, Klein W (2015) Amyloid β oligomers in Alzheimer's disease pathogenesis, treatment, and diagnosis. *Acta Neuropathologica* 1–24.

Vitkova A, Evstatieva L (2002) Plants rich in inulin from family Asteraceae. *Godishnik na Sofiskiya Universitet "Sv Kliment Okhridski", Kniga* **2**: 107–111.

Voss K, Combs B, Patterson KR, Binder LI, Gamblin TC (2012) Hsp70 alters tau function and aggregation in an isoform specific manner. *Biochemistry* **51**: 888–898.

Vowels JJ, Thomas JH (1992) Genetic analysis of chemosensory control of dauer formation in *Caenorhabditis elegans*. *Genetics* **130**: 105–123.

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ (2002) Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation *in vivo*. *Nature* **416**: 535–539.

Walter J, van Echten-Deckert G (2013) Cross-talk of membrane lipids and Alzheimer-related proteins. *Molecular Neurodegeneration* **8**: 34–45.

Wan L, Nie G, Zhang J, Luo Y, Zhang P, Zhang Z, Zhao B (2011) β-Amyloid peptide increases levels of iron content and oxidative stress in human cell and *Caenorhabditis elegans* models of Alzheimer disease. *Free Radical Biology and Medicine* **50**: 122–129.

Wang CY, Kao TC, Lo WH, Yen GC (2011) Glycyrrhizic acid and 18β-glycyrrhetinic acid modulate lipopolysaccharide-induced inflammatory response by suppression of NF-κB through PI3K p110δ and p110γ inhibitions. *Journal of Agricultural and Food Chemistry* **59**: 7726–7733.

Wang JZ, Grundke-Iqbal I, Iqbal K (2007) Kinases and phosphatases and tau sites involved in Alzheimer neurofibrillary degeneration. *European Journal of Neuroscience* **25**: 59–68.

Wang R, Shen X, Xing E, Guan L, Xin L (2013a) *Scutellaria baicalensis* stem-leaf total flavonoid reduces neuronal apoptosis induced by amyloid β-peptide (25-35). *Neural Regeneration Research* **8**: 1081–1090.

Wang R, Zhang CY, Bai LP, Pan HD, Shu LM, Kong AN, Leung EL, Liu L, Li T (2015) Flavonoids derived from liquorice suppress murine macrophage activation by up-regulating heme oxygenase-1 independent of Nrf2 activation. *International Immunopharmacology* **28**: 917–924.

Wang W, Hu X, Zhao Z, Liu P, Hu Y, Zhou J, Zhou D, Wang Z, Guo D, Guo H (2008) Antidepressant-like effects of liquiritin and isoliquiritin from *Glycyrrhiza uralensis* in the forced swimming test and tail suspension test in mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* **32**: 1179–1184.

Wang X, Su B, Lee HG, Li X, Perry G, Smith MA, Zhu X (2009) Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. *Journal of Neuroscience* **29**: 9090–9103.

Wang X, Zhang H, Chen L, Shan L, Fan G, Gao X (2013b) Liquorice, a unique "guide drug" of traditional Chinese medicine: a review of its role in drug interactions. *Journal of Ethnopharmacology* **150**: 781–790.

Wang Y, Oh SW, Deplancke B, Luo J, Walhout AJ, Tissenbaum HA (2006) *C. elegans* 14-3-3 proteins regulate life span and interact with SIR-2.1 and DAF-16/FOXO. *Mechanisms of Ageing and Development* **127**: 741–747.

Ward S (1973) Chemotaxis by the nematode *Caenorhabditis elegans*: identification of attractants and analysis of the response by use of mutants. *Proceedings of the National Academy of Sciences* **70**: 817–821.

Ward S, Thomson N, White JG, Brenner S (1975) Electron microscopical reconstruction of the anterior sensory anatomy of the nematode *Caenorhabditis elegans*. *Journal of Comparative Neurology* **160**: 313–337.

Ware RW, Clark D, Crossland K, Russell RL (1975) The nerve ring of the nematode *Caenorhabditis elegans*: sensory input and motor output. *The Journal of Comparative Neurology* **162**: 71–110.

Watson DJ, Selkoe DJ, Teplow DB (1999) Effects of the amyloid precursor protein $Glu^{693} \rightarrow Gln$ 'Dutch' mutation on the production and stability of amyloid β-protein. *Biochemical Journal* **340**: 703–709.

Watt AD, Perez KA, Rembach A, Sherrat NA, Hung LW, Johanssen T, McLean CA, Kok WM, Hutton CA, Fodero-Tavoletti M, Masters CL, Villemagne VL, Barnham KJ (2013) Oligomers, fact or artefact? SDS-PAGE induces dimerization of β-amyloid in human brain samples. *Acta Neuropathologica* **125**: 549–564.

Weidemann A, König G, Bunke D, Fischer P, Salbaum JM, Masters CL, Beyreuther K (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* **57**: 115–126.

Weingarten MD, Lockwood AH, Hwo SY, Kirschner MW (1975) A protein factor essential for microtubule assembly. *Proceedings of the National Academy of Sciences of the United States of America* **72**: 1858–1862.

Weller RO, Massey A, Newman TA, Hutchings M, Kuo YM, Roher AE (1998) Cerebral amyloid angiopathy: amyloid β accumulates in putative interstitial fluid drainage pathways in Alzheimer's disease. *American Journal of Pathology* **153**: 725–733.

Wessel D, Flügge UI (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Analytical Biochemistry* **138**: 141–143.

White J, Southgate E, Thomson J, Brenner S (1986) The structure of the nervous system of the nematode *C. elegans*. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences* **314**: 1–340.

Williams TI, Lynn BC, Markesbery WR, Lovell MA (2006) Increased levels of 4-hydroxynonenal and acrolein, neurotoxic markers of lipid peroxidation, in the brain in Mild Cognitive Impairment and early Alzheimer's disease. *Neurobiology of Aging* **27**: 1094–1099.

Wink M (2003) Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* **64**: 3–19.

Wink M (2008) Evolutionary advantage and molecular modes of action of multi-component mixtures used in phytomedicine. *Current Drug Metabolism* **9**: 996–1009.

Wolkow CA, Muñoz MJ, Riddle DL, Ruvkun G (2002) Insulin receptor substrate and p55 orthologous adaptor proteins function in the *Caenorhabditis elegans daf-2*/insulin-like signaling pathway. *Journal of Biological Chemistry* **277**: 49591–49597.

Wu Y, Cao Z, Klein WL, Luo Y (2010) Heat shock treatment reduces beta amyloid toxicity *in vivo* by diminishing oligomers. *Neurobiology of Aging* **31**: 1055–1058.

Wu Y, Wu Z, Butko P, Christen Y, Lambert M, Klein W, Link C, Luo Y (2006) Amyloid-β-induced pathological behaviors are suppressed by *Ginkgo biloba* extract EGb 761 and ginkgolides in transgenic *Caenorhabditis elegans*. *The Journal of Neuroscience* **26**: 13102–13113.

Wu YP, Meng XS, Bao YR, Wang S (2013) Pharmacokinetic study of four flavones of *Glycyrrhiza* in rat plasma using HPLC-MS. *Journal of Ethnopharmacology* **148**: 266–270.

Xian YF, Lin ZX, Ip SP, Su ZR, Chen JN, Lai XP (2013) Comparison the neuropreotective effect of Cortex Phellodendri chinensis and Cortex Phellodendri amurensis against β-amyloid-induced neurotoxicity in PC12 cells. *Phytomedicine* **20**: 187–193.

Yamazaki T, Koo EH, Selkoe DJ (1996) Trafficking of cell-surface amyloid β-protein precursor. II. Endocytosis, recycling and lysosomal targeting detected by immunolocalization. *Journal of Cell Science* **109**: 999–1008.

Yan LJ (2014) Positive oxidative stress in aging and aging-related disease tolerance. *Redox Biology* **2c**: 165–169.

Yan R, Bienkowski MJ, Shuck ME, Miao H, Tory MC, Pauley AM, Brashier JR, Stratman NC, Mathews WR, Buhl AE, Carter DB, Tomasselli AG, Parodi LA, Heinrikson RL, Gurney ME (1999) Membrane-anchored aspartyl protease with Alzheimer's disease β-secretase activity. *Nature* **402**: 533–537.

Yan S, Li Z, Li H, Arancio O, Zhang W (2014) Notoginsenoside R1 increases neuronal excitability and ameliorates synaptic and memory dysfunction following amyloid elevation. *Scientific Reports* **4**: 6352–6359.

Yang AJ, Chandswangbhuvana D, Margol L, Glabe CG (1998) Loss of endosomal/lysosomal membrane impermeability is an early event in amyloid Aβ1–42 pathogenesis. *Journal of Neuroscience Research* **52**: 691–698.

Yang EJ, Min JS, Ku HY, Choi HS, Park Mk, Kim MK, Song KS, Lee DS (2012) Isoliquiritigenin isolated from *Glycyrrhiza uralensis* protects neuronal cells against glutamate-induced mitochondrial dysfunction. *Biochemical and Biophysical Research Communications* **421**: 658–664.

Yang EJ, Park GH, Song KS (2013a) Neuroprotective effects of liquiritigenin isolated from licorice roots on glutamate-induced apoptosis in hippocampal neuronal cells. *Neurotoxicology* **39**: 114–123.

Yang G, Wang Y, Tian J, Liu JP (2013b) Huperzine A for Alzheimer's disease: a systematic review and meta-analysis of randomized clinical trials. *PLoS One* **8**: e74916.

Yang L, Hao J, Zhang J, Xia W, Dong X, Hu X, Kong F, Cui X (2009a) Ginsenoside Rg3 promotes β-amyloid peptide degradation by enhancing gene expression of neprilysin. *Journal of Pharmacy and Pharmacology* **61**: 375–380.

Yang WJ, Li DP, Li JK, Li MH, Chen YL, Zhang PZ (2009b) Synergistic antioxidant activities of eight traditional Chinese herb pairs. *Biological and Pharmaceutical Bulletin* **32**: 1021–1026.

Yang Y, Bai L, Li X, Xiong J, Xu P, Guo C, Xue M (2014) Transport of active flavonoids, based on cytotoxicity and lipophilicity: an evaluation using the blood-brain barrier cell and Caco-2 cell models. *Toxicology in Vitro* **28**: 388–396.

Yatin SM, Varadarajan S, Link CD, Butterfield DA (1999) In vitro and *in vivo* oxidative stress associated with Alzheimer's amyloid β-peptide (1–42). *Neurobiology of Aging* **20**: 325–330.

Yu W, Polepalli J, Wagh D, Rajadas J, Malenka R, Lu B (2012) A critical role for the PAR-1/MARK-tau axis in mediating the toxic effects of Aβ on synapses and dendritic spines. *Human Molecular Genetics* **21**: 1384–1390.

Yu WH, Cuervo AM, Kumar A, Peterhoff CM, Schmidt SD, Lee JH, Mohan PS, Mercken M, Farmery MR, Tjernberg LO, Jiang Y, Duff K, Uchiyama Y, Näslund J, Mathews PM, Cataldo AM, Nixon RA (2005) Macroautophagy—a novel β-amyloid peptide-generating pathway activated in Alzheimer's disease. *The Journal of Cell Biology* **171**: 87–98.

Yu X, An L, Wang Y, Zhao H, Gao C (2003) Neuroprotective effect of *Alpinia oxyphylla* Miq. fruits against glutamate-induced apoptosis in cortical neurons. *Toxicology Letters* **144**: 205–212.

Yu Y, Ye RD (2015) Microglial Aβ receptors in Alzheimer's disease. *Cellular and Molecular Neurobiology* **35**: 71–83.

Yuan SM, Gao K, Wang DM, Quan XZ, Liu JN, Ma CM, Qin C, Zhang LF (2011) Evodiamine improves congnitive abilities in SAMP8 and $APP^{swe}/PS1^{\Delta E9}$ transgenic mouse models of Alzheimer's disease. *Acta Pharmacologica Sinica* **32**: 295–302.

Zamberlan DC, Arantes LP, Machado ML, Golombieski R, Soares FA (2014) Diphenyl-diselenide suppresses amyloid-β peptide in *Caenorhabditis elegans* model of Alzheimer's disease. *Neuroscience* **278**: 40–50.

Zempel H, Mandelkow EM (2012) Linking amyloid-β and tau: amyloid-β induced synaptic dysfunction via local wreckage of the neuronal cytoskeleton. *Neuro-Degenerative Diseases* **10**: 64–72.

Zempel H, Thies E, Mandelkow E, Mandelkow EM (2010) Aβ oligomers cause localized Ca^{2+} elevation, missorting of endogenous tau into dendrites, tau phosphorylation, and destruction of microtubules and spines. *Journal of Neuroscience* **30**: 11938–11950.

Zeng L, Zhang RY, Meng T, Lou ZC (1990) Determinatin of nine flavonoids and coumarins in licorice root by high-performance liquid chromatography. *Journal of Chromatography A* **513**: 247–254.

Zeng LF, Wang NS, Wang Q, Zou YP, Liang ZH, Kong LS, Wu HT, Liao NY, Liang XW, Mo YS (2015) Oral Chinese herbal medicine for kidney nourishment in Alzheimer's disease: a systematic review of the effect on MMSE index measures and safety. *Complementary Therapies in Medicine* **23**: 283–297.

Zeng LH, Zhang HD, Xu CJ, Bian YJ, Xu XJ, Xie QM, Zhang RH (2013) Neuroprotective effects of flavonoids extracted from licorice on kainate-induced seizure in mice through their antioxidant properties. *Journal of Zhejiang University Science B* **14**: 1004–1012.

Zhang J, Zhen YF, Pu Bu Ci R, Song LG, Kong WN, Shao TM, Li X, Chai XQ (2013a) Salidroside attenuates β amyloid-induced cognitive deficits *via* modulating oxidative stress and inflammatory mediators in rat hippocampus. *Behavioural Brain Research* **244**: 70–81.

Zhang L, Yu H, Zhao X, Lin X, Tan C, Cao G, Wang Z (2010) Neuroprotective effects of salidroside against β-amyloid-induced oxidative stress in SH-SY5Y human neuroblastoma cells. *Neurochemistry International* **57**: 547–555.

Zhang SQ, Obregon D, Ehrhart J, Deng J, Tian J, Hou H, Giunta B, Sawmiller D, Tan J (2013b) Baicalein reduces β-amyloid and promotes nonamyloidogenic amyloid precursor protein processing in an Alzheimer's disease transgenic mouse model. *Journal of Neuroscience Research* **91**: 1239–1246.

Zhang YW, Thompson R, Zhang H, Xu H (2011) APP processing in Alzheimer's disease. *Molecular Brain* **4**: 3–15.

Zhang Z, Li G, Szeto SS, Chong C, Quan Q, Huang C, Cui W, Guo B, Wang Y, Han Y, Michael Siu KW, Lee SM, Chu IK (2015) Examining the neuroprotective effects of protocatechuic acid and chrysin on *in vitro* and *in vivo* models of Parkinson's disease. *Free Radical Biology and Medicine* **84**: 331–343.

Zhang ZY, Li C, Zug C, Schluesener HJ (2014) Icariin ameliorates neuropathological changes, TGF-β1 accumulation and behavioral deficits in a mouse model of cerebral amyloidosis. *PLoS One* **9**: e104616.

Zhao H, Wang SL, Qian L, Jin JL, Li H, Xu Y, Zhu XL (2013) Diammonium glycyrrhizinate attenuates $A\beta_{1–42}$-induced neuroinflammation and regulates MAPK and NF-κB pathways *in vitro* and *in vivo*. *CNS Neuroscience & Therapeutics* **19**: 117–124.

Zhao H, Zhang X, Chen X, Li Y, Ke Z, Tang T, Chai H, Guo AM, Chen H, Yang J (2014) Isoliquiritigenin, a flavonoid from licorice, blocks M2 macrophage polarization in colitis-associated tumorigenesis through downregulating PGE2 and IL-6. *Toxicology and Applied Pharmacology* **279**: 311–321.

Zhao WQ, Lacor PN, Chen H, Lambert MP, Quon MJ, Krafft GA, Klein WL (2009) Insulin receptor dysfunction impairs cellular clearance of neurotoxic oligomeric Aβ. *Journal of Biological Chemistry* **284**: 18742–18753.

Zhao Z, Wang W, Guo H, Zhou D (2008) Antidepressant-like effect of liquiritin from *Glycyrrhiza uralensis* in chronic variable stress induced depression model rats. *Behavioural Brain Research* **194**: 108–113.

Zheng SQ, Ding AJ, Li GP, Wu GS, Luo HR (2013) Drug absorption efficiency in *Caenorhbditis elegans* delivered by different methods. *PLoS One* **8**: e56877.

Zhong SZ, Ge QH, Li Q, Qu R, Ma SP (2009) Peoniflorin attentuates $A\beta_{1–42}$-mediated neurotoxicity by regulating calcium homeostasis and ameliorating oxidative stress in hippocampus of rats. *Journal of the Neurological Sciences* **280**: 71–78.

Zhu X, Castellani RJ, Takeda A, Nunomura A, Atwood CS, Perry G, Smith MA (2001) Differential activation of neuronal ERK, JNK/SAPK and p38 in Alzheimer disease: the 'two hit' hypothesis. *Mechanisms of Ageing and Development* **123**: 39–46.

Zhu X, Lee HG, Perry G, Smith MA (2007) Alzheimer disease, the two-hit hypothesis: an update. *Biochimica et Biophysica Acta* **1772**: 494–502.

Zhu X, Raina AK, Perry G, Smith MA (2004) Alzheimer's disease: the two-hit hypothesis. *Lancet Neurology* **3**: 219–226.