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


Conference abstracts


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

v
# Table of contents

## 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
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td><em>Carlina acaulis</em></td>
<td>108</td>
</tr>
<tr>
<td>4.5.1</td>
<td>Isolation of Carlina oxide</td>
<td>108</td>
</tr>
<tr>
<td>4.5.2</td>
<td>Dichlormethane extract of <em>Carlina acaulis</em> but not Carlina oxide has an effect against Aβ toxicity</td>
<td>108</td>
</tr>
<tr>
<td>4.5.3</td>
<td>Carlina oxide exhibits <em>in vivo</em> antioxidant activity</td>
<td>109</td>
</tr>
<tr>
<td>4.5.4</td>
<td>Toxicity of <em>Carlina acaulis</em> and Carlina oxide</td>
<td>110</td>
</tr>
<tr>
<td>4.6</td>
<td>Conclusion</td>
<td>111</td>
</tr>
</tbody>
</table>

**References**

113
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAP-1</td>
<td>AGE-1 adaptor protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>beta-amyloid</td>
</tr>
<tr>
<td>ABAD</td>
<td>Aβ-binding alcohol dehydrogenase</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholine esterase</td>
</tr>
<tr>
<td>AChEI</td>
<td>acetylcholine esterase inhibitor</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADAM</td>
<td>a disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>AGE-1</td>
<td>ageing alteration 1</td>
</tr>
<tr>
<td>AICD</td>
<td>APP intracellular C-terminal domain</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APOE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>APL-1</td>
<td>APP-like 1</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BACE1</td>
<td>β-site APP-cleaving enzyme 1</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>Car0</td>
<td>residual extract of <em>Carlina acaulis</em> after isolation of Carlina oxide</td>
</tr>
<tr>
<td>CarOx</td>
<td>Carlina oxide fraction isolated from the dichlormethane extract of <em>Carlina acaulis</em></td>
</tr>
<tr>
<td>CGC</td>
<td>Caenorhabditis Genetics Center</td>
</tr>
<tr>
<td>CI</td>
<td>chemotaxis index</td>
</tr>
<tr>
<td>DAF</td>
<td>abnormal dauer formation</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPPH*</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>mean effective concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td><em>exempli gratia</em></td>
</tr>
<tr>
<td>EGCG</td>
<td>(−)-epigallocatechin gallate</td>
</tr>
<tr>
<td>eV</td>
<td>electron volt</td>
</tr>
<tr>
<td>FOXO</td>
<td>forkhead box O</td>
</tr>
<tr>
<td>FTT-1</td>
<td>14-3-3 family protein 1</td>
</tr>
<tr>
<td>FYN</td>
<td>FYN proto-oncogene</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GA</td>
<td>glycyrrhizic acid</td>
</tr>
<tr>
<td>GAA</td>
<td>glycyrrhizic acid monoammonium</td>
</tr>
<tr>
<td>GLC</td>
<td>gas-liquid chromatography</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GRA</td>
<td>glycyrrhetinic acid</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HSF-1</td>
<td>heat shock factor 1</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>ILS</td>
<td>insulin-like signalling</td>
</tr>
<tr>
<td>INS</td>
<td>insulin related peptide</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate, protein in <em>H. sapiens</em></td>
</tr>
<tr>
<td>IST-1</td>
<td>insulin receptor substrate, protein in <em>C. elegans</em></td>
</tr>
<tr>
<td>JNK-1</td>
<td>c-Jun N-terminal kinase 1</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
</tr>
<tr>
<td>LG</td>
<td>liquiritigenin</td>
</tr>
<tr>
<td>LRP1</td>
<td>low density lipoprotein receptor-related protein 1</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>mA</td>
<td>milliamperes</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>mAU</td>
<td>arbitrary unit</td>
</tr>
<tr>
<td>[M−H]&lt;sup&gt;+&lt;/sup&gt;</td>
<td>negatively charged molecular ion</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometer</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells</td>
</tr>
<tr>
<td>NGM</td>
<td>nematode growth medium</td>
</tr>
<tr>
<td>mm</td>
<td>nanometre</td>
</tr>
<tr>
<td>NMD</td>
<td>non-sense mediated mRNA decay</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NRF2</td>
<td>nuclear factor erythroid 2-like 2</td>
</tr>
<tr>
<td>p</td>
<td>probability</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate buffered saline containing 0.05 % Tween® 20</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDK-1</td>
<td>3-phosphoinositide-dependent kinase-1</td>
</tr>
<tr>
<td>PHA-4</td>
<td>defective pharynx development 4</td>
</tr>
<tr>
<td>pH</td>
<td>potential of hydrogen</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol-3,4,5-trisphosphat</td>
</tr>
<tr>
<td>PLSD</td>
<td>protected least significant difference</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PrP</td>
<td>prion protein</td>
</tr>
<tr>
<td>PSEN</td>
<td>presenilin</td>
</tr>
<tr>
<td>PT₅₀</td>
<td>mean time of paralysis</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue</td>
</tr>
<tr>
<td>RAGE</td>
<td>advanced glycosylation end product-specific receptor</td>
</tr>
<tr>
<td>rbcL</td>
<td>ribulose bisphosphate carboxylase large chain</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>retention time</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>sAPPα</td>
<td>soluble APP ectodomain released by α-secretase</td>
</tr>
<tr>
<td>sAPPβ</td>
<td>soluble APP ectodomain released by β-secretase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SIR-2.1</td>
<td>yeast silent information regulator related 2.1</td>
</tr>
<tr>
<td>SKN-1</td>
<td>skinhead 1</td>
</tr>
<tr>
<td>SMG-1</td>
<td>suppressor with morphogenetic effect on genitalia</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SGK-1</td>
<td>serum- and glucocorticoid-inducible kinase 1</td>
</tr>
<tr>
<td>spp.</td>
<td>species</td>
</tr>
<tr>
<td>ssp.</td>
<td>subspecies</td>
</tr>
<tr>
<td>TCM</td>
<td>Traditional Chinese Medicine</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylediamine</td>
</tr>
<tr>
<td>ThT</td>
<td>thioflavin T</td>
</tr>
<tr>
<td>ThS</td>
<td>thioflavin S</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)- aminomethane</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
</tbody>
</table>
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 (PT50) 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 PT50). 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 de-localisation. 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


Für die vorliegende Arbeit wurde der Modellorganismus Caenorhabditis elegans benutzt. In einem Screening von 55 Extrakan 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 (PT50) um 1,8 h verlängert wurde, und zeigte antioxidative Wirkung in dem Hitzeschock-Protein (HSP)-Expressions-Test.


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 Carlinoaoxid als den Hauptbestandteil dieses Extraks. Carlinoaoxid als Reinsubstanz zeigte in dem Paralyse-Test eine geringere Wirksamkeit als der Extrakt, aber war für die antioxidative Wirkung verantwortlich. Der Extrakt und das Carlinoaoxid 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.

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.
1. Introduction

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-
1.1. Alzheimer’s disease

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).

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 Aβ1–40, followed by a smaller amount of Aβ1–42. Also N-terminal truncated forms (Aβ40/42) 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 Aβ1–42, plaques in the walls of leptomeningeal and intracortical blood vessels (a sign of cerebral amyloid angiopathy often found in AD brains) contain both Aβ1–40 and Aβ1–42 (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. Aβ1–42 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 (Aβ17–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 APP770 transcript) that increases the production of both Aβ1–40 and Aβ1–42 (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, 1999; Pasalar et al., 2002; Terreni et al., 2002) and increase the Aβ1–42/Aβ1–40 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 APP770 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 homeostasis, 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; Nilserth 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; Nilserth 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 Aβ1–40 is rapidly degraded. Aβ1–42 is more resistant to degradation and tends to accumulate (Burick 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 tangles (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 A\(\beta\) oligomers (Ding & Johnson, 2008). As for A\(\beta\), 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 A\(\beta\) toxicity (Roberson et al., 2007). A\(\beta\) 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 excitotoxicity and seizures in AD models (Ittner et al., 2010). Tau itself can also be phosphorylated by FYN that is activated by A\(\beta\) 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 A\(\beta\) in accordance with the amyloid cascade hypothesis. What remains unclear are the mechanisms by which A\(\beta\) leads to changes in tau. A\(\beta\) 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 A\(\beta\) levels (Bright et al., 2015), suggesting a more complex interaction between these proteins. While most of the treatment strategies today aim at A\(\beta\) 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 A\(\beta\) (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 A\(\beta\) or tau tangles, suggesting the possibility of other pathogenic mechanisms. Oxidative stress and
1. Introduction

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$_2$O). 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$_2$O$_2$), another ROS that is further reduced to H$_2$O 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):

\begin{align*}
\text{Fenton reaction:} & \quad \text{Me}^{n+} + \text{H}_2\text{O}_2 \rightarrow \text{Me}^{(n+1)+} + \text{OH}^\bullet + \text{OH}^- \\
\text{Haber-Weiss reaction:} & \quad \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}^\bullet + \text{OH}^- + \text{O}_2
\end{align*}

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$_2$O$_2$, 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 antioxidative 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).
1. Introduction

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$_2^-$ 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., 2015). 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
1.1. Alzheimer’s disease

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. Aβ1–42 is lower in patients with AD whereas total and phosphorylated tau levels are higher than in controls. Especially the ratios Aβ1–42/Aβ1–40 and Aβ1–42/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
1. Introduction

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 AChEIs 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 a 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,
1. Introduction

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
sarcotesta is removed and the gametophytes, also called ‘nuts’, are briefly steamed or boiled before baking them dry. This drug, bái guò, 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 wù), Epimedium spp. (yín yáng huò), or Phellodendron spp. (huáng bái) (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 (wū zhù yú), Magnolia officinalis (hòu pò), Paeonia lactiflora (chí sháo), Panax ginseng (rén shén), or Verbena officinalis (mà biān cáo) 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.

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

<sup>1</sup>Naturally occurring mouse line with accelerated senescence and several features similar to AD.

<sup>2</sup>Doubly transgenic mice expressing APP with the Swedish mutation and PSEN1 with M146L mutation.

<sup>3</sup>Transgenic mice expressing APP with the Swedish mutation.
<table>
<thead>
<tr>
<th>Plant</th>
<th>Drug</th>
<th>Model</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cnidium monnieri</em></td>
<td>osthole</td>
<td>APP/PS1 mice&lt;sup&gt;4&lt;/sup&gt;</td>
<td>memory enhancing, enhanced neurogenesis in dentate gyrus, neurotrophic</td>
<td>Liu &lt;i&gt;et al.&lt;/i&gt; (2015b)</td>
</tr>
<tr>
<td><em>Coptis chinensis</em></td>
<td>berberine</td>
<td>TgCRND8 mice&lt;sup&gt;5&lt;/sup&gt;, cell culture</td>
<td>memory enhancing, decreased Aβ levels, anti-inflammatory, decreased phosphorylation of APP and tau</td>
<td>Durairajan &lt;i&gt;et al.&lt;/i&gt; (2012)</td>
</tr>
<tr>
<td><em>Eleutherococcus senticosus</em></td>
<td>water and methanol extracts of rhizome methanol extract of rhizome, isolated compounds</td>
<td>rat cortical primary neuron cultures treated with Aβ&lt;sub&gt;25–35&lt;/sub&gt;</td>
<td>protective against axonal and dendritic atrophy, against cell death, and against synapse loss</td>
<td>Tohda &lt;i&gt;et al.&lt;/i&gt; (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rat cortical primary neuron cultures treated with Aβ&lt;sub&gt;25–35&lt;/sub&gt;</td>
<td>protective against axonal and dendritic atrophy</td>
<td>Bai &lt;i&gt;et al.&lt;/i&gt; (2011)</td>
</tr>
<tr>
<td><em>Epimedium brevicornum</em></td>
<td>icariin</td>
<td>APP/PS1-21 mice&lt;sup&gt;6&lt;/sup&gt;</td>
<td>rescues nesting behaviour, reduced Aβ deposition, anti-inflammatory, memory enhancing, reduced insoluble Aβ and APP levels, increased neurogenesis</td>
<td>Zhang &lt;i&gt;et al.&lt;/i&gt; (2014)</td>
</tr>
<tr>
<td></td>
<td>icariin</td>
<td>Tg2576</td>
<td>memory enhancing, reduced insoluble Aβ and APP levels, increased neurogenesis</td>
<td>Li &lt;i&gt;et al.&lt;/i&gt; (2015)</td>
</tr>
<tr>
<td><em>Evodia rutaecarpa</em></td>
<td>evodiamine</td>
<td>APP/PS1, SAMP8 mice</td>
<td>memory enhancing, increased glucose uptake in the brain, anti-inflammatory</td>
<td>Yuan &lt;i&gt;et al.&lt;/i&gt; (2011)</td>
</tr>
<tr>
<td></td>
<td>EGb761®, individual compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EGb761®, ginkgolides, bilobalide, flavonoids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ginkgolides A, B, C, and bilobalide</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>4</sup>Doubly transgenic mice expressing APP with the Swedish mutation and PSEN1 with exon 9 deletion.

<sup>5</sup>Transgenic mice expressing APP with the Swedish mutation and V717F mutation.

<sup>6</sup>Doubly transgenic mice expressing APP with the Swedish mutation and PSEN1 with L166P mutation.
## Table 1.1 – Continued from previous page

<table>
<thead>
<tr>
<th>Plant</th>
<th>Drug</th>
<th>Model</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ginkgo biloba</em></td>
<td>EGb761®</td>
<td>TgCRND8 mice, cell culture</td>
<td>memory enhancing, anti-inflammatory, induces autophagy, reduces Aβ deposits</td>
<td>Liu <em>et al.</em> (2015d)</td>
</tr>
<tr>
<td><em>Magnolia officinalis</em></td>
<td>magnolol, honkiol, ethanol extract of bark, 4-O-methylhonkiol, ethanol extract of bark</td>
<td>cell culture, mice treated with Aβ1-42, cell culture, Aβ1-42, BACE1, Tg2576</td>
<td>protective against apoptotic cell death, interfere with increases in calcium and ROS concentration, memory enhancing, protective against neuronal cell death, antioxidant, inhibited Aβ aggregation, BACE1 activity, and BACE1 expression, memory enhancing, decreases Aβ1-42 levels, reduces expression of APP and BACE1</td>
<td>Hoi <em>et al.</em> (2010), Lee <em>et al.</em> (2010a), Lee <em>et al.</em> (2012c)</td>
</tr>
<tr>
<td><em>Paeonia lactiflora</em></td>
<td>paeoniflorin, paeoniflorin, isolated lignans</td>
<td>rats treated with Aβ1-42, microglial cell culture treated with Aβ1-42, Aβ1-42</td>
<td>memory enhancing, antioxidant, protective against apoptotic cell death, interferes with increase in calcium, reduced secretion of inflammatory mediators and chemokines, suppressed chemotaxis to Aβ1-42, inhibit Aβ1-42 aggregation</td>
<td>Zhong <em>et al.</em> (2009), Liu <em>et al.</em> (2015a), Liu <em>et al.</em> (2015e)</td>
</tr>
<tr>
<td><em>Panax ginseng</em></td>
<td>extract from HerbPharm, pure compounds</td>
<td>cell culture, Tg2576 mice</td>
<td>reduce Aβ levels, ginsenosides Re, Rg1, and Rg3 selectively reduce Aβ1-42 levels</td>
<td>Chen <em>et al.</em> (2006)</td>
</tr>
<tr>
<td>Plant</td>
<td>Drug</td>
<td>Model</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------------</td>
<td>--------------------------------------------</td>
<td>---------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td><em>Panax ginseng</em></td>
<td>ginsenoside Rg3</td>
<td>cell culture</td>
<td>reduces A(\beta) levels, enhances neprilysin gene expression</td>
<td>Yang <em>et al.</em> (2009a)</td>
</tr>
<tr>
<td></td>
<td>fermented water extract of</td>
<td>cell culture, scopolamin-induced amnesic</td>
<td>memory enhancing, reduces A(\beta_{1-42}) level</td>
<td>Kim <em>et al.</em> (2013)</td>
</tr>
<tr>
<td></td>
<td>root</td>
<td>mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ginsenoside compound K</td>
<td>mouse primary astrocyte culture</td>
<td>promotes A(\beta) clearance through enhancing autophagy</td>
<td>Guo <em>et al.</em> (2014a)</td>
</tr>
<tr>
<td><em>Panax notoginseng</em></td>
<td>panaxynol, panaxydol</td>
<td>rat cortical primary neuron cultures treated with A(\beta_{25-35})</td>
<td>protective against apoptotic cell death, interfere with increases in calcium and ROS concentration</td>
<td>Nie <em>et al.</em> (2008)</td>
</tr>
<tr>
<td></td>
<td>quercetin</td>
<td>cell culture, A(\beta_{1-40}), scopolamin-induced amnesic mice</td>
<td>oestrogenic activity, antioxidant, inhibits A(\beta) aggregation, protective against apoptotic cell death, interferes with increase in calcium, memory enhancing</td>
<td>Choi <em>et al.</em> (2010)</td>
</tr>
<tr>
<td></td>
<td>notoginsenoside R1</td>
<td>cell culture</td>
<td>protective against apoptotic cell death and mitochondrial damage, antioxidant</td>
<td>Ma <em>et al.</em> (2014)</td>
</tr>
<tr>
<td></td>
<td>notoginsenoside R1</td>
<td>mouse hippocampal primary neuron cultures, acute brain slices, APP/PS1 mice</td>
<td>memory enhancing, increases intrinsic excitability of hippocampal neurons, rescues A(\beta)-induced decline in long term potentiation</td>
<td>Yan <em>et al.</em> (2014)</td>
</tr>
<tr>
<td><em>Phellodendron chinense / amurense</em></td>
<td>ethanol extract of cortex</td>
<td>cell culture</td>
<td>protective against apoptotic cell death, <em>P. amurense</em> is more potent than <em>P. chinense</em></td>
<td>Xian <em>et al.</em> (2013)</td>
</tr>
<tr>
<td><em>Polygonum multiflorum</em></td>
<td>water extract of roots</td>
<td>mice treated with A(\beta_{25-35}) <em>Drosophila melanogaster</em> expressing A(\beta_{1-42}), cell culture</td>
<td>memory enhancing, inhibits AChE, antioxidant ameliorated neurological phenotypes, protective against cell death, antioxidant</td>
<td>Um <em>et al.</em> (2006)</td>
</tr>
<tr>
<td></td>
<td>ethanol extract of roots, 2,3,5,4'-tetrahydroxy-stilbene-2-O-(\beta)-D-glucoside</td>
<td></td>
<td></td>
<td>Liu <em>et al.</em> (2015c)</td>
</tr>
<tr>
<td><em>Punica granatum</em></td>
<td>pomegranate juice</td>
<td>Tg2576 mice</td>
<td>improved learning, reduced A(\beta) levels</td>
<td>Hartman <em>et al.</em> (2006)</td>
</tr>
<tr>
<td><em>Rhodiola rosea</em></td>
<td>salidroside</td>
<td>cell culture</td>
<td>protective against apoptotic cell death, antioxidant</td>
<td>Zhang <em>et al.</em> (2010)</td>
</tr>
</tbody>
</table>

Continued on next page
### 1. Introduction

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. (Arecales). This palm tree is native to south and southeast 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 (bìn láng), baked, or charred. Bìn 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).

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

Table 1.1 – Continued from previous page
1.2. Medicinal plants

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 cāo (the drug consisting of dried roots of either three *Glycyrrhiza* species) is one of

![Figure 1.5: Selected compounds from *Alpinia oxyphylla* and *Areca catechu*.](image1)

![Figure 1.6: *Glycyrrhiza glabra* (picture taken in the botanical garden of University of Würzburg, June 2008) and the drug gān cāo, dried root from *Glycyrrhiza uralensis*.](image2)
1. Introduction

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 glycyrrhetic 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 phospohoinositide 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-
1.2. Medicinal plants

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 E2 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 (Dordević et al., 2012; Herrmann et al., 2011a; Jović et al., 2012; Schmidt-Thomé, 1950). Furthermore, antioxidant (Dordević et al., 2007, 2012), anti-inflammatory, anti-ulcer (Dordević 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 acaenthifolia 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 (Dordević et al., 2012; Schilcher & Hagels, 1990; Stojanović-
1. Introduction

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 Rhabdidae. 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.
1.3. Caenorhabditis elegans

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.
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 PIP₃. This is antagonized by the phosphatase DAF-18. PIP₃ 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 (PIP₃) 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 PIP₃ 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*.

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

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, motor-, 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).
1. Introduction

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 Aβ1–42. Further examination of this model, however, showed that Aβ fibrils were formed intracellularly and the peptide produced was Aβ3–42, 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.
1.4. Objectives

- **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
- 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
2. Material and methods

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
<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile (ACN)</td>
<td>J.T. Baker</td>
</tr>
<tr>
<td>Acrylamide/bisacrylamide (30:1)</td>
<td>Roth</td>
</tr>
<tr>
<td>Actin antibody C11</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Agarose</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ammonium acetate (NH₄Ac)</td>
<td>Grüssing</td>
</tr>
<tr>
<td>Ammonium persulfate (APS)</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>Bacto™ Agar</td>
<td>Beckton, Dickinson and Company</td>
</tr>
<tr>
<td>Bacto™ Tryptone</td>
<td>Beckton, Dickinson and Company</td>
</tr>
<tr>
<td>Bacto™ Yeast Extract</td>
<td>Beckton, Dickinson and Company</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>Merck</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Beta-amyloid 1-42, human</td>
<td>PeptaNova</td>
</tr>
<tr>
<td>Beta-amyloid monoclonal antibody 6E10, horse radish peroxidase (HRP) labelled</td>
<td>Signet</td>
</tr>
<tr>
<td>Bromphenolblue</td>
<td>Fulka</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂)</td>
<td>Merck</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Citric acid monohydrate</td>
<td>Applichem</td>
</tr>
<tr>
<td>Complete PCR reaction buffer, 10 x</td>
<td>Bioron</td>
</tr>
<tr>
<td>Coomassie blue G-250</td>
<td>Fulka</td>
</tr>
<tr>
<td>Copper sulphate pentahydrate (CuSO₄·5H₂O)</td>
<td>Grüssing</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>Grüssing</td>
</tr>
<tr>
<td>Deoxynucleotides</td>
<td>Bioron</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>VWR</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate (K₂HPO₄)</td>
<td>Merck</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate (Na₂HPO₄)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate</td>
<td>Applichem</td>
</tr>
<tr>
<td>(−)-Epigallocatechin gallate (EGCG)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Berkel</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>Serva</td>
</tr>
<tr>
<td>Ethylacetate</td>
<td>University Heidelberg</td>
</tr>
</tbody>
</table>
### 2. Material and methods

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic acid (HCOOH)</td>
<td>Merck</td>
</tr>
<tr>
<td>Glycerin</td>
<td>AppliChem</td>
</tr>
<tr>
<td>Glycin</td>
<td>AppliChem</td>
</tr>
<tr>
<td>Glycyrrhizic acid</td>
<td>Northeast Forestry University, China</td>
</tr>
<tr>
<td>Glycyrrhizic acid monoammonium</td>
<td>Northeast Forestry University, China</td>
</tr>
<tr>
<td>Glycyrrhethinic acid</td>
<td>Northeast Forestry University, China</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>AnalA R Normapur</td>
</tr>
<tr>
<td>Isoliquiritigenin</td>
<td>Phytolab</td>
</tr>
<tr>
<td>Iron sulphate septahydrate (FeSO$_4$$ \cdot 7$H$_2$O)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Juglone (5-hydroxy-1,4-naphthochinon)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Liquiritigenin</td>
<td>Phytolab</td>
</tr>
<tr>
<td>Methanol</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Magnesium sulphate (MgSO$_4$)</td>
<td>Merck</td>
</tr>
<tr>
<td>Milk powder</td>
<td>Granovita</td>
</tr>
<tr>
<td>Manganese chloride tetrahydrate (MnCl$_2$$ \cdot 4$H$_2$O)</td>
<td>Merck</td>
</tr>
<tr>
<td>Nitocellulose blotting membrane</td>
<td>Satorius</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>Air Liquide</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Phosphoric acid</td>
<td>Roth</td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Primers ITS4, ITS5</td>
<td>NWG</td>
</tr>
<tr>
<td>Protease inhibitor cocktail Complete Mini</td>
<td>Roche</td>
</tr>
<tr>
<td>PST I</td>
<td>Fermentas</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>Merck</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate (KH$_2$PO$_4$)</td>
<td>Applichem</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>Applichem</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>Applichem</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>Merck</td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium hypochlorite (NaClO)</td>
<td>Grüssing</td>
</tr>
<tr>
<td>Spectra™ Multicolor low range protein ladder</td>
<td>Fermentas</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>Bioron</td>
</tr>
</tbody>
</table>
2.1. Material

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$7H$_2$O) Merck

2.1.4 Buffers, solutions, media

Phosphate buffered saline (PBS), 1 ×
- 140 mM NaCl
- 2.7 mM KCl
- 10 mM Na$_2$HPO$_4$
- 1.8 mM KH$_2$PO$_4$

pH was adjusted to 7.4 with HCl.

4 % Paraformaldehyde (PFA) solution

50 ml H$_2$O 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$_2$O. 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$_2$HPO$_4$
- 6 g KH$_2$PO$_4$
- 1 ml 5 mg/ml Cholesterol in ethanol
- H$_2$O to 1 litre

The solution was autoclaved for 15 min at 125 °C.
2. Material and methods

**Trace metals solution**

- 1.86 g Disodium EDTA
- 0.69 g FeSO$_4$$\cdot$7H$_2$O
- 0.2 g MnCl$_2$$\cdot$4H$_2$O
- 0.29 g ZnSO$_4$$\cdot$7H$_2$O
- 0.025 g CuSO$_4$$\cdot$5H$_2$O
- H$_2$O 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$_2$O 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$_2$HPO$_4$
- 108.3 g KH$_2$PO$_4$
- H$_2$O to 1 litre

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

NGM
- 17 g Agar
- 3 g NaCl
- 2.5 g Tryptone
- 975 ml H$_2$O

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$_2$O

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$_2$HPO$_4$
- 3 g KH$_2$PO$_4$
- 1 ml 1 M MgSO$_4$
- H$_2$O 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$_2$O

The solution was autoclaved for 15 min at 125 °C.
2. Material and methods

**LB Agar**
- 10 g Tryptone
- 5 g Yeast extract
- 10 g NaCl
- 15 g Agar
- 950 ml H$_2$O

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$_2$O

**TAE-Buffer, 50 ×**
- 2420 g Tris
- 186 g EDTA
- 571 ml Acetic acid
- H$_2$O to 10 litre

**Bradford reagent**
- 25 mg Coomassie blue G-250
- 12.5 ml Ethanol
- 25 ml Phosphoric acid
- H$_2$O to 250 ml

**Coomassie solution**
- 0.5 % Coomassie blue G-250
- 50 % Ethanol
- 10 % Acetic acid
- 39.5 % H$_2$O
2.1. Material

Destaining solution

- 20 % Ethanol
- 10 % Acetic acid
- 70 % H₂O

4 × Laemmli buffer

- 5 ml 1 M Tris, pH 6.8
- 40 ml Glycerin
- 5 g SDS
- 5 mg Bromphenolblue
- H₂O to 100 ml

10 % β-Mercaptoethanol were added prior to use.

Gels for SDS-PAGE

<table>
<thead>
<tr>
<th>Component</th>
<th>15 % resolving gel</th>
<th>5 % stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>2.3 ml</td>
<td>3.4 ml</td>
</tr>
<tr>
<td>30 % Acrylamide mix</td>
<td>5 ml</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>2.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>1 M Tris (pH 6.8)</td>
<td>-</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>0.1 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>10 % APS</td>
<td>0.1 ml</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>4 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

Cathode buffer

- 12.11 g Tris
- 17.92 g Tricine
- 1 g SDS
- 1 l H₂O

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

Anode buffer, 5 ×

- 121.1 g Tris
- 500 ml H₂O

The pH was adjusted to 8.9 with HCl, then water was added to 1 l.
2. Material and methods

**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).

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

*Continued on next page*
Table 2.1 – Continued from previous page

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

2.1.6 *Caenorhabditis elegans* strains

The *C. elegans* strains used in this work (Tab. 2.2) and their food source *Escherichia 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.
2. Material and methods

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Description from CGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>wildtype</td>
<td><em>C. elegans</em> var. <em>bristol</em>. Isolated from mushroom compost near Bristol, England by L.N. Staniland.</td>
</tr>
<tr>
<td>CL2006</td>
<td><em>dvIs2</em></td>
<td><em>dvIs2</em> [pCL12(unc-54::Aβ1–42) + pRF4(rol-6(su1006))]. Adult onset paralysis and egg-laying deficiency when raised at 20 °C.</td>
</tr>
<tr>
<td>CL4176</td>
<td><em>smg-1(cc546) I; dvIs27 X</em></td>
<td><em>dvIs27</em> [pAF29(myo-3::Aβ1–42::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.</td>
</tr>
<tr>
<td>CL802</td>
<td><em>smg-1(cc546) I; rol-6(su1006) II</em></td>
<td>Temperature sensitive with roller phenotype.</td>
</tr>
<tr>
<td>CL2355</td>
<td><em>smg-1(cc546) I; dvIs50 I</em></td>
<td><em>dvIs50</em> [pCL45(snb-1::Aβ1–42::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.</td>
</tr>
<tr>
<td>PD8120</td>
<td><em>smg-1(cc546) I</em></td>
<td>Temperature sensitive.</td>
</tr>
<tr>
<td>TJ375</td>
<td><em>gpIs1</em></td>
<td><em>gpIs1</em> [hsp-16.2::GFP]. Insertion not mapped. GFP fluorescence after &gt; 1 hour heat shock at 35 °C.</td>
</tr>
<tr>
<td>TJ356</td>
<td><em>zIs356 IV</em></td>
<td><em>zIs356</em> [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.</td>
</tr>
</tbody>
</table>

### 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 dichloromethane 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
2. Material and methods

<table>
<thead>
<tr>
<th>Time in min</th>
<th>% H₂O with 3 % acetic acid</th>
<th>% ACN</th>
<th>Flow rate in ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>80</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>50</td>
<td>50</td>
<td>1.2</td>
</tr>
<tr>
<td>25</td>
<td>20</td>
<td>80</td>
<td>1.2</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>80</td>
<td>1.2</td>
</tr>
</tbody>
</table>

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

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 gàn cào 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 x reaction buffer, 0.5 µl 20 mg/ml BSA, 0.2 µl Taq polymerase, 16.3 µl H₂O, 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.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>94 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>38 cycles:</td>
<td></td>
</tr>
<tr>
<td>94 °C</td>
<td>45 s</td>
</tr>
<tr>
<td>45 °C</td>
<td>50 s</td>
</tr>
<tr>
<td>72 °C</td>
<td>50 s</td>
</tr>
<tr>
<td>72 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>4 °C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

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 NH₄Ac 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 H₂O 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:

\[
\text{% DPPH}\text{• scavenging effect} = \frac{A_0 - A_1}{A_0} \times 100
\]

A₀: absorbance of DPPH•
A₁: 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.

<table>
<thead>
<tr>
<th>GenBank accession nr.</th>
<th>Species</th>
<th>Encoded region</th>
</tr>
</thead>
<tbody>
<tr>
<td>JF778868</td>
<td><em>G. inflata</em></td>
<td>5.8S rRNA, partial sequence; ITS2; 26S rRNA, partial sequence</td>
</tr>
<tr>
<td>AB280740</td>
<td><em>G. inflata</em></td>
<td>ITS1; 5.8S rRNA; ITS2</td>
</tr>
<tr>
<td>AY065623</td>
<td><em>G. glabra</em></td>
<td>18S rRNA, partial sequence; ITS1; 5.8S rRNA; ITS2; 26S rRNA, partial sequence</td>
</tr>
<tr>
<td>JF778867</td>
<td><em>G. glabra</em></td>
<td>5.8S rRNA, partial sequence; ITS2; 26S rRNA, partial sequence</td>
</tr>
<tr>
<td>HE602402</td>
<td><em>G. glabra</em></td>
<td>ITS1; 5.8S rRNA; ITS2</td>
</tr>
<tr>
<td>AB280739</td>
<td><em>G. glabra</em></td>
<td>ITS1; 5.8S rRNA; ITS2</td>
</tr>
<tr>
<td>JF421504</td>
<td><em>G. uralensis</em></td>
<td>5.8S rRNA, partial sequence; ITS2; 26S rRNA, partial sequence</td>
</tr>
<tr>
<td>JF778869</td>
<td><em>G. uralensis</em></td>
<td>5.8S rRNA, partial sequence; ITS2; 26S rRNA, partial sequence</td>
</tr>
<tr>
<td>HQ229003</td>
<td><em>G. uralensis</em></td>
<td>5.8S rRNA, partial sequence; ITS2; 26S rRNA, partial sequence</td>
</tr>
<tr>
<td>GQ246129</td>
<td><em>G. uralensis</em></td>
<td>ITS1; 5.8S rRNA; ITS2</td>
</tr>
<tr>
<td>AB280738</td>
<td><em>G. uralensis</em></td>
<td>ITS1; 5.8S rRNA; ITS2</td>
</tr>
<tr>
<td>EU418258</td>
<td><em>G. uralensis</em></td>
<td>ITS1, partial sequence; 5.8S rRNA; ITS2, partial sequence</td>
</tr>
<tr>
<td>AB649775</td>
<td><em>G. uralensis</em></td>
<td>18S rRNA, partial sequence; ITS1; 5.8S rRNA; ITS2; 26S rRNA, partial sequence</td>
</tr>
<tr>
<td>JF421503</td>
<td><em>G. uralensis</em></td>
<td>5.8S rRNA, partial sequence; ITS2; 26S rRNA, partial sequence</td>
</tr>
<tr>
<td>GQ246133</td>
<td><em>G. astragalina</em></td>
<td>ITS1; 5.8S rRNA; ITS2</td>
</tr>
<tr>
<td>GQ246134</td>
<td><em>G. astragalina</em></td>
<td>ITS1; 5.8S rRNA; ITS2</td>
</tr>
</tbody>
</table>

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 (OD600 ≈ 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 prepared 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-lysine 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
2. Material and methods

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 fluorescence 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 3.2: 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), 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](image)

**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](image)

**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).
3. Results

**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 cáo. 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.

|            | 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: glycyrrhetic acid. Values given in µg/ml.

<table>
<thead>
<tr>
<th></th>
<th>LG</th>
<th>ILG</th>
<th>GA</th>
<th>GRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water extract</td>
<td>3.0</td>
<td>0.9</td>
<td>54.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Methanol extract</td>
<td>9.4</td>
<td>1.8</td>
<td>64.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Dichlormethane extract</td>
<td>2.9</td>
<td>1.5</td>
<td>4.6</td>
<td>4.1</td>
</tr>
</tbody>
</table>

**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 + COOH]+, 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−C7O2H5]+) and m/z = 297 due to additional loss of the apiace residue ([M−C7O2H4−C5O4H8]+). 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+ + 2Na+ + 2HCOOH]−, 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
3. Results

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–C₇O₂H₅]+, 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 than 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+2Na+]−, m/z = 865), adducts with formic acid ([M−H+2COOH]−, m/z = 911), combinations of
both kinds of adducts ([M−2H+Na+]−, m/z = 888; [M−2H+Na+2 •COOH]−, m/z = 933; [M−2H+Na+2 •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_{2}O]− (m/z = 803), [M−H−H_{2}O−CO_{2}]− (m/z = 759) and the fragment without one glucuronic acid moiety ([M−C_{6}O_{6}H_{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_{2}O−CO_{2}−C_{6}O_{6}H_{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.
3. Results

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).

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

(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 an acetoxyl group (\(\text{CH}_2\text{COO}^-\)). Other peaks in this spectrum correspond to sodium and formic acid adducts ([M−2H⁺+Na⁺]⁻, m/z = 901; [M−3H⁺+2Na⁺]⁻, m/z = 923; [M−2H⁺+Na⁺+\(^+\text{COOH}\)]⁻, m/z = 946; [M−3H⁺+2Na⁺+\(^+\text{HCOOH}\)]⁻, m/z = 969; [M−2H⁺+Na⁺+2 \(^+\text{COOH}\)]⁻, 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.
The next substance that could be identified is licorice saponin G2 (Tab. 3.3). 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). Again, adducts with sodium ($[M-2H^++Na^+]^-$, m/z = 859; $[M-3H^++2Na^+]^-$, m/z = 881) and formic acid ($[M-3H^++2Na^++HCOOH]^-$, m/z = 927; $[M-4H^++3Na^++2HCOOH]^-$, 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), is licorice saponin J2 which was also observed by Farag et al. (2012) and Montoro et al. (2011). Its MS spectrum (Fig. 3.15) shows a $[M-H]^-$ peak with m/z = 823, sodium ($[M-2H^++Na^+]^-$, m/z = 845; $[M-3H^++2Na^+]^-$,
3. Results

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 subtracted.

m/z = 867) and formic acid adducts ([M−H]+HCOOH]−, m/z = 868; [M−2H+2 *COOH]−, m/z = 913), and combination of both ([M−3H+2Na+2 *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−CO2]−) 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 subtracted.

strong absorption at 356 nm, pointing to a conjugated $\pi$-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+−OCH3+H]+, m/z = 337) or, to a lesser extent, only the methyl group ([M−H+−CH3]+, m/z = 352), loss of carbon dioxide from the lactone group ([M−H+−CO2]+, m/z = 323), and loss of the isoprenyl group ([M−H+−C5H9]+, 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
3. Results

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 subtracted.

A small peak with m/z = 338 indicating the loss of a methyl group ([M–H+–*CH3]–). 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 an isoprenyl group ([M–H+–*C5H9]– or [M–C5H9+]–, respectively). The masses 298 and 297 could arise from a partial loss of this group ([M–H+–*C4H7]– and [M–2H+–C4H7–], respectively). There are several substances with the corresponding molecular mass of 354, such as licochalcon D and G, licoflavonol, gancaonin I, dehydroglyasperin C, or glycybenzo-furan. All of them have an 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 subtracted.
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.
molecules detected here. Substances 13 and 14 could be gancaonin I, dehydrogysasperin 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, \([M-H−'CH_3]^+\)), \(307\) (additional loss of carbon dioxide, \([M−CH_3−CO_2]^−\)), \(295\) (loss of a methyl and part of an isoprenyl group, \([M−H−'CH_3−'C_4H_7]^−\)) and \(282\) (loss of a methyl and an isoprenyl group, \([M−CH_3−'C_5H_9]^−\)). Glycyrol has an 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 \(\pi\)-electron system of

**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 subtracted.
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–C₅H₉+] , m/z = 283) and a methyl group ([M–H–°CH₃]– , 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–C₅H₉H₂O]–). 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–C₅H₉+]–). 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
3.4. Glycyrrhiza uralensis

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−C5H9−C5H9]+•−).

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](image-url)

*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.](image)

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 Aβ1-42 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.](image)

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 PT50. The effect for the methanol extract was dose-dependent, 200 µg/ml increased PT50 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 PT50 by 1.8 h (p = 0.02), but the control EGCG had the highest effect with 2.7 h increase in PT50 (p = 0.03). From the pure substances only ILG had a significant effect with 1.2 h increase in PT50 (p < 0.01). Notably, both controls treated either with water or with 1 % methanol had 2.7 h difference in their PT50 values.
3. Results

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) \(P_{50}\) 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](image_url)

**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. 3.24B). 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-
3. Results

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.
3.4 Glycyrrhiza uralensis

Treatment 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 LLG (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
3.4. *Glycyrrhiza uralensis*

**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.](image-url)
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).

![GLC-MS total ion current of dichloromethane extract of C. acaulis and corresponding MS spectra.](image)

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 (Dordević *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.](image)
3.5.2 Paralysis assay

Figure 3.33: Results of the paralysis assay for *C. acaulis* extract (Carlina), the extract without Carlina oxide (CarO) 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, CarO 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 CarO (*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.](image-url)
3. Results

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• scavenging**

The *in vitro* antioxidant activity was tested via DPPH• 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• scavenging activity of C. acaulis](image)

**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).
3.5. *Carlina acaulis*

![Image of plants and bar graph](image)

**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).
3. Results

**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 guŏ. Other compounds remain in the nuts or are produced during the preparation of bái guŏ. To our knowledge there are to date no studies of the effects of *G. biloba* seeds or bái guŏ 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 panaxyol 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 bái), *A. galanga*, and *Coptis* spp. (huáng lián). *P. amurense* and *P. chinense*, both species used in the TCM drug huáng bái, 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 Aβ25–35. 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 lián. The *Phellodendron* species also contain berberine, although
in smaller amounts than Coptis spp. (Van Wyk et al., 2015). Therefore, the effect of huáng bái 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 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 Aβ1–42 ([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 cāo 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 nu-
cleotides 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 identi-
cal 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 chalcones 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 consist-
ent 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 differ-
ent 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 aglycones 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 < 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.
4. Discussion

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 activity. 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
4.4. Glycyrrhiza uralensis

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).
4. Discussion

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 \textit{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 \textit{C. acaulis} was further studied in the \textit{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. \textit{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. \textit{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 \textit{in vivo} antioxidant activity

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

The extract has an EC$_{50}$ of 122 µg/ml in the DPPH$^*$ assay (Fig. 3.35). Dordević \textit{et al.} have reported an EC$_{50}$ of 13.6 µl/ml for the essential oil of \textit{C. acanthifolia} (Dordević \textit{et al.}, 2007) and 208 and 155 µg/ml for the methanol extracts of \textit{C. acaulis} and \textit{C. acanthifolia} roots, respectively (Dordević \textit{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. Dordević \textit{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.
4. Discussion

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 bursa bursa* (Herrmann et al., 2011a) and 0.1 ml of the oil can be deadly to rats (Schmidt-Thomé, 1950). This poses the question, 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.

Dordević 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. Dordević 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 hormeric 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 multi-target 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 hormeric 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


References


References


Cruts M, Theuns J, Van Broeckhoven C (2012) Locus-specific mutation databases for neurodegener-


Daigle I, Li C (1993) apl-1, a Caenorhabditis elegans gene encoding a protein related to the human


Davidson JS, Baumgarten IM (1988) Glycyrrhetinic acid derivatives: a novel class of inhibitors of


Deibel MA, Ehmann WD, Markesbery WR (1996) Copper, iron, and zinc imbalances in severely de-

Denzer I, Münch G, Pischetsrieder M, Friedland K (2016) S-allyl-l-cysteine and isoliquiritigenin im-

References


References


References


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.


References


References


References


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.


References


References


References


References


