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Gene Regulatory Effects of *Ginkgo biloba* Extract and Its Flavonol and Terpenelactone Fractions in Mouse Brain

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Summary The standardised *Ginkgo biloba* extract EGb761 is known for its potential beneficial effects in the prevention and therapy of neurodegenerative disorders including Alzheimer's disease (AD). However, the molecular mechanisms and the specific role of its constituents are largely unknown. The aim of the present feeding trial was to investigate the effects of EGb761 and its major constituents on the expression of genes encoding for proteins involved in the pathogenesis of AD in mouse brain. Six month old C57B6 mice were fed semi synthetic diets enriched with either EGb761 or one of its main fractions, flavonols and terpenelactones, respectively, over a period of 4 weeks. Thereafter, mRNA of α-secretase, neprilysin, amyloid precursor protein (App), App binding protein-1 and acetylcholine esterase was quantified in hippocampus and cortex. EGb761 and its flavonol fraction had no effects on relative mRNA levels of the respective genes in mouse brain. However, the terpenelactone fraction significantly decreased the mRNA levels of App in the hippocampus. Taken together, a 4 week dietary treatment with EGb761 or its main fractions had only moderate effects on mRNA levels of AD related genes in cortex and hippocampus of mice.

Key Words: *Ginkgo biloba*, flavonols, terpenelactones, amyloid beta precursor protein, Alzheimer's disease

Introduction

EGb761 is an extract from the leaves of the *Ginkgo biloba* tree and standardised by its two main groups of constituents, the flavonols (22–27%) and terpenelactones (5–7%) [1] (Fig. 1). The extract is widely administered for the prevention and therapy of disturbances in vigilance, memory, cognitive functions and dementias including Alzheimer's disease (AD) [2, 3]. Both, the flavonols and terpenelactones are thought to be, at least partly, responsible for the pharmacological activity of EGb761 [4]. However, the molecular mechanisms involved and the specific role of the respective constituents are largely unknown. Bastianetto and coworkers have shown a protective effect of EGb761 and its flavonol fraction against nitric oxide induced toxicity in hippocampal cells whereas the terpenelactone fraction failed to exhibit any effect [5]. In a study with neuronal PC12 cells, the *Ginkgo biloba* terpenelactone bilobalide diminished the cytotoxic effects of Amyloid β (Aβ) fragments that play a decisive role in AD pathology [6, 7]. Other studies also found evidence that the terpenelactone fraction of EGb761 might be more relevant for the neuroprotective activities of the extract [7–10]. A gene array study suggested that the

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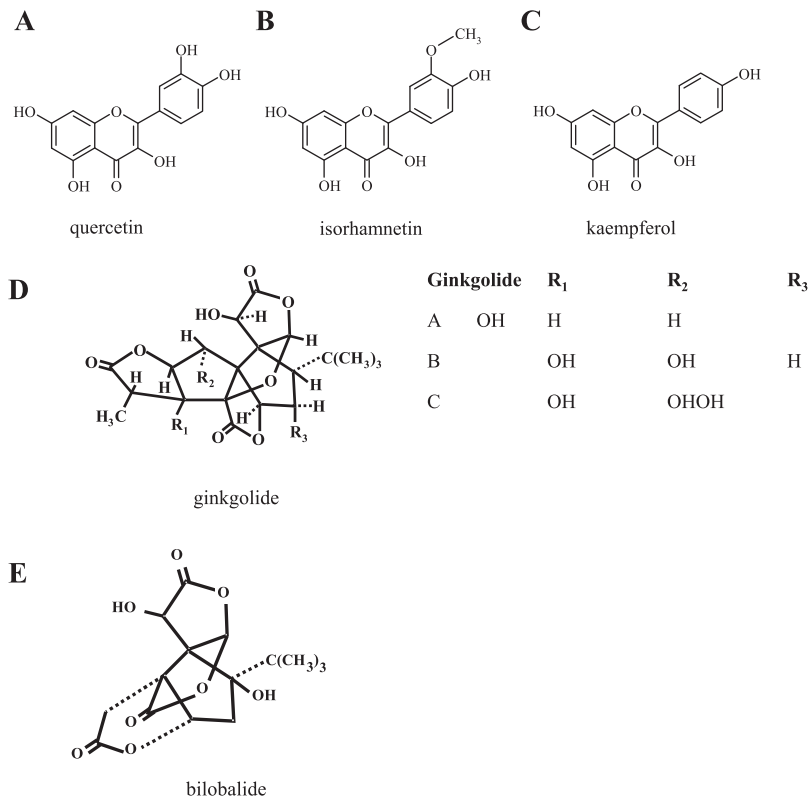E-mail: wolffram@aninut.uni-kiel.de

Fig. 1. Structure of the main flavonolsquercetin (A), isorhamnetin (B) and kaempferol (C) and the main terpenelactones, the ginkgolides (D) and bilobalide (E), present in EGb761 and its main fractions used (according to [*1*]).

gene-regulatory activity of *Ginkgo biloba* on gene expression in the brain could be involved in its neuroprotective activity [*11*]. A study by Watanabe *et al.* showed that in cortex and hippocampus of mice fed a diet supplemented with EGb761 several genes were up regulated, among them genes encoding for transthyretin (Ttr), prolactin and growth hormone [*11*]. These genes could be molecular targets of EGb761 in improving neurological and cognitive functions [*7, 11–13*]. However, it is still unclear which compounds of EGb761 are responsible for this effect. Thus, in the present study we investigated the effect of diets supplemented with EGb761 or with its flavonol and terpenelactone fractions on the mRNA levels of genes involved in the pathogenesis of AD in mice. Due to their prominent role in AD pathology, especially genes involved in amyloid-precursor protein (App) metabolism were analysed. These include App itself, a protease that cleaves APP (Adam10), the Aβ-degrading protease neprilysin (Nep), and App binding protein 1 (Appbp1), a protein that induces apoptosis and is increased in human AD brains [*14–17*]. Acetylcholine esterase (Ache) mRNA levels were also investigated because increasing the synaptic acetylcholine concentration by inhibiting Ache enzyme activity is one of the current strategies in AD therapy [*18*].

Materials and Methods

Source of extracts

Three different extracts of *Ginkgo biloba* leaves were used in the present study: the standardised *Ginkgo biloba* extract EGb761 and two extracts containing either the flavonol or the terpenelactone fraction of EGb761, respectively (Table 1). All extracts were a kind gift from Dr. Schwabe pharmaceuticals (Karlsruhe, Germany).

Animals

Eighty female C57B6 mice aged 6 months (Charles River Breeding Laboratories, Sulzfeld, Germany) were randomly allocated to four dietary groups ($n = 20$ each, body weight 24.5 ± 1.2 g; means ± SEM). Five mice per cage were housed and maintained under standard conditions (23 ± 1°C, 55% relative humidity, 14 h light/10 h dark cycle). The mice were fed a commercial semi-synthetic low-flavonoid diet (C1000 code #100E, Altromin; Lage, Germany) enriched either with no extract (control diet, C), with 300 mg EGb761/kg diet (G), 300 mg flavonol extract/kg diet (F) or 100 mg terpenelactone extract/kg diet (T). Prior to the dietary intervention, mice were adapted to the control diet for one week. The animals had *ad libitum* access to the diets and tap water. Body weights were recorded weekly.

Table 1. Amount of flavonols, ginkgolides and bilobalide in the used extract EGb761 (G) and in two extract fractions containing either its flavonols (F) or terpenelactones (T)

	G	F	T
	Amount (% w/w)*		
Σ **Flavonols**	**24.4**	**25**	#
Quercetin	10.9	#	0.19
Kaempferol	11.2	#	0.18
Isorhamnetin	2.3	#	n.d.†
Σ **Terpenoids**	**6**	**<1**	**38.27**
Bilobalide	3.02	#	21.44
Ginkgolid A	1.28	#	6.35
Ginkgolid B	0.55	#	3.76
Ginkgolid C	1.15	#	6.72

*Data provided by Schwabe pharmaceuticals (Karlsruhe, Germany). Data is shown as % w/w to the whole extract. #not determined. †n.d.: not detectable.

After 4 weeks on the experimental diets, mice were euthanatized and blood was collected in heparinised tubes (Sarstedt, Nuembrecht, Germany). Blood samples were taken from all animals per dietary group (n = 20) and pooled group-wise. Brain cortices and hippocampi for PCR analyses were rapidly dissected, immediately suspended in RNAlater™ RNA stabilisation reagent (Qiagen, Hilden, Gemany) and incubated over night. Hippocampus tissue samples of all animals per cage were pooled. RNA sample preparation was performed as previously described [19]. RNA integrity was checked by denaturating agarose gel electrophoresis and ethidium bromide staining. Total RNA (0.5 μg per lane) was separated on a 2% agarose gel (agarose neeo, Carl Roth, Karlsruhe, Germany). Total RNA degradation was evaluated from the ratio between 18S to 28S rRNA peak areas quantified with the ChemiDoc XRS using QuantityOne™ software (version 4.6.5; BioRad Laboratories, Munich, Germany). A ratio of 0.5 ± 0.075 was accepted.

Animal care and experimental procedures were conducted according to the German Guidelines and Regulation on Animal Care (Deutsches Tierschutzgesetz, 2006) and were approved by the University of Kiel Committee on Animal Care.

Determination of flavonoid and terpenelactone content in plasma

Plasma was obtained after centrifugation (2000 × g for 10 min, 4°C) and stored at −80°C until analysis. Flavonols were analysed in plasma aliquots by HPLC with postcolumn derivatisation as described previously [20]. Plasma samples were treated with β-glucuronidase/sulfatase (Carl Roth) prior to extraction of flavonols.

Terpenelactones were analysed by Dr. Schwabe pharmaceuticals. Plasma aliquots were acidified with HCl (0.1N) and analysed using a GC/MS method according to Fourtallin and Biber & Koch with minor modifications [21, 22].

Determination of mRNA levels

Primer pairs for target genes were designed to the corresponding sequence of *Mus musculus* mRNA sequences using primer3 software (http://fokker.wi.mit.edu/primer3/input.htm). Primer sequences are provided in Table 2. Primer pairs were obtained from MWG (Ebersberg, Germany).

QuantiTect™ Primer Assay (Qiagen) was used for 18S rRNA amplification, with a product of 149 bp. One-step quantitative reverse transcriptase polymerase chain reaction (One-Step qRT-PCR) was performed as previously described by Augustin [19]. All runs were performed at an annealing temperature of 55°C (Adam10: 58°C). Quantification and melting curves of the amplified PCR products were analysed using the RotorGene3000™ software (Corbett Lifescience, Sydney, Australia). Data was normalized by dividing the expression level of each target gene by the level of 18S rRNA.

Immunoblotting

Hippocampi for protein analysis were pooled per cage, immediately frozen in liquid nitrogen and stored at −80°C until analysis. For isolation of membrane-bound proteins Radio Immuno Precipitation Assay (RIPA)-lysis-buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium

Table 2. Nucleotide sequences of PCR primers used in real-time qRT-PCR

target gene and mRNA sequence	sense	antisense	product
Ache (acetylcholine esterase); NM_009599	ccaccgatcctctggacgag	cgctcctgcttgctatagtg	112 bp
Adam10 (a disintegrin and metallopeptidase 10); NM_007399	ccatgctcatggaagacagtt	ccttcttcaccataaatatgtcca	144 bp
App (amyloid beta precursor protein); NM_007471.2	ccgttgcctagttggtgagt	gctcttctcgctgcatgtc	142 bp
Appbp1 (amyloid beta precursor binding protein 1); NM_144931.2	gctgccaggtattggatcat	gctcggttcttgccaatact	108 bp
Nep (neprilysin), NM_008604	cattttgaccagcctcgact	ggcaaactttgttcctgacg	137 bp
Ttr (transthyretin); NM_013697	ggaagacacttggcattcc	tctctcaattctgggggttg	153 bp

deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing protease-inhibitors (Complete Mini, Roche, Mannheim, Germany) was used. Tissue samples $(25 \pm 5$ mg) were homogenized in RIPA-buffer (200 µl per 5 mg) with a rotor-stator homogenizer for 45 s, agitated for 2 h at 4°C, and centrifuged (20 min, $15300 \times g$, 4°C). The supernatant was removed and the protein amount was measured with the BCA kit (Pierce Biotechnology, Rockford, IL). Protein homogenates were diluted 1:1 with Laemmli sample buffer (BioRad Laboratories) containing 5% β-mercaptoethanol and denaturated for 5 min at 95°C. Electrophoresis was performed on 10% Tris-HCl gel (15 µg protein per lane; Criterion Gel System, BioRad) and proteins were transferred onto Immobilion P-PVDF membranes (Millipore, Bedford, MA). Membranes were blocked in 3% non-fat milk (Biomol, Hamburg, Germany) in Tris-buffered saline containing 0.5% Tween 20 (TBST) at room temperature for 6 h. Subsequently, blots were incubated with the primary antibodies for 12 h at room temperature followed by incubation with horse-radish peroxidase-conjugated secondary antibodies (1:10000; Abcam, Cambridge, UK) for 1 h at room temperature. The following primary antibodies were used: monoclonal anti-APP (clone 22C11; 1:2000; Chemicon, Temecula, CA), and rabbit polyclonal anti-actin as internal control (1:2000; Abcam). All antibodies were diluted in TBST. Immuno-detection analyses were performed with the ImmunStar™ WesternC Kit (BioRad). All quantitative analyses were performed with the ChemiDoc XRS using QuantityOne™ software (version 4.6.5; BioRad).

Statistical analysis

Statistical calculations were conducted with Graphpad Prism 4 software (Graphpad Software Inc., San Diego, CA). Data of target gene mRNA levels were analysed by one-way ANOVA followed by Dunnetts multiple comparison test for pair wise comparison of group means. Data of target protein levels were analysed by non parametric Mann-Whitney-U test. Data are presented as mean ± SEM, and significance was accepted at $p<0.05$. Plasma flavonoid and terpenoid concentrations were not statistically analysed since pooled plasma samples were used.

Results

Plasma concentration of flavonols and terpenelactones

In plasma of control mice, concentrations of the main *Ginkgo* flavonols quercetin, kaempferol, and isorhamnetin were 0.069 µmol/l, 0.037 µmol/l and 0.005 µmol/l, respectively. The respective concentrations for quercetin, kaempferol and isorhamnetin in mice treated with either EGb761 or its flavonol fraction were 0.108 µmol/l, 0.121 µmol/l, and 0.108 µmol/l (EGb761) or 0.075 µmol/l, 0.080 µmol/l, and 0.098 µmol/l (flavonol fraction), respec-

tively. Thus, in both treatment groups the total concentration of flavonols was increased more than twofold compared to controls. In contrast, mice treated with the terpenelactone fraction had similar plasma flavonol levels as control animals (0.073 µmol/l, 0.056 µmol/l, and 0.009 µmol/l for quercetin, kaempferol, and isorhamnetin, respectively).

Plasma concentrations of the terpenelactones ginkgolide A, ginkgolide B, and bilobalide were below the limit of detection (<0.02 µmol/L plasma) in control mice and in mice treated with the flavonol fraction. In contrast, animals that had received either EGb761 or its terpenelactone fraction had terpenoid concentrations in plasma that were clearly above the detection limit (in mice treated with EGb761: 0.07 µmol/l bilobalide, 0.396 µmol/l ginkgolide A and 0.164 µmol/l ginkgolide B; in mice treated with terpene-lactones: 0.235 µmol/l bilobalide, 0.429 µmol/l ginkgolide A and 0.291 µmol/l ginkgolide B).

mRNA levels of Alzheimer's Disease relevant genes

The mRNA levels of the housekeeping gene 18S rRNA were not significantly different between the different treatments (data not shown). Neither EGb761 nor its flavonol fraction significantly affected the mRNA levels of the investigated target genes in mouse cortex (Table 3). In cortex of mice treated with the terpenelactone fraction, the expression of Ttr was increased, but this was not significant, probably due to the high inter-individual variation. Furthermore, other genes investigated including Ache, Adam10, Appbp1 and Nep were not significantly affected by the terpenelactone fraction. The increase in Nep mRNA level by ~50% in the EGb761 group was also not significant (Table 3).

In hippocampus, neither EGb761 nor its flavonol fraction affected the mRNA level of App (Table 4). In contrast, App expression in hippocampi of mice treated with the terpenelactone fraction was significantly lower than in control animals. However, EGb761 and both of its fractions failed to alter mRNA expression of Ache, Adam10, Appbp1, Nep or Ttr significantly (Table 4). In accordance with cortex, EGb761 seemed to increase Nep mRNA levels in hippocampus; however, this was also not significant. Interestingly, mRNA levels of Ttr relative to 18S rRNA were 50–200 fold higher in hippocampus compared to cortex (data not shown).

Protein levels of amyloid precursor orotein

Since the terpenelactone fraction significantly down-regulated App mRNA levels in the hippocampus, protein levels of App was also analysed by Western blotting. In accordance with the mRNA data, terpenelactone fraction also decreased the protein expression of App by ~20% in hippocampus compared to control animals, but this was not significant (data not shown).

Table 3. Relative mRNA levels of AD relevant genes in the cortex of mice fed either a control diet or a diet enriched with EGb761 or with its flavonol or terpenelactone fraction over a period of 4 weeks.

target gene	C	G	F	T
Ache	1.00 ± 0.13	0.73 ± 0.06	0.83 ± 0.10	0.79 ± 0.06
Adam10	1.00 ± 0.05	0.94 ± 0.03	1.11 ± 0.05	1.01 ± 0.03
App	1.00 ± 0.03	0.98 ± 0.05	1.00 ± 0.06	1.03 ± 0.03
Appbp1	1.00 ± 0.04	1.02 ± 0.07	1.02 ± 0.06	1.00 ± 0.03
Nep	1.00 ± 0.14	1.53 ± 0.25	1.22 ± 0.25	1.19 ± 0.18
Ttr	1.00 ± 0.40	0.54 ± 0.23	0.85 ± 0.38	2.4 ± 0.76

Data are expressed as mean ± SEM and are relative to control. C, control; G, EGb761; F, flavonol fraction; T, terpenelactone fraction; n = 10 in each group.

Table 4. Relative mRNA levels of AD relevant genes in the hippocampus of mice fed either a control diet or a diet enriched with EGb761 or with its flavonol or terpenelactone fraction over a period of 4 weeks.

target gene	C	G	F	T
Ache	1.00 ± 0.09	1.06 ± 0.14	1.36 ± 0.10	1.18 ± 0.12
Adam10	1.00 ± 0.05	1.00 ± 0.05	0.97 ± 0.05	0.90 ± 0.10
App	1.00 ± 0.06	0.88 ± 0.06	0.82 ± 0.06	0.73 ± 0.07*
Appbp1	1.00 ± 0.11	1.18 ± 0.10	1.18 ± 0.12	0.94 ± 0.05
Nep	1.00 ± 0.18	1.72 ± 0.39	1.05 ± 0.16	1.21 ± 0.28
Ttr	1.00 ± 0.32	1.64 ± 0.72	1.22 ± 0.40	1.16 ± 0.50

Data are expressed as mean ± SEM and are relative to control. C, control; G, EGb761; F, flavonol fraction; T, terpenelactone fraction; n = 4; *p<0.05.

Discussion

The present study examined the effects of the *Ginkgo biloba* extract EGb761 and its flavonol and terpenelactone fractions on brain mRNA levels of genes involved in the pathogenesis of AD. Adult C57B6 mice were treated over a period of 4 weeks with diets enriched with either the whole extract, or with its flavonol or terpenelactone fraction. The increase in plasma flavonol and/or terpenelactone concentrations in the respective dietary groups demonstrated the absorption of these constituents from the diets. This finding is in accordance with previous data from humans [21] and laboratory animals [22, 23].

It is still largely unknown if and to what extent EGb761 constituents may cross the blood-brain barrier. De Boer *et al.* could detect quercetin, a major constituent of EGb761, in brain tissue of rats [24]. This indicates absorption and distribution of at least some of the major constituents of EGb761 to the brain. EGb761 has been shown to inhibit generation of Aβ from APP and Aβ aggregation, which are crucial processes in AD pathogenesis [25–27]. Furthermore, App protein levels in cortex and hippocampus were decreased in rats after oral treatment with *Ginkgo biloba* extract for 28 weeks [28]. In the present study, neither dietary EGb761 nor its flavonol fraction affected App mRNA levels in cortex and hippocampus after 4 weeks of treatment. The different

outcome between the two studies may be explained by the different duration of the dietary treatment (4 vs 28 weeks) and/or by species differences (rat vs mice). However, dietary treatment with the terpenelactone fraction led also in our study to a significant decrease in App mRNA, at least in hippocampus. Several studies reported that in particular the terpenelactones could be the most relevant constituents with regard to the neuroprotective properties of EGb761 [6–10]. In this context, Ahlemeyer and Kriegelstein analysed data from *in vitro* and *in vivo* studies regarding potential beneficial effects of EGb761; they concluded that the terpenelactones including ginkgolide B and bilobalide may be the most effective constituents of EGb761 against oxidative damage in the brain [9]. It has to be taken into account that in our study the active ingredients in the terpenelactone enriched diet were twofold higher compared to the EGb761 enriched diet (Table 1). Thus, EGb761 could be equally effective as the terpenelactone fraction when higher doses of EGb761 are administered.

mRNA levels of other genes apart from App, including Ache, Adam10, Appbp1 and Nep, were not significantly altered by EGb761 or by its main fractions after 4 weeks of dietary treatment in our study. However, we found a slight but non significant increase in hippocampus Ttr mRNA levels in the EGb761 group. Furthermore, the diet supplemented with terpenelactones increased also mRNA levels of

Ttr in cortex, although this was not significant again. Several studies demonstrate a potential beneficial effect of transthyretin against Aβ toxicity and plaque formation in murine models for AD [*29–32*]. It has also been shown that brains of AD patients have decreased levels of transthyretin [*32*]. In a previous study, Watanabe *et al.* observed a significant increase in Ttr mRNA levels in hippocampus after a 4 week dietary treatment with EGb761 [*11*]. Our results cannot fully support this latter observation. Interestingly, Sousa *et al.* have shown that Ttr mRNA might exclusively occur in the choroids plexus [*33*]. These authors generated tissue samples via micro dissection and detected Ttr mRNA neither in the cortex nor in the hippocampus of wild-type mice and transgenic mice models of AD. They stated that the reason for the detection of Ttr mRNA in other brain tissues such as cortex and hippocampus could only be due to tissue contamination [*33*]. Thus, the high variation in Ttr mRNA levels in cortex and hippocampus in our study could be due to a possible contamination of theses samples with choroid plexus. This could also explain the difference between our data and that of Watanabe *et al.* [*11*]. Thus, further studies are warranted to reveal the impact of EGb761 on Ttr expression especially in the choroid plexus.

Regarding the time dependency of the development of any effects of EGb761 within the brain, a definitive statement is not possible. On the one hand, Watanabe *et al.* [*11*] have shown that EGb761 significantly influences gene expression in brains of adult mice after 4 weeks of dietary treatment, whereby most of the affected genes were associated with neuroprotective properties (e.g. growth hormone or τ-protein). On the other hand, we have also recently shown in a transgenic mouse model for AD (Tg2576 mice) that only long-term treatment (16 months, old mice) decreased App mRNA and protein levels; in the same trial, a 4 week treatment (young mice) did not affect mRNA levels of genes investigated [*19*, *34*]. This study, however, indicates that not only the duration of the treatment with *Ginkgo biloba*, but also the (biological) age of the animals might influence results. In this context, a study by Williams *et al.* has shown an age-related effect of a short-term treatment with EGb761 (30 days) on synaptic plasticity and excitability. Only in old mice (aged 18–24 months) the beneficial effects of EGb761 on investigated parameters were significant. The authors mentioned that the absence of effects of EGb761 in young mice (aged 2–3 months) may indicate that EGb761's influence is only expressed in a system where normal homeostatic processes are undergoing age-related decline and where an increase in the range of synaptic plasticity could prove beneficial [*35*]. Thus, further studies are required to elucidate the possible age-related and/or time-dependent effects of EGb761 on gene expression.

Taken together, our results indicate that a 4 week dietary treatment with EGb761 or its flavonol and terpenelactone fractions induces only moderate effects on the expression of genes relevant for Alzheimer disease in healthy mice. However, terpenelactones seem to be able to decrease the expression of amyloid precursor protein. This could lead to a reduced generation of the neurotoxic Aβ. To test this hypothesis, long-term studies in aged mice are warranted to investigate the effect of terpenelactones on Aβ generation and clearance in a mouse model for AD.

Acknowledgments

Abbreviations

Aβ, amyloid beta; Ache, acetylcholine esterase; AD, Alzheimer's Disease; Adam10, a disintegrin and metalloprotease-10; App, Amyloid Precursor protein; Appbp1, amyloid precursor protein binding protein 1; C, control diet; F, flavonol fraction supplemented diet; EGb761, standardised *Ginkgo biloba* extract; G, EGb761 supplemented diet; T, terpenelactone fraction supplemented diet; Nep, neprilysin; Ttr, transthyretin.

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