Extracts of *Glycyrrhiza uralensis* and Isoliquiritigenin Counteract Amyloid-*β* Toxicity in *Caenorhabditis elegans*

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Abstract
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Alzheimer's disease is a rising threat for modern societies as more and more people reach old age. To date, there is no effective treatment for this condition. In this study, we investigated the potential of *Glycyrrhiza uralensis* to counteract amyloid-*β* toxicity, one of the key features of Alzheimer's disease. An LC-MS/MS analysis revealed glycyrrhizic acid and glycosylated forms of isoliquiritigenin and liquiritigenin as major constituents of water and methanol extracts of *G. uralensis*. These extracts and the pure compounds were tested for their activity in two *Caenorhabditis elegans* models of amyloid-*β* aggregation and amyloid-*β* toxicity, respectively. The number of amyloid-*β* aggregates decreased by 30% after treatment with isoliquiritigenin, the methanol extract could reduce the number by 14%, liquiritigenin and glycyrrhizic acid by 15%, and the aglycon of glycyrrhizic acid, glycyrrhetinic acid, by 20%. Both extracts and isoliquiritigenin also showed significant activity against acute amyloid-*β* toxicity in transgenic *C. elegans* that express human amyloid-*β* peptides, delaying the paralysis in this model by 1.8 h and 1.1 h, respectively. We conclude that secondary compounds of *G. uralensis* may become interesting drug candidates for the treatment of Alzheimer's disease, which, however, need further analysis in other model systems.

Abbreviations
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A*β*:	amyloid-beta
AD:	Alzheimer's disease
EGCG:	(−)-epigallocathechin gallate
GA:	glycyrrhizic acid
GUE:	Glycyrrhiza uralensis extract
GRA:	glycyrrhetinic acid
ILG:	isoliquiritigenin
LG:	liquiritigenin
NGM:	nematode growth medium
NMDA:	N-methyl-D-aspartate
PT_{50}:	median paralysis time
TCM:	traditional Chinese medicine

Introduction
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AD occurs with a prevalence of 5% in people over 60 and the risk increases with age. About 36 million people were estimated to suffer from this disease in 2010 according to Alzheimer's disease International [1]. Despite these facts, there is still no effective cure for this disease today. Therefore, it is important to continue research for new possible disease modulating substances. Rich sources for potential therapeutic compounds are medicinal plants and their secondary metabolites, which have evolved as a means of protection against herbivores and microbes for plants producing them [2].

Glycyrrhiza species (eng. licorice; Fabaceae) have been known as medicinal plants and sweets for centuries both in Europe and Asia. The traditional uses include conditions of the respiratory system and the gastrointestinal tract [3]. Licorice is also used in many formulations of TCM and is the most frequently used herb in the Chinese Formulae Database [4]. Several studies indicate that *Glycyrrhiza* can be beneficial in the treatment of AD [5–7]. The most abundant secondary metabolite found in *Glycyrrhiza* species is the triterpene saponin GA. GA and its aglycone GRA have been shown to have anti-inflammatory and neuroprotective effects [8,9]. Next to the triterpenes, the plant also produces many flavonoids. These polyphenols can interact with biomolecules via their phenolic hydroxyl groups and thereby modify the function of many proteins. The flavonoid ILG shows anti-inflammatory [10–12] and neuroprotective ef-
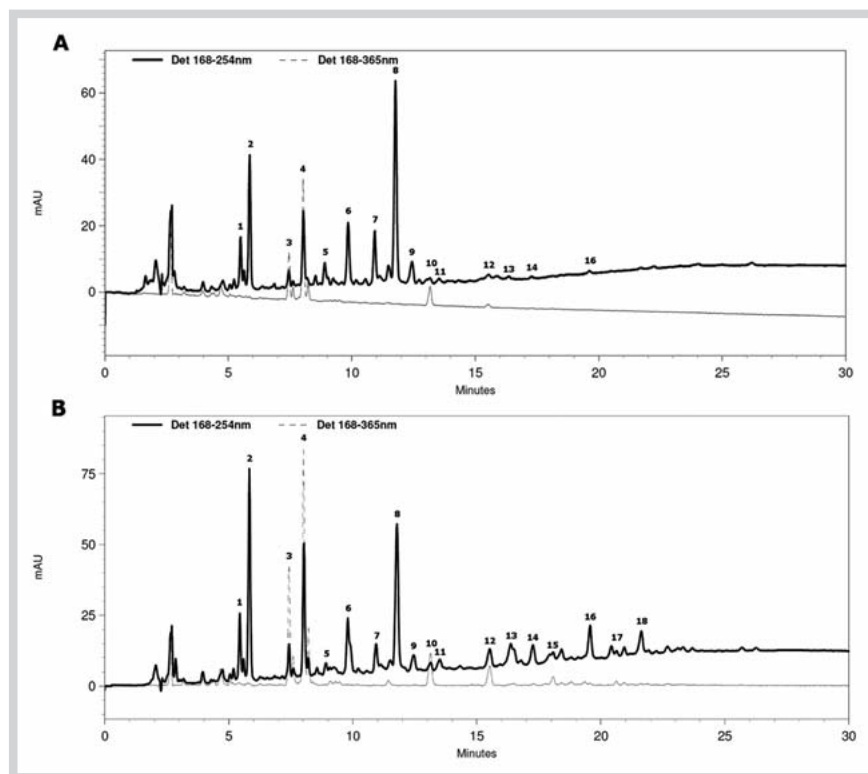
Fig. 1 HPLC profile of water (**A**) and methanol (**B**) extracts of *Glycyrrhiza uralensis* recorded at 254 and 365 nm. Peak numbers correspond to compounds listed in ▸ **Table 1**.

fects [13, 14]. It is an NMDA receptor antagonist [15]. Additionally, ILG and its isomer LG exhibit antidepressant properties in mice [16] and both have been shown to inhibit neurotoxicity caused by Aβ in rat neurons [17, 18].

Aβ is one of the key proteins in AD and a potential drug target [19]. This peptide of 38–43 amino acids can take different conformations, build aggregates, and interact with cellular processes. Monomeric Aβ is not very stable in aqueous solutions and builds oligomers, which are toxic to cells [20]. Further aggregation leads to so-called senile plaques that are abundant in the brains of AD patients.

To better understand Aβ aggregation and its toxic effect *in vivo*, transgenic *Caenorhabditis elegans* models have been developed [21]. These worms express the human $A\beta_{3-42}$ peptide in their muscles, where it aggregates and forms plaques. The toxicity to the surrounding muscle cells manifests in a paralysis phenotype. This *in vivo* model can assess the bioavailability of the compounds in contrast to *in vitro* aggregation experiments or toxicity assays with cell cultures. In the present study, the effect of *Glycyrrhiza uralensis* Fisch. ex DC and its secondary metabolites on Aβ aggregation and acute Aβ toxicity were studied using transgenic *C. elegans* strains.

Results
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The HPLC analysis revealed several compounds (▸ **Fig. 1**), 18 of which were further analyzed (▸ **Table 1**). Substances **1–9, 11, 12, 15**, and **18** could be identified according to their mass spectra and to published data for GUEs [22–24]. GA and glycosylated forms of LG and ILG are among the most abundant compounds in this extract. In the water extract (▸ **Fig. 1 A**), fewer compounds were found. The more lipophilic substances **15, 17**, and **18** were missing; **12, 14**, and **16** were only present in traces. Also, the oth-

er compounds were less concentrated in the water extract, except for the saponins **7** and **8** that have a slightly higher abundance in the water extract compared to the methanol extract.

CL2006, a transgenic *C. elegans* strain that constitutively expresses the human Aβ peptide, was used to test the effect on Aβ aggregation *in vivo*. Aβ aggregates can be visualized by thioflavin S staining. ▸ **Fig. 2** shows typical pictures for the thioflavin S staining of a control (**A**) and a GUE-treated worm (**B**). GUE (500 µg/mL) and the pure substances GA, GRA, LG, and ILG (50 µg/mL) all significantly reduced the number of Aβ aggregates (▸ **Fig. 2 C**). The effect of ILG (30% reduction) was similar to the positive control EGCG (a polyphenol from green tea) (100 µg/mL) that reduced the number of Aβ aggregates by 35%. GUE, GA, GRA, and LG had a weaker effect (a reduction by 14% for GUE, 15% for GA and LG, and 20% for GRA). For all treatments, the low concentrations had no significant effect; a significant effect was reached at a concentration of 50 µg/mL for GRA, LG, and ILG and at a concentration of 500 µg/mL for GUE, as can be seen on ▸ **Fig. 2 D**. Therefore, the treatments can be considered dose-dependent.

The paralysis assay with strain CL4176 reveals the effect of the treatment on Aβ toxicity. Since the methanol that was used as a solvent for methanol GUE, ILG, LG, GRA, and GA had a paralysis delaying effect itself, the results of the compounds were compared to a methanol-treated control (▸ **Fig. 3 A, B**). EGCG and water GUE that were solved in water were compared to a water-treated control (▸ **Fig. 3 C**). EGCG was used as a positive control in a concentration of 100 µg/mL. It increased the median time to paralysis (PT_{50}) by 2.7 h (▸ **Table 2**). Treatments with 500 µg/mL methanol GUE, 200 µg/mL GUE (both methanol and water), and 50 µg/mL ILG could also significantly increase the time until paralysis compared to the control. The water extract was less effective than the positive control, but with a 1.7 h increase in PT_{50}, it was more effective than the same amount of methanol extract.

Nr.	RT in min	λ_{max}	$[M - H]^-$	Daughter ions	Tentative identification
1	5.4	198, 214, 277, 310	549	255, 429	liquiritin apioside
2	5.8	199, 215, 277, 311	417	255	liquiritin
3	7.4	206, 362	549	225, 417	isoliquiritin apioside
4	8.0	202, 361	417	255	isoliquiritin
5	8.9	214	983	821, 803, 351	licorice saponin A3
6	9.8	199, 215, 276, 311	879	351, 861, 703, 817	22-acetoxyglycyrrhizin
7	10.9	215	837	351, 661, 819, 775	licorice saponin G2
8	11.8	218, 250	821	351, 803, 645, 759	glycyrrhizic acid
9	12.4	217	821	351, 803, 645, 759	licorice saponin H2
10	13.1	218, 370	821	351, 510, 645, 777	
11	13.5	218	823	351, 805, 647	licorice saponin J2
12	15.5	218, 348	367	298, 337	glycycoumarin
13	16.3	219	353	298, 284	
14	17.3	221, 287	353	297, 285	
15*	18.1	221, 347	365	307, 295, 350	glycyrol
16	19.6	221	351	283, 265, 307	
17*	20.6	219, 292	423	229, 193	
18*	21.6	222, 268	421	284	

Table 1 Retention times, absorption maxima, and results of the MS/MS analysis of *G. uralensis* extract.
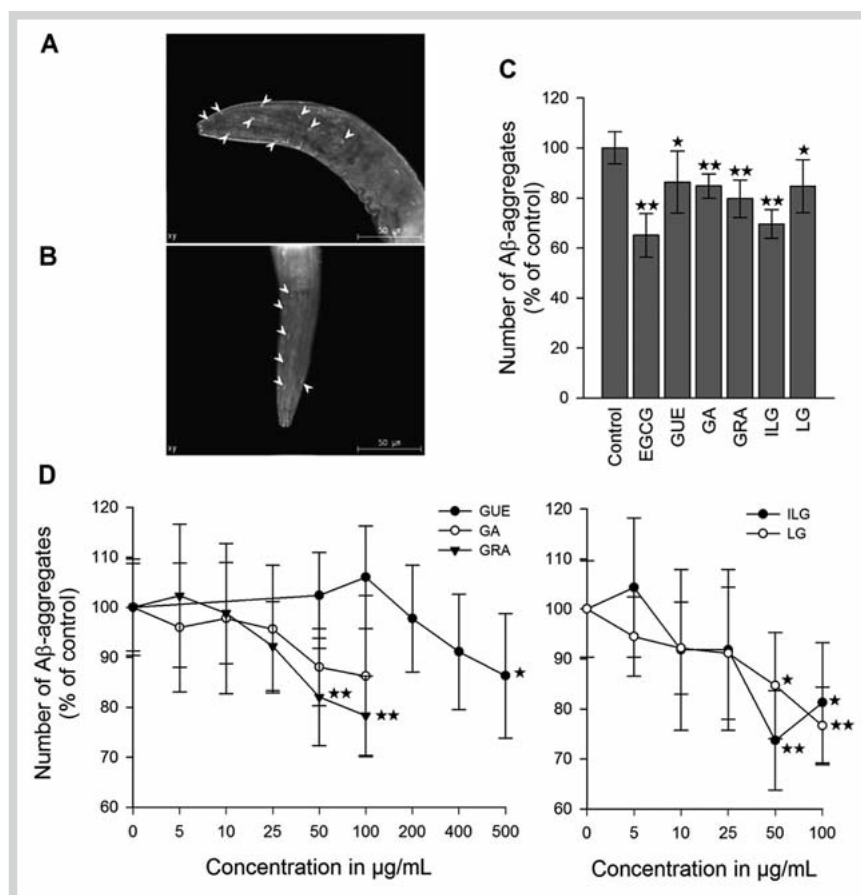
* Only present in the methanol extract

Fig. 2 Effects of *G. uralensis* extract, glycyrrhizic acid, liquiritigenin, and isoliquiritigenin on the amyloid-β aggregation in *C. elegans* strain CL2006. **A, B** Typical images of the methanol-treated control worm (**A**) and a worm treated with 500 µg/mL methanol GUE (**B**), both stained with thioflavin S. Arrowheads point out the Aβ plaques. **C** Reduction in number of Aβ aggregates relative to the control. Control treated with 1% methanol, positive control with 100 µg/mL EGCG, samples for GUE with 500 µg/mL methanol extract, and other samples with 50 µg/mL of respective substance. **D** Dose-dependence of the treatments. *P < 0.05, **p < 0.01 compared to control.

Treatments with LG, GRA, and GA showed no significant increase. The control strain CL802 showed no paralysis when treated with extracts or pure compounds.

Discussion

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All the tested substances had a significant effect on Aβ aggregation and lowered the number of plaques in the *C. elegans* strain CL2006, but the effects were much weaker in the test for acute Aβ toxicity. Only GUE and ILG could delay the paralysis in CL4176. In this *C. elegans* strain, amyloid toxicity was observed despite the small number of amyloid deposits, suggesting that
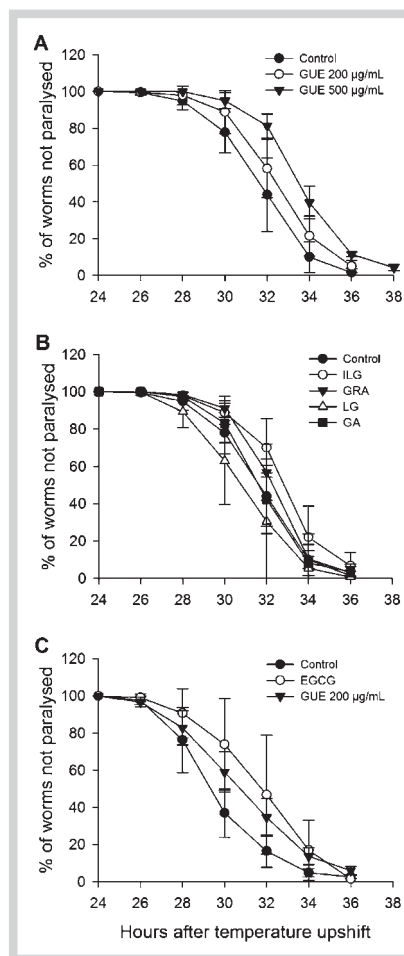
Fig. 3 Results of the paralysis assay in CL4176. **A,B** Paralysis curves for worms treated with the methanol extract of *G. uralensis* (200 and 500 µg/mL) (**A**), ILG, GRA, LG, and GA (50 µg/mL each) (**B**). Control treated with 1% methanol. **C** Paralysis curves for EGCG (100 µg/mL) and the water extract of *G. uralensis* (200 µg/mL); the control was treated with water.

Table 2 Delay of paralysis in *C. elegans* strain CL4176.

Treatment	$PT_{50} \pm SD$	Significance
1% methanol	33.7 ± 0.6	
200 µg/mL GUE	34.4 ± 0.7	p < 0.05
500 µg/mL GUE	35.5 ± 0.4	p < 0.01
50 µg/mL GA	33.7 ± 0.6	
50 µg/mL GRA	34.3 ± 0.7	
50 µg/mL LG	32.8 ± 1.5	
50 µg/mL ILG	34.8 ± 0.7	p < 0.05
Water	30.9 ± 0.8	
100 µg/mL EGCG	33.6 ± 1.7	p < 0.01
200 µg/mL GUE	32.7 ± 0.8	p < 0.05

PT_{50} values in hours after temperature upshift, calculated from paralysis curves (○ **Fig. 3**); p values compared to the respective control (methanol or water)

the toxicity was exerted by Aβ oligomers [25]. Possibly, the other tested substances are only able to destabilize fibrillar Aβ, resulting in a decreased number of plaques, but they fail to interact with the toxic oligomers. To confirm the interactions with different forms of Aβ, further studies have to be performed.

Along with a possible direct interaction of GUE and its constituents with Aβ, the effects seen can also be explained by the supporting nature of the drugs on the innate defensive mechanisms of the worms. Cohen et al. have proposed that there are two possibilities in *C. elegans* to deal with toxic Aβ oligomers on a molecular level: deposition in less toxic plaques or disaggregation and degradation [26]. Based on this hypothesis, the observation that GUE and ILG reduce both toxicity and the number of Aβ aggregates can be interpreted as an enhancement of the latter pathway involving HSF-1. This transcription factor is highly conserved and has an ortholog in humans. Since clearance of Aβ is decreased in patients with Alzheimer's disease [27], a stimulation of the degradation is a promising therapeutic strategy, and the results shown here suggest that GUE or ILG might be potential drugs.

The results for ILG are in accordance with earlier results in cell cultures [18]. Interestingly, the isomer LG fails to delay the paralysis in CL4176, although it could prevent apoptosis of cultured rat neurons at even lower concentrations than ILG [17]. The suggested mechanism of action for LG involves the estrogen receptor β and Notch-2 signaling, resulting in lower astrogliosis [28]. Although *C. elegans* expresses homologues of an estrogen receptor and Notch-2, the nervous system of *C. elegans* is much simpler. For example, it does not contain astrocytes. Therefore, the inability of this study to reproduce the positive effects of LG seen in mice and cell cultures might be due to lack of a corresponding target or pathway in the chosen model organism.

The beneficial effect of ILG against Aβ toxicity might be achieved by NMDA receptor antagonism, antioxidative, or antiinflammatory properties of this compound. The reports about antioxidant properties of ILG are somewhat inconsistent. ILG has poor radical scavenging activity compared to other phenolics in licorice, but it can prevent LDL oxidation [29] and has shown beneficial effects in *in vivo* experiments [30]. Thus, the antioxidant properties in the given model system should be determined before any conclusions about the involvement of this mechanism can be made. It has also been shown that ILG can inhibit Aβ aggregation [31]. Possibly, all these effects contribute to the observed results, but the exact mechanism of action needs further elucidation.

ILG was only found in traces in GUE, but its glycosylated forms isoliquiritin and isoliquiritin apioside were far more abundant. Suggesting that the effect of GUE is based on ILG, it is possible that the glycosylated forms found in the extract have similar effects as ILG or are cleaved either by the *Escherichia coli* used as a food source for *C. elegans* or by the enzymes of *C. elegans* itself. Still there is the possibility that this compound alone is not responsible for the effect observed by the treatment with GUE. Therefore, some other not yet tested substances in this extract might either have a toxicity ameliorating effect against Aβ themselves or can synergistically contribute to the effect of ILG.

The observation that the water extract had an even stronger effect than the methanol extract against the paralysis supports the suggestion that ILG is not the only active component of the extracts. The water extract contains less derivatives of ILG, but equal or slightly higher amounts of saponins. Saponins can enhance the effect of a drug by making the cell membranes more permeable and allowing the active substances to reach their intracellular targets. On the other hand, they can be active themselves, although GA, the only saponin tested here, could not counteract the Aβ toxicity alone. The possible synergistic effects of *Glycyrrhiza* saponins need to be further investigated.

The effects of GUE and ILG were quite low given the relatively high drug concentrations, which may be due to an incomplete absorption of the drugs into the body of *C. elegans*. Studies in mice and rats have shown a substantial absorbance for ILG, the major active component of GUE in current experiments, however, the bioavailability in these studies accounted only for 22–34% [32,33]. According to Zheng et al., the amount of drugs absorbed by *C. elegans* is similar to that found in mice [34], and therefore

the low bioavailability may also account for the low effects observed in the present study. It is also possible that part of the drugs were metabolized by the bacteria used as a food source, thereby lowering the effective drug concentration in the experiments. For further studies in *C. elegans*, it is therefore recommended to use dead bacteria.

In conclusion, it can be stated that ILG and GUE exhibit significant effects, counteracting the pathological effects of Aβ in *C. elegans*. While this nematode is a good tool for identifying new drug candidates and studying specific targets, it lacks the complexity of the vertebrate body. Therefore, ILG and possibly other constituents of *G. uralensis* should be further studied as possible drug candidates against AD in vertebrate systems.

Materials and Methods
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Chemicals
EGCG (from green tea, purity ≥ 95%) and thioflavin S were purchased from Sigma-Aldrich Co. ILG (purity > 99%), GA (purity 75%), and glycyrrhetinic acid (GRA) (purity > 96%) were isolated by Prof. Dr. Yujie Fu. LG (purity > 98%) was a kind gift from Dr. Qiujun Lu, Wangjing Science and Technology Park. The purity of ILG, GA, GRA, and LG was confirmed by HPLC as described in [35].

Plant material
Dried roots of *G. uralensis* were purchased in China and provided to our Institute by Prof. Dr. Thomas Efferth. The authenticity of the plant material was confirmed by DNA barcoding of the ITS sequence (GenBank accession number KM588200). Voucher specimens with the registration number P6873 are deposited at the Department of Biology, Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Germany.

Fifty g of the dried plant material were pulverized and extracted with 300 ml of water or methanol at moderate heat for 4 h. The water extract was lyophilized (DER 13.8 : 1), the methanol extract was reduced in a rotary evaporator to dryness (DER 11.1 : 1), and both extracts were stored at −20 °C.

LC-MS/MS analysis of the extract
The composition of the methanol extract was analyzed on an LCQ-Duo ion trap mass spectrometer with an ESI source (ThermoQuest) coupled to a Beckman Gold HPLC system (solvent module 125P, PDA detector 168) with a LiChroCART RP18 column (5 μm, 250 × 4 mm, Merck). A gradient of water and acetonitrile with 0.1% formic acid each was applied from 20% to 80% ACN in 20 min and isocratic for 10 min with the latter conditions. The flow rate was 1 mL/min throughout the whole run. The absorption maxima were determined in background-subtracted spectra by 32 Karat™ software (Beckman Coulter, Inc.). The MS operated in the negative mode with a capillary voltage of −10 V, a source temperature of 200 °C, and high purity nitrogen as a sheath and auxiliary gas at a flow rate of 80 and 40 (arbitrary units), respectively. The ions were detected in a mass range of 50–2000 *m/z*. Major peaks were identified by comparison with published data [22–24].

Caenorhabditis elegans strains and culturing conditions
The *C. elegans* strains CL2006 (constitutively expressing Aβ in its muscle cells) [36], CL4176 (a temperature inducible strain expressing Aβ in its muscle cells) [25], and CL802 (standard control for CL4176) were obtained from *Caenorhabditis* Genetics Center.

The worms were kept on NGM at 20 °C (CL2006) or 16 °C (CL4176 and CL802) and fed with *E. coli* OP50. For all experiments, *C. elegans* eggs, gained from gravid hermaphrodites by sodium hypochlorite treatment, were used to obtain an age-synchronized population. The Aβ aggregation assay was conducted in S-medium with *E. coli* OP50 (1×10^9 cells/mL) as a food source [37].

Amyloid-β aggregation in *Caenorhabditis elegans* CL2006
The worms were treated with 500 μg/mL methanol extract of *G. uralensis* or 50 μg/mL pure substances (solved in methanol) on day two after hatching. The concentrations were chosen based on a dose-dependence experiment, where various concentrations between 5–100 μg/mL for pure compounds and 50–500 μg/mL for the extract were tested. A treatment with 100 μg/mL EGCG served asa positive control and the solvent methanol (1% of the final volume) as a negative control. On day six, the worms were fixed, followed by thioflavin S staining of the Aβ aggregates as described before [38,39]. In contrast to the original method, 0.013% of thioflavin S in 50% ethanol was used. The Aβ aggregates in the head region of the worms were quantified using Nikon Eclipse Ni-E with an FITC filter and 40× objective. Pictures were acquired with a DS-Qi1Mc camera, and deconvolution was performed with Huygens software by SVI.

Paralysis assay in *Caenorhabditis elegans* CL4176
The paralysis assay was conducted as described by Dostal and Link [40]. The methanol extract of *G. uralensis* (200 μg/mL and 500 μg/mL), the water extract of *G. uralensis* (200 μg/mL), or one of the pure substances, ILG, LG, GA, or GRA (50 μg/mL), was added to each NGM plate. EGCG (100 μg/mL) and the respective solvent (1%) were used as controls. The worms were kept at 16 °C for 36 h, and then the temperature was upshifted to 25 °C to induce the transgene expression. Paralysis was scored every 2 h for 12 h or, in the case of GUE 500 μg/mL, for 14 h starting at 24 h after the temperature upshift. Worms who failed to move as a response to a touch with a platin wire were counted as paralyzed.

Statistical analysis
Data are expressed as the mean ± SD of at least three independent experiments (n = 4 for the Aβ aggregation experiments, n = 3 for the paralysis assay). With the data from the paralysis assays, a survival analysis using the life table method was conducted to calculate the median paralysis times (PT_{50}). Differences were analyzed in StatView software using ANOVA and Dunnet's test with $p < 0.05$ as the threshold for significance.

Conflict of Interest

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The authors declare no conflict of interest.

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