Einfluss von Flavonoiden und Stilbenen auf oxidativen Stress und Lebensspanne im Modellorganismus *Caenorhabditis elegans*

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Abkürzungsverzeichnis

AGE	Advanced Glycation Endproduct
AGE-1	Ageing Alteration-1 (PI3K-Homolog)
AKT-1/-2	Serin/Threonin Kinasen der AKT-Familie
AMP	Adenosinmonophosphat
AMPK	Adenosinmonophosphat-abhängige Kinase
ATP	Adenosintriphosphat
Ca^{2+}	Kalziumion
C. elegans	*Caenorhabditis elegans*
COMT	Catechol-O-Methyltransferase
CTL	Katalase
DAF-16	Dauer Formation Abnormal-16 (FoxO Homolog; Transkriptionsfaktor)
DAF-18	Phosphatase und Tensin Homolog
DAF-2	Dauer Formation Abnormal-2 (Insulin/ IGF-1 Rezeptor Homolog)
D. melanogaster	*Drosophila melanogaster*
DNS	Desoxyribonukleinsäure
E. coli	*Escherichia coli*
EGCG	Epigallocatechingallat
FG	Frischgewicht
FoxO	Forkhead Box O Transkriptionsfaktor
GCS	Glutamylcysteinylsynthetase
GFP	Grün fluoreszierendes Protein
GPx	Glutathionperoxidase
GST	Glutathion-S-Transferase
GSS	Glutathionsynthase
HSF-1	Hitzeschockfaktor-1 (Transkriptionsfaktor)
HSPs	Hitzeschockproteine
IC50	Halbmaximale Inhibitorische Konzentration
IGF-1	Insulin ähnlicher Wachstumsfaktor-1
IIS	Insulin / IGF-1 ähnlicher Signalweg
Keap1	Kelch-like erythroid cell-derived protein with CNC homology associated protein1
lof	loss of function
LPH	Laktase-Phlorizin-Hydrolase
MAPK	Mitogen-aktivierte Proteinkinase
µM	mikromolar
mtDNS	mitochondriale DNS

mTOR	mechanistic Target of Rapamycin
NAD^+	Nicotinamidadenindinukleotid (oxidierte Form)
NADPH	Nicotinamidadenindinucleotidphosphat (reduzierte Form)
NF_KB	Nuclear factor kappa B
N. furzeri	*Nothobranchius furzeri*
NO	Stickstoffmonoxid
Nrf2	Nuclear factor erythroid 2-related factor 2 (Transkriptionsfaktor)
PDK-1	Phosphoinositid abhängige Proteinkinase-1
PI3K	Phosphatidylinositol-3-Kinase
RNA	Ribonucleic acid
RNAi	RNA vermittelte Interferenz
RNS	Ribonukleinsäure
ROS	Reaktive Sauerstoffspezies
SAMP8	Senescence Accelerated Mouse Prone-8
S. cerevisiae	*Saccharomyces cerevisiae*
SGK-1	Serum-Glukokortikoid-regulierte Kinase-1
SGLT1	Natriumabhängiger Glukosetransporter
SIR-2.1	Sirtuin-2.1 (NAD^+-abhängige Deacetylase)
SKN-1	Skinhead-1 (Homolog des humanen Nrf2) Transkriptionsfaktor
SOD	Superoxiddismutase
SULT	Sulfotransferase
TEAC	Trolox Equivalent Antioxidative Capacity
TNF-α	Tumornekrosefaktor- α
TSG	2,3,5,4′-Tetrahydroxystilben-2-O-β-D-glucosid
UGT	UDP-Glucuronosyltransferasen
UV	Ultraviolett
vs.	versus

1. Einleitung

1.1 Sekundäre Pflanzenstoffe

Alle Pflanzen erzeugen durch ihren Metabolismus primäre und sekundäre Stoffwechselprodukte. Während primäre Metabolite essenziell für das Überleben der Pflanzen notwendig sind, zählen sekundäre Pflanzenstoffe zu den nicht essenziellen Sekundärmetaboliten. Die Mehrzahl der sekundären Pflanzenstoffe entsteht durch fünf Wege, die die benötigten Vorläufer für die Synthese bereitstellen: Acetyl-Koenzym A (Polyketide wie Anthrachinone und Flavonoide), aktives Isopren (Terpenoide), Shikimat (aromatische Aminosäuren, Zimtsäuren, Tannine, Indole und Isoquinolinalkaloide), Glykolyse (Zucker, Gallat) und der Zitratzyklus (Alkaloide) (Wink, 2016). Sekundäre Pflanzenstoffe besitzen wichtige physiologische Eigenschaften, die das Überleben der Pflanzen begünstigen. Sie bieten Schutz gegen Herbivore durch einen abstoßenden Geschmack oder toxische Wirkungen oder dienen der Abwehr von schädlichen Mikroorganismen durch antivirale, antibakterielle oder antimykotische Eigenschaften. Darüber hinaus erzeugen sie einen Schutz gegen UV-Strahlung oder dienen als Farbstoffe zum Anlocken von Tieren zur Bestäubung oder zur Verteilung von Samen (siehe Tab. 1.1). Die Klasse der sekundären Pflanzenstoffe ist vielfältig und umfasst bisher mehr als 200000 identifizierte Substanzen (Wink, 2016). Die von der Anzahl umfangreichsten Gruppen der sekundären Pflanzenstoffe sind die Alkaloide (ca. 27000 Verbindungen), die Terpene (ca. 16000 Verbindungen) und die Phenole und Polyphenole (ca. 6800 Verbindungen) (Wink, 2015).

Tabelle 1.1: Geschätzte Anzahl identifizierter phenolischer und polyphenolischer Verbindungen und ihre Hauptwirkung in Pflanzen. (Modifiziert nach Wink, 2015). Aktivität: – keine oder sehr wenige Sekundärmetabolite (SM) aktiv; ++ viele SM aktiv; +++ die meisten SM aktiv; ++++ fast alle SM aktiv.

Klasse	Geschätzte Anzahl an Strukturen	Toxisch oder abstoßend für Herbivoren	Anti-mikrobielle Aktivität	Lockstoff für Bestäuber oder Fruchtverteiler
Phenylpropanoide, Coumarine, Lignane	**2000**	**+++**	**+++**	**++**
Flavonoide, Anthocyane, Tannine	**4000**	**+++**	**+++**	**++**
Polyketide (Anthrachinone)	**800**	**++++**	**+++**	**-**

Polyphenole werden definiert als Klasse von pflanzlichen Sekundärmetaboliten, die in ihrer Grundstruktur aus mehr als einem phenolischen Ring bestehen, keine Stickstoff basierte funktionelle Gruppe besitzen und ausschließlich über den von Shikimat ausgehenden Phenylpropanoid- und/ oder Polyketidweg hergeleitet werden (Quideau, 2013). Die Unterteilung der Polyphenole erfolgt in Subklassen und basiert auf ihrer jeweiligen Struktur. Zu diesen in drei Subklassen eingeteilten Polyphenolen zählen Lignane, Flavonoide und Stilbene, wobei auf die beiden letzteren nachfolgend näher eingegangen wird.

1.1.1 Flavonoide

Die Klasse der Flavonoide ist gekennzeichnet durch die gemeinsame Grundstruktur, bestehend aus zwei aromatischen (A und B) sowie einem O-heterozyklischen Ring (C) (Abb. 1.1). Mit einem Anteil von etwa 60% stellen Flavonoide den größten Teil der Polyphenole dar (Woodward et al., 2018). Eine große strukturelle Vielfalt der Flavonoide ergibt sich aus Modifikationen des Grundgerüsts durch Substitution mit unterschiedlichen funktionellen Gruppen, wie z.B. Hydroxyl-, Methyl- oder Prenylgruppen, durch die Bindung mit Zuckern (Flavonoidglykoside) oder durch Konjugation der aromatischen Ringe. Aufgrund des Oxidationstatus und der Anwesenheit von funktionellen Gruppen am heterozyklischen C-Ring und der Verbindung zum aromatischen B-Ring erfolgt die Einteilung der Flavonoide in sechs Klassen: Flavonole, Flavanole, Flavanone, Flavone, Anthocyane und Isoflavonoide (Watzl & Rechkemmer, 2001; Beecher, 2003) (Tab. 1.2).

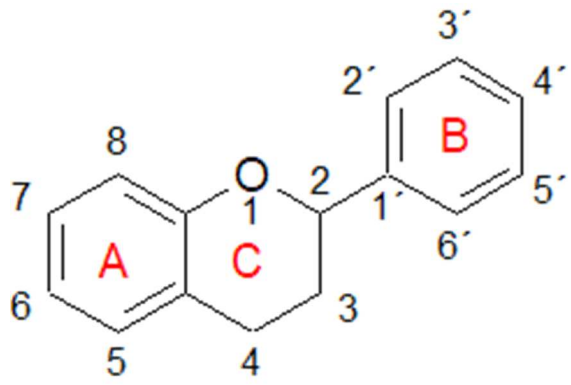

Abbildung 1.1: Grundstruktur der Flavonoide. Das Grundgerüst der Flavonoide besteht aus zwei aromatischen Ringen (A und B) und einem O-heterozyklischen Ring (C), die zusammen das Flavan-Grundgerüst darstellen.

Tabelle 1.2: Beispiele verschiedener Flavonoide und Stilbene und ihr Vorkommen in Nahrungsmitteln und Medizinalpflanzen. (Modifiziert nach Watzl & Rechkemmer, 2001; Büchter et al., 2013; Żołnierczyk et al., 2015).

Substanzklasse	Beispiele	Vorkommen
Flavonole	Myricetin	Trauben, Rotwein
	Quercetin	Zwiebeln
Flavanole	Catechin	Rotwein, Äpfel
	Epicatechingallat	Grüner Tee
Flavanone	Naringenin	Grapefruit
	Isoxanthohumol	Hopfen, *Sophora flavescens*
Flavone	Luteolin	Sellerie
	Apigenin	Paprika
Anthocyane	Malvidin	Blaue Trauben
	Cyanidin	Kirschen
Isoflavonoide	Genistein	Sojabohnen
	Daidzein	
Stilbene	Resveratrol	Trauben, Wein
	2,3,5,4'-Tetrahydroxystilben-2-O- β -D-glucosid	*Polygonum multiflorum*

Flavonoide treten in Pflanzen hauptsächlich als Glykoside (mit Ausnahme der Flavanole) auf, wobei mehr als 80 verschiedene Zucker in Flavonoidglykosiden nachgewiesen wurden (Watzl & Rechkemmer, 2001). Die Glykolysierung erhöht die Wasserlöslichkeit, so dass die Lagerung in den Zellvakuolen erleichtert wird. In Pflanzen erfüllen Flavonoide wichtige physiologische Prozesse, z.B. dienen sie als Lockstoff durch Färbung von Blüten und Früchten, sind beteiligt an Wachstum und Entwicklung, wirken als Metaboliten bei der Stressantwort (Wunden, Nahrungsmangel, Temperaturstress), besitzen antioxidative Eigenschaften und schützen gegen UV-Strahlung. Darüber hinaus erhöhen sie die Resistenz gegen Krankheiten, da sie antivirale, antibakterielle und antimykotische Eigenschaften besitzen (Miranda et al., 2012).

1.1.2 Flavonole

Die am weitesten verbreiteten Flavonoide in Pflanzen stellen die Flavonole dar, deren höchste Konzentration in Blättern von z.B. Blattgemüse und der Haut von Früchten vorzufinden ist und nahezu ubiquitär in pflanzlichen Nahrungsmitteln auftreten, wobei typische Vertreter dieser Gruppe Isorhamnetin, Kaempferol, Quercetin und Myricetin sind (Beecher, 2003; Miranda et al., 2012). Die Klasse der Flavonole ist gekennzeichnet durch die Struktur des heterozyklischen C-Rings, der eine 2-3 Doppelbindung, eine 3-Hydroxyl- und eine 4-Oxogruppe besitzt. Eine große Vielfalt an Verbindungen entsteht durch die Anzahl und Art der funktionellen Gruppen in den aromatischen Ringen A und B. Fünf der in dieser Arbeit verwendeten Substanzen gehören zur Klasse der Flavonole: Quercetin (2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxychromen-4-on), Myricetin (3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-on) und seine Derivate Laricitrin (2-(3,4-Dihydroxy-5-methoxyphenyl)-3,5,7-trihydroxychromen-4-on), Syringetin (3,5,7-Trihydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)chromen-4-on) und Myricetintrimethylether (3,5,7-Trihydroxy-2-(3,4,5-trimethoxyphenyl)chromen-4-on) (Abb. 1.2).

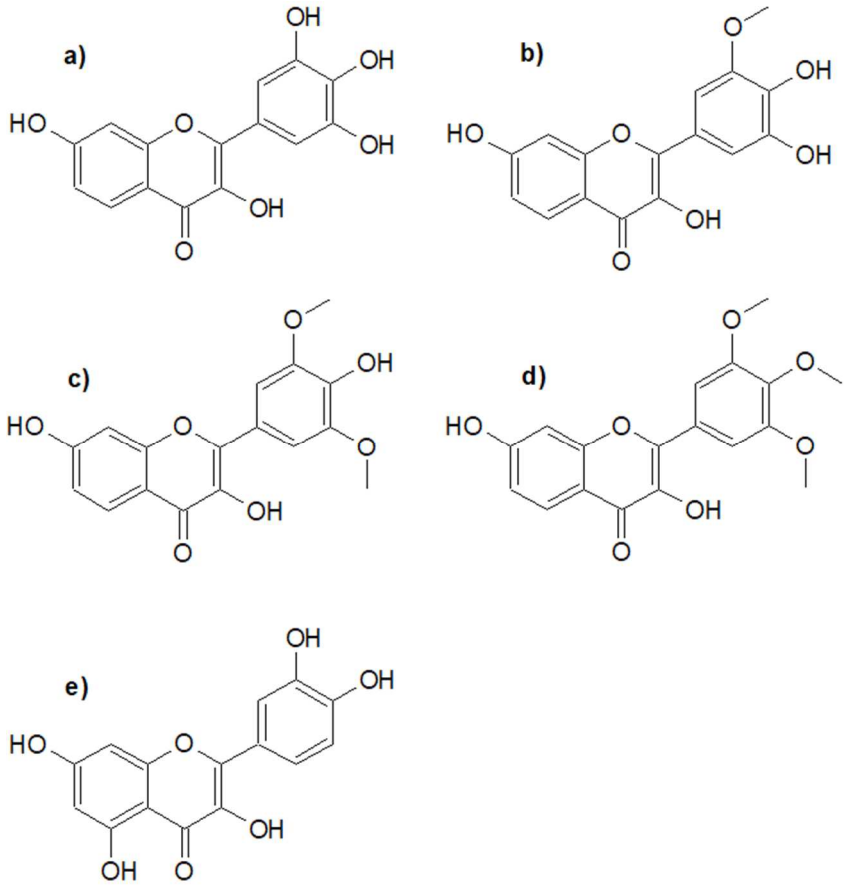

Abbildung 1.2: Strukturformeln der in dieser Arbeit untersuchten Flavonole. a) Myricetin; b) Laricitrin; c) Syringetin; d) 3´,4´,5´ Myricetintrimethylether; e) Quercetin

1.1.3 Flavanone

Die Gruppe der Flavanone ist gekennzeichnet durch eine 4-Oxogruppe und eine C2-C3 Doppelbindung des heterozyklischen C-Rings. Typische Vertreter dieser Substanzklasse sind Eriodictyol, Hesperitin und Naringenin, welche hauptsächlich in Zitrusfrüchten anzutreffen sind (Beecher, 2003). Ein weiterer Vertreter dieser Gruppe ist das prenylierte Flavanon Isoxanthohumol (7-Hydroxy-2-(4-hydroxyphenyl)-5-methoxy-8-(3-methylbut-2-enyl)-2,3-dihydrochromen-4-on), das in geringen Konzentrationen (0,008% der Trockenmasse) in den weiblichen Blüten des Hopfens *Humulus lupulus* und in der Wurzel von *Sophora flavescens*, einer in der traditionellen chinesischen Medizin verwendeten Pflanze, vorkommt. In der menschlichen Ernährung wird Isoxanthohumol hauptsächlich durch den Konsum von Bier aufgenommen, in dem durch den Brauprozess das hauptsächlich in Hopfen vorkommende prenylierte Chalkon Xanthohumol durch Isomerisierung in Isoxanthohumol überführt wird (Żołnierczyk et al., 2015). Die Struktur des Isoxanthohumol ist in der Abbildung 1.3 dargestellt.

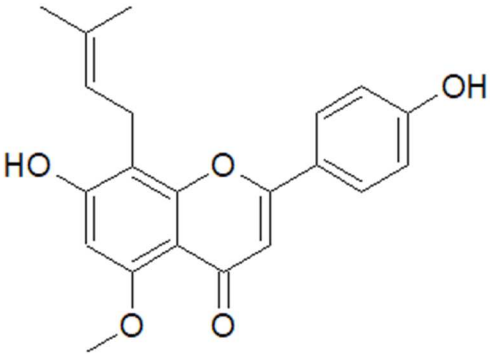

Abbildung 1.3: Strukturformel des prenylierten Flavanons Isoxanthohumol.

1.1.4 Stilbene

Stilbene sind eine Gruppe von polyphenolischen Verbindungen, die durch ein 1, 2 Diphenylethylen Grundgerüst charakterisiert werden. Ihre Biosynthese erfolgt aus p-Coumaroyl-CoA und drei Malonyl-CoA Einheiten durch das Enzym Stilbensynthase (Almagro et al., 2013; Chong, 2009). Stilbene gehören zu den so genannten Phytoalexinen, niedermolekularen Substanzen mit antimikrobiellen Wirkungen, welche in den Pflanzen nach biotischem und abiotischem Stress gebildet werden (Jeandet, 2015). Typische Vertreter dieser Klasse sind das 3,5,4'-Trihydroxystilben, besser bekannt unter dem Trivialnamen Resveratrol (5-[(E)-2-(4-Hydroxyphenyl)ethenyl]benzen-1,3-diol) und Pterostilben (4-[(E)-2-(3,5-Dimethoxyphenyl)ethenyl]phenol), welche durch das Vorkommen in Trauben und Wein (*Vitis vinifera*) weitläufig in der menschlichen Ernährung vorzufinden sind. Weitere Stilbene sind häufig Derivate des Resveratrol, die sich durch weitere funktionelle Gruppen (z.B. Hydroxyl-, Methoxy- oder Prenylgruppen) oder durch Glykosylierung ergeben (Shen et al., 2013). Zu den Resveratrol-Glykosiden zählt z.B. das 2,3,5,4'-Tetrahydroxystilben-2-O-β-D-glucosid (TSG; (2S,3R,4S,5S,6R)-2-[2,4-Dihydroxy-6-[(E)-2-(4-hydroxyphenyl)ethenyl]phenoxy]-6-(hydroxymethyl)oxan-3,4,5-triol). In der traditionellen chinesischen Medizin werden Wurzelextrakte des vielblättrigen Knöterichs (*Polygonum multiflorum*) verwendet, zu dessen Inhaltsstoffen unterschiedliche Stilbene gehören, u.a. Resveratrol und TSG (Zhu et al., 2012; Lin et al., 2014) (Abb. 1.4).

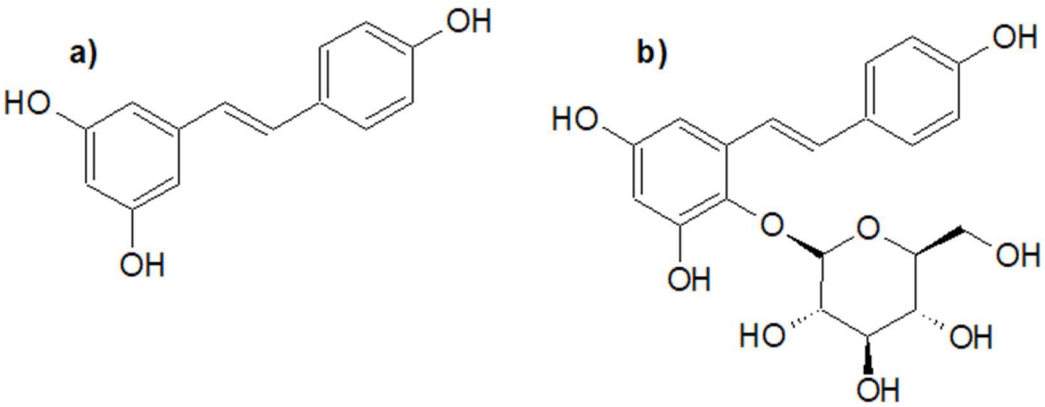

Abbildung 1.4: Strukturformeln der Stilbene Resveratrol und TSG. a) Resveratrol; b) TSG (2,3,5,4'-Tetrahydroxystilbene-2-O-β-D-glucosid)

Durch ihre für den Menschen potentiell gesundheitsförderlichen Wirkungen besteht großes Interesse daran, weitere Stilbenderivate auch chemisch zu synthetisieren und es wurden die neu synthetisierten Derivate Trans-3,5-dimethoxy-4-fluoro-4´-hydroxystilben, Trans-4´-hydroxy-3,4,5-trifluorostilben, Trans-2,5-dimethoxy-4´-hydroxystilben, und Trans-2,4´,5-trihydroxystilben von Prof. Dr. Csuk vom Lehrstuhl für Organische und Bioorganische Chemie der Martin-Luther Universität zur Verfügung gestellt. Die Strukturformeln der Stilbenderivate sind in Fischer et al. (2017) dargestellt.

1.2 Aufnahme und Bioverfügbarkeit von Polyphenolen

Aufgrund ihrer ubiquitären Verbreitung werden Polyphenole in der menschlichen Ernährung hauptsächlich durch den Verzehr von Nahrungsmitteln pflanzlichen Ursprungs aufgenommen, (Obst und Gemüse, Kräuter, Tee, Säfte, Wein, Bier). Darüber hinaus erfolgt eine Aufnahme durch den Einsatz als pflanzliche Arzneimittel, wie dies z.B. seit langem in der traditionellen chinesischen Medizin der Fall ist. Eine weitere bedeutende Quelle ist der steigende Konsum von pflanzlichen Extrakten oder von Reinsubstanzen als Nahrungsergänzungsmittel.

1.2.1 Polyphenole in Nahrungsmitteln und ihre Aufnahme in der menschlichen Diät

In westlichen Populationen (Europa und USA) beträgt die durchschnittliche Aufnahme von Flavonolen, Flavanonen, Flavanolen und Isoflavonen etwa 100- 150 mg pro Tag, wobei die Gesamtaufnahme von Polyphenolen ca. 1 g pro Tag erreichen kann (Manach et al., 2004). Die im Plasma vorzufindenden Konzentrationen von Flavonoiden und der auftretenden Metaboliten nach einmaligem Konsum der Reinsubstanz, einem Pflanzenextrakt oder eines Nahrungsmittels bzw. eines Getränks reichen dabei von 0 bis 4 µmol/L bei einer Aufnahme von 50 mg Aglykon Äquivalenten, wobei die Plasmakonzentration der verschiedenen Flavonoide in der folgenden Reihenfolge ansteigt: Anthocyanine und Galloylcatechine< Flavonole< Flavanone< Flavanole< Isoflavone (Manach et al., 2005). Für das am ausgiebigsten untersuchte Stilben Resveratrol wurden in Humanstudien nach einmaliger oraler Aufnahme von 25 mg Resveratrol Plasmakonzentrationen von <10 ng/mL Resveratrol bzw. 400-500 ng/mL (entspricht ca. 2 µmol/L) Resveratrolmetabolite nachgewiesen (Walle, 2011). Der Gehalt verschiedener Polyphenole in Lebensmitteln umfasst eine große Bandbreite und reicht von wenigen Milligramm bis zu mehreren Gramm pro Liter oder pro Kilogramm des Frischgewichts. Eine Auswahl weit verbreiteter Lebensmittel und deren Gehalt an Flavonoiden und Stilbenen ist in der Tabelle 1.3 dargestellt.

Tabelle 1.3: Gehalt von Flavonoiden und Stilbenen in Nahrungsmitteln. (Modifiziert nach Manach et al., 2004; Lee & Rennaker, 2007; Żołnierczyk et al., 2015; El Khawand et al., 2018)

Substanzklasse	Vorkommen in Nahrungsmitteln	Gehalt in mg/L oder mg/kg Frischgewicht
Anthocyane	Beeren und dunkle Trauben	250 -7500 mg/kg
	Rotwein	200 -350 mg/L
Flavonole	gelbe Zwiebeln	350 - 1200 mg/kg
	Grünkohl	300 - 600 mg/kg
	Tee	20 - 45 mg/L
Flavone	Petersilie	240 - 1850 mg/kg
	Sellerie	20 - 140 mg/kg
Isoflavone	Sojaprodukte	80 - 1800 mg/kg
Flavanole	Schokolade	460 - 610 mg/kg
	Bohnen	350 - 550 mg/kg
	Tee	60 - 800 mg/L
	Rotwein	80 - 300 mg/L
Flavanone	Säfte aus Zitrusfrüchten	50 - 680 mg/L
	Bier	0,04 - 3,44 mg/L
Stilbene	Trauben	0,16 - 245 mg/kg
	Wein	0,05 - 30,9 mg/L

1.2.2 Bioverfügbarkeit von Polyphenolen

Die meisten Daten zu pharmakologischen Wirkungen von Polyphenolen stammen aus Studien, in denen die Aglykone, d.h. die nicht glykosylierten Formen einer Substanz, untersucht wurden. In Pflanzen und somit auch in der menschlichen Ernährung sind jedoch vorwiegend mit verschiedenen Zuckern konjugierte Verbindungen anzutreffen. Nach dem Verzehr findet im Dünndarm eine Hydrolyse von Glukose-, Arabinose- oder Xyloseresten durch β-Glukosidasen, wie der Laktase-Phlorizin-Hydrolase (LPH) statt, die auf der Bürstensaummembran lokalisiert sind und eine passive Diffusion in die Enterocyten gewährleisten. Darüber hinaus werden glykosylierte Polyphenole durch den natriumabhängigen Glukosetransporter SGLT 1 in die Zellen des Dünndarms transportiert und durch zelluläre β-Glukosidasen hydrolysiert. Eine Hydrolysierung von Polyphenolrhamnosiden erfolgt im Dickdarm, durch die bakterielle α-Rhamnosidase der Darmflora, und ermöglicht dort die Diffusion in die intestinalen Zellen. Nach Metabolisierung durch Methyltransferasen (Catechol-O-Methyltransferase, COMT), UDP-Glucuronosyltransferasen (UGT) oder

Sulfotransferasen (SULT) erfolgt über die Portalvene die Weitergabe in den Blutkreislauf. Weiterhin wird eine Aufnahme über den Interzellularraum der Enterocyten diskutiert. Die Elimination der Polyphenole geschieht über die Leber bzw. die Galle und die Nieren (Scalbert & Williamson, 2000; Terahara, 2015). Eine schematische Aufnahme von Flavonoiden ist in der Abbildung 1.5 dargestellt.

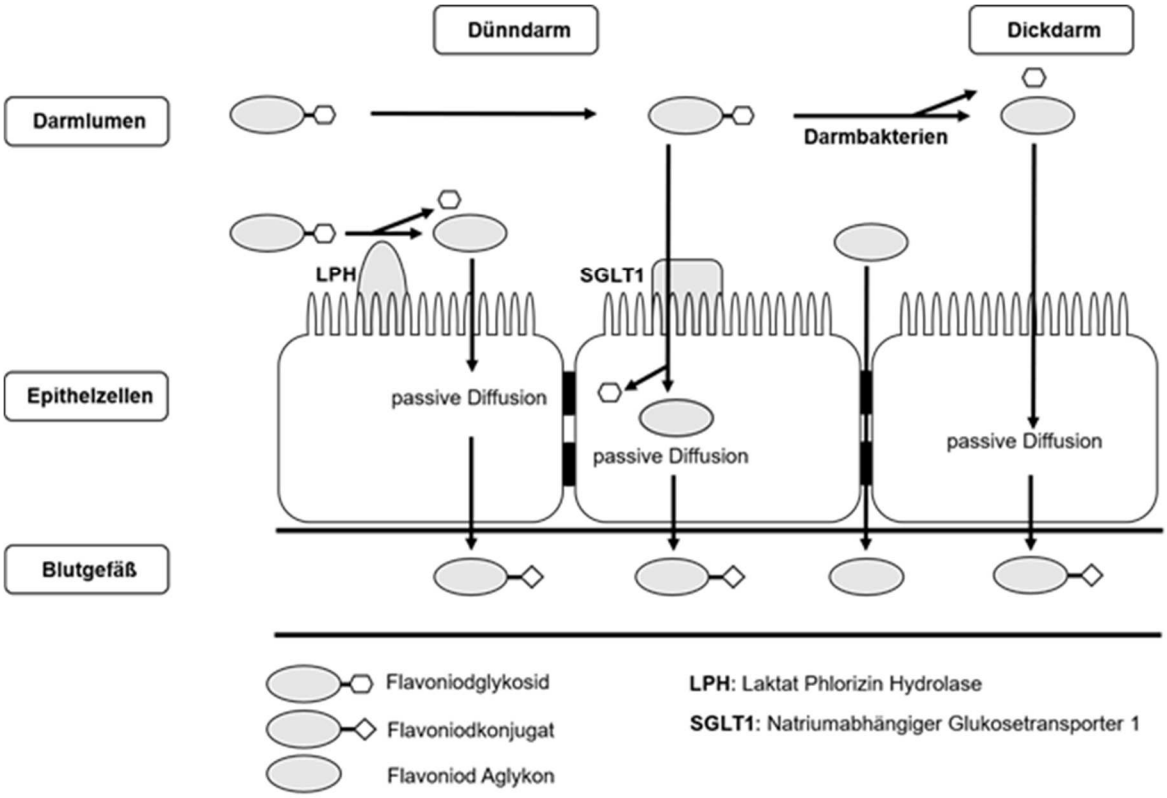

Abbildung 1.5: Schematische Darstellung der intestinalen Aufnahme von Flavonoiden. (Modifiziert nach Terahara, 2015)

1.3 Physiologische Wirkungen von Polyphenolen

Das ursprüngliche Interesse an der Untersuchung von Polyphenolen und insbesondere von Flavonoiden entstand durch das so genannte „french paradox“. Es besagt, dass die französische Bevölkerung weniger an kardiovaskulären Erkrankungen leidet, obwohl sie sich in ihrer Ernährung nicht besonders von anderen Europäern unterscheidet. Diese Beobachtung wurde auf den regelmäßigen und moderaten Konsum von Rotwein zurückgeführt (Renaud & de Lorgeril, 1992). Ebenso wurde postuliert, dass eine mediterrane Diät, die sich durch einen hohen Anteil an Obst und Gemüse und den mäßigen Konsum von Wein auszeichnet, das Risiko von Herzerkrankungen senkt (Zern & Fernandez, 2005). Für diese positiven und gesundheitsförderlichen Wirkungen werden zum Teil die über die Nahrung aufgenommenen Flavonoide verantwortlich gemacht. Es konnten vornehmlich in *in vitro* Untersuchungen für einzelne Flavonoide antiinflammatorische, antiatherosklerotische, antithrombogene, antivirale und antikanzerogene Wirkungen nachgewiesen werden (Übersicht in Nijveldt et al., 2001).

Teilweise sind diese protektiven Wirkungen der Flavonoide auf ihre Eigenschaften als direkte Radikalfänger zurückzuführen. Flavonoide besitzen die Fähigkeit, Elektronen der substituierten Hydroxylgruppen (vor allem durch die Catechol-Gruppe des B-Rings) für die Reaktion mit Radikalen zur Verfügung zu stellen und sie so zu stabilisieren. Zusätzliche antioxidative Funktionalität entsteht durch eine 2,3 Doppelbindung zusammen mit einer 4-oxo-Gruppe im heterozyklischen C-Ring, da sie verantwortlich ist für eine Elektronendelokalisation des B-Rings und zur Stabilisierung der entstehenden Phenoxylradikale beiträgt. Weiterhin können eine 3-OH und 5-OH Gruppe in Kombination mit der 4-oxo-Funktion als Chelatbildner wirken und Übergangsmetallionen komplexieren, wodurch die Bildung der sehr reaktiven Hydroxylradikale verhindert werden kann (Rice-Evans et al., 1996; Williams et al., 2004).

Flavonoide beeinflussen darüber hinaus unterschiedliche Signalwege, wie z.B. die Mitogen-aktivierte Kinase Kaskade (MAPK) oder den Insulin/ IGF-1 ähnlichen Signalweg, sie modulieren zelluläre Transportvorgänge, z.B. als Inhibitor der Glukoseaufnahme, oder sie inhibieren die Aktivität von Transkriptionsfaktoren. Der Einfluss der Flavonoide auf unterschiedliche Signalwege ist häufig darin begründet, dass sie Phosphorylierungsvorgänge von Signalmolekülen modulieren können und auf diese Weise die Aktivität von Kinasen ändern, so dass es letztendlich zu einer veränderten Genexpression kommen kann (Dong et al., 1997; Park, 1999; Briviba et al., 2002; Williams et al., 2004; Cameron et al., 2008).

1.3.1 Bioaktivität der Flavonoide Myricetin und Isoxanthohumol

Für das Flavonol Myricetin konnte in humanen HepG2 Zellen gezeigt werden, dass es redox-sensitive Signalwege innerhalb von Zellen aktiviert. Myricetin stimuliert die Expression von Nrf2 (Nuclear factor erythroid 2-related factor 2), inhibiert die Ubiquitin vermittelte Degradation des Transkriptionsfaktors Nrf2, und moduliert die Aktivität des Nrf2 Inhibitors Keap1 (kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1) (Qin et al., 2013). Myricetin besitzt chemopräventive Eigenschaften, da es das Absterben von Pankreaskrebszellen durch die Inhibition der PI3K (Phosphatidylinositol-3-Kinase) Signaltransduktion induziert und es schützt, unter physiologischen Konzentrationen, vor Peroxynitrit-induzierten DNS-Strangbrüchen (Phillips et al., 2011; Chen et al., 2011). Weiterhin zeigte Myricetin in einem Mausmodell eine Verringerung von UVB-induzierten Hautkrebstumoren, durch eine kompetitive Verdrängung von ATP an der Kinase Fyn (Jung et al., 2008). Myricetin beeinflusst den Glukosemetabolismus in Säugern, da an einem Modell mit insulinresistenten Ratten gezeigt wurde, dass die Applikation des Flavonols die Phosphorylierung des Insulin Rezeptors, des Insulin Rezeptor Substrat-1, sowie von Akt und dem Akt-Substrat AS160 in Skelettmuskelzellen induziert, mit der Folge einer verstärkten Translokation des Glukosetransporter-4 an die Zellmembran (Tzeng et al., 2004). Im Modellorganismus *C. elegans* zeigten Grünz et al. eine Verlängerung der Lebensspanne der Tiere nach Behandlung mit Myricetin, eine nukleäre Translokation des Transkriptionsfaktors DAF-16 und eine erhöhte Promoteraktivität der Superoxiddismutase-3 (*sod-3*) (Grünz et al., 2012).

Dem prenylierten Flavanon Isoxanthohumol werden, antiinflammatorische, chemopräventive und östrogene Eigenschaften zugeschrieben. *In vitro* zeigte Isoxanthohumol z.B. entzündungshemmende Wirkungen durch eine Verringerung der pro-inflammatorischen Faktoren TNF-α und NF_KB in humanen glatten Muskelzellen der Aorta und humanen Endothelzellen der Nabelvene (Negrão et al., 2013). Studien zeigten, dass verschiedene Krebszelllinien durch Isoxanthohumol zum Absterben gebracht wurden, wie Brustkrebszellen (MCF-7), Prostatakrebszellen (PC-3, DU145) oder Kolonkrebszellen (HT-29) (Tronina et al., 2013; Delmulle et al., 2006). Isoxanthohumol zeigt eine schwache östrogene Aktivität, wird allerdings durch die bakterielle Darmflora in das stärkere Phytoöstrogen 8-Prenylnaringenin metabolisiert, wobei Phytoöstrogene wahrscheinlich als selektive Östrogen-Rezeptor Modulatoren agieren (Possemiers et al., 2006; Monteiro et al., 2007). Darüber hinaus werden Hopfenextrakte, welche reich an prenylierten Flavonoiden und prenylierten Chalkonen sind, häufig in der Behandlung menopausaler Beschwerden eingenommen (Krause et al., 2014).

1.3.2 Bioaktivität der Stilbene Resveratrol und TSG

Das Stilben Resveratrol wurde in zahlreiche Studien untersucht, welche antioxidative, chemopräventive, kardioprotektive antiinflammatorische und ant-ageing Effekte beschreiben (Baur & Sinclair, 2006). Jang et al. fanden, dass Resveratrol *in vitro* drei wichtige Schritte in der Tumorgenese inhibiert (Initiation, Promotion und Progression) und *in vivo* im Mausmodell das Auftreten und die Anzahl von Tumoren in einem Hautkrebsmodell reduziert (Jang et al., 1997). Es konnte gezeigt werden, dass Resveratrol Sirtuine (NAD^+-abhängige Deacetylasen) aktivieren kann und die Lebensspanne der Hefe *S. cerevisiae* verlängert (Howitz et al., 2003). Auch in höher entwickelten Organismen wie *C. elegans*, *D. melanogaster* und der kurzlebigen Fischspezies *N. furzeri* wurde für Resveratrol eine Verlängerung der Lebensspanne beschrieben (Bauer et al., 2004; Viswanathan et al., 2005; Valenzano et al., 2006).

Das Resveratrolderivat TSG zeigte in Studien antioxidative, neuroprotektive antiinflammatorische, sowie anti-ageing Effekte. Antioxidative und antiinflammatorische Eigenschaften von TSG wurden durch Chen et al. und Ryu et al. bei der Analyse von Substanzen des in der traditionellen chinesischen Medizin verwendeten vielblättrigen Knöterichs (*Polygonum multiflorum*) beschrieben und protektive Wirkungen bei experimentell induzierter Colitis, durch eine Verringerung von freien Sauerstoff- und Stickstoffradikalen, wurden gezeigt (Chen et al., 1999; Ryu et al., 2002; Wang et al., 2008). Neuroprotektive Effekte zeigten sich z.B. durch die Inhibition des Absterbens von humanen Neuroblastomzellen oder die Verbesserung des Erinnerungsvermögens sowie eine Verringerung der Bildung von Amyloidplaques, die mit der Alzheimerschen Krankheit assoziiert sind (Sun et al., 2011; Zhou et al., 2015; Yin et al., 2018). In einem schnell alternden Mausmodell (Senescence Accelerated Mouse-Prone 8, SAMP8) konnten Zhou et al. eine Verlängerung der Lebensspanne der Tiere, sowie erhöhte Protein Level von neuralem Klotho und reduzierte neurale Konzentrationen von Insulin, Insulinrezeptor, IGF-1, and IGF-1 Rezeptor im Gehirn durch TSG zeigen (Zhou et al., 2015).

1.4 Reaktive Sauerstoffspezies, oxidativer Stress und Mechanismen zum Schutz vor oxidativem Stress

Die Gruppe der reaktiven Sauerstoffspezies (*reactive oxygen species*, ROS) umfasst verschiedene radikalische und nicht radikalische Vertreter. Zu den ROS mit radikalischem Charakter zählt das kurzlebige, sehr reaktionsfreudige Superoxidradikalanion ($O_2^{\bullet -}$), welches bei der Aufnahme eines einzelnen Elektrons (e^-) durch molekularen Sauerstoff (O_2) entsteht. Durch weitere Aufnahme von einem Elektron und zwei Protonen (H^+) entsteht das nicht radikalische Wasserstoffperoxid (H_2O_2). Das oxidativ wirksame H_2O_2 kann in Anwesenheit von redoxaktiven Metallionen wie Eisen oder Kupfer über die Fenton-Reaktion die sehr reaktiven Hydroxylradikale ($OH^{\bullet}$) bilden. Eine schematische Darstellung der Reaktionen ist in der Abb. 1.6 gezeigt. Des Weiteren entstehen Peroxyl- ($RO_2^{\bullet}$) und Alkoxylradikale ($RO^{\bullet}$) durch den Aufbruch organischer Peroxide (Halliwell & Cross, 1994).

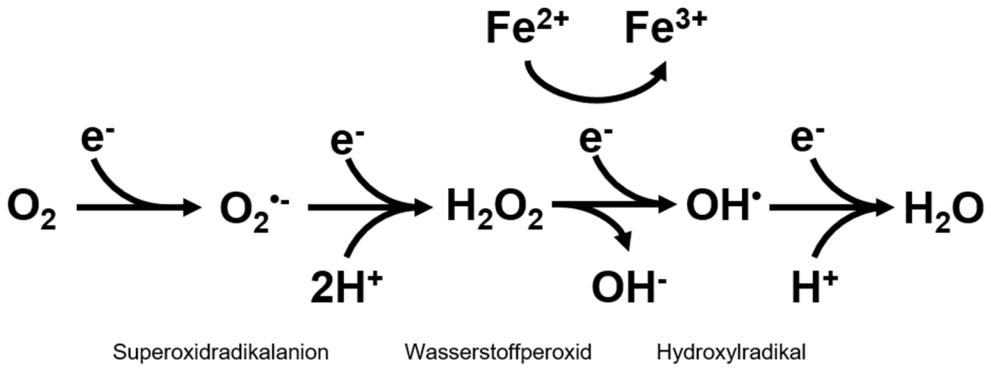

Abbildung 1.6: Schema der Entstehung von radikalen Sauerstoffspezies aus molekularem Sauerstoff durch die schrittweise Aufnahme von Elektronen. (Modifiziert nach Ames et al., 1993)

1.4.1 Entstehung und Bedeutung von ROS im zellulären Kontext

ROS entstehen in Zellen sowohl durch endogene als auch durch exogene Faktoren. Vier endogene Quellen werden als Hauptverursacher für die Entstehung von ROS in Zellen angesehen. Mitochondrien verursachen durch die oxidative Phosphorylierung in den Komplexen der Atmungskette eine sukzessive Übertragung von vier Elektronen auf molekularen Sauerstoff, wodurch dieser zu Wasser reduziert wird. Während dieses Vorgangs

treten jedoch auch unvollständige Reaktionen auf, so dass ROS als Zwischenprodukte der mitochondriellen Atmungskette auftreten (Beckman & Ames, 1998). Eine weitere Quelle der ROS-Produktion stellen Peroxisomen dar (Fahl et al., 1984; Reddy & Rao, 1989). Diese Zellorganellen produzieren als Nebenprodukt der ß-Oxidation von Fettsäuren Wasserstoffperoxid. Weiterhin generieren mikrosomale Enzyme der Cytochrom P-450 Oxidasen während der Metabolisierung von Xenobiotika reaktive Sauerstoffspezies (Gottlieb, 2003). Die letzte bedeutende Quelle für die Entstehung von ROS sind Enzyme wie die NADH-Oxidase. Mit Hilfe dieses Enzyms können phagozytische Zellen Pathogene angreifen und auf diese Weise zur unspezifischen Immunabwehr beitragen (Babior, 1984).

Neben diesen endogenen Quellen entstehen ROS aber auch durch den Einfluss exogener Quellen wie Strahlung, Hitze oder Chemikalien (Halliwell & Gutteridge, 1984; Ames et al., 1993). Ionisierende Strahlung (ubiquitäre Hintergrundstrahlung) führt im wässrigen Milieu der Zelle zu einer kontinuierlichen Produktion von Hydroxylradikalen durch die homolytische Spaltung der O-H Bindung und eine Exposition gegenüber Hitze führt beispielsweise zu einer Konversion des Enzyms Xanthin Dehydrogenase in die Oxidase-Form und trägt so zur Bildung von ROS bei (Halliwell & Cross, 1994; Flanagan et al., 1998). Zusätzlich weisen Mitochondrien bei Hitzestress eine erhöhte Produktion von ROS auf. In Folge der erhöhten Temperatur schlägt die geregelte Einelektronenübertragung in der mitochondrialen Atmungskette in zunehmendem Maße fehl und es entstehen vermehrt Superoxidradikalanionen und Wasserstoffperoxid (Flanagan et al., 1998; Davidson & Schiestl, 2001).

In physiologischen Konzentrationen wird ROS auch eine bedeutende Rolle in der Kontrolle von essenziellen zellulären Funktionen zugesprochen. Dazu zählen Prozesse wie die Verstärkung der Signaltransduktion von membranständigen Rezeptoren (Epidermaler Wachstumsfaktor Rezeptor EGFR oder Insulinrezeptor), die Inhibition von Proteintyrosinphosphatasen, die Aktivierung von MAPK-Kaskaden, ebenso wie die Regulation von redoxsensitiven Transkriptionsfaktoren. ROS üben aber auch eine Funktion als *second messenger* aus, indem sie beispielsweise in Folge von Ligand-Rezeptor Interaktionen gebildet werden können und so die Aktivität bestimmter Zielmoleküle wie z.B. Transkriptionsfaktoren stimulieren. Neben der direkten Interaktion von ROS mit Molekülen ist ein Einfluss auf die Signaltransduktion ebenso durch die Regulation der Redox-Homöostase möglich (Dröge, 2002).

1.4.2 Oxidativer Stress

Unter physiologischen Bedingungen befinden sich Zellen in einem Redox-Gleichgewicht, d.h. Entstehung und Elimination von ROS halten sich in der Waage. Dieses Gleichgewicht zwischen Oxidantien und Antioxidantien versucht die Zelle aufrechtzuerhalten, so dass keine gravierenden Schäden entstehen. Bestimmte Stressreize, wie etwa Strahlung, Hitze oder verschiedene Chemikalien, können jedoch zu einer Verschiebung auf die pro-oxidative Seite und somit zu einer Imbalance der Redox-Homöostase führen. Im Jahre 1985 wurde dieses Phänomen von Helmut Sies als oxidativer Stress bezeichnet und definiert als: „eine Störung in der Balance von Prooxidans und Antioxidans zugunsten des erstgenannten“ (Sies, 1985). Später wurde diese Definition erweitert zur „Imbalance zwischen Oxidantien und Antioxidantien zugunsten der Oxidantien, welche zu einer Störung der Redox-Signalleitung und Kontrolle und/ oder molekularem Schaden führt“ (Sies; 2015).

Reaktive Sauerstoffspezies sind im Stande zelluläre Makromoleküle, wie beispielsweise Nukleinsäuren, Proteine oder Lipide oxidativ zu schädigen. Eines der Ziele für Angriffe durch reaktive Sauerstoffspezies stellen Nukleinsäuren dar. Die Reaktion mit ROS kann zu Einzel- und Doppelstrangbrüchen des Nukleinsäuregrundgerüsts sowie Vernetzungen mit anderen Molekülen führen, ebenso wie zur Bildung von modifizierten DNS-Basen. Diese schädlichen Veränderungen in der Struktur der DNS können zu Mutationen und zu Instabilitäten des Genoms führen und zur Entstehung von Krebs beitragen (Ames et al., 1993; Beckman & Ames, 1998). Mutationen der mitochondrialen DNS (mtDNS) treten im Alter z.B. verstärkt auf, da die mtDNS noch anfälliger für Schäden durch reaktive Sauerstoffspezies ist als genomische DNS (Lee & Wei, 2007). Einerseits ist es aufgrund der räumlichen Nähe der mtDNS zu den Enzymen der mitochondrialen Atmungskette als bedeutender ROS-Quelle wahrscheinlicher, dass eine Reaktion stattfindet, andererseits ist die Kapazität der DNS-Reparatur in den Mitochondrien sehr limitiert und es fehlt der Schutz durch Histone (Lee & Wei, 2007; Ott et al., 2007). Diese erhöhte Sensitivität gegenüber oxidativen Schäden führt zu einer sich selbst verstärkenden Schleife, bei der eine initiale ROS-induzierte Schädigung der mtDNS zu einer erhöhten Produktion von ROS führt, die wiederum weitere Schäden an den Mitochondrien verursacht (Balaban et al., 2005). Als Folge kann es zu einer gestörten Stöchiometrie der Komponenten der mitochondrialen Atmungskette kommen, so dass nicht nur vermehrt ROS produziert und oxidativer Stress verstärkt werden, die Energiegewinnung in den Mitochondrien nimmt ebenfalls ab. Zusammengenommen werden diese mitochondrialen Dysfunktionen als einer der wichtigsten Faktoren bei den Prozessen des Alterns betrachtet (Ott et al., 2007).

Proteine stellen ein weiteres Ziel für schädliche Reaktionen mit reaktiven Sauerstoffspezies dar. Es tritt eine Vielzahl von Proteinmodifikationen auf, die durch Reaktionen mit ROS hervorgerufen werden, wie z.B. die Oxidation von Sulfhydrylgruppen, Peptidfragmentierung

oder auch Vernetzungen zwischen den Proteinen (Beckman & Ames, 1998). Diese schädlichen Modifikationen an Proteinen können zu einem teilweisen oder kompletten Verlust der Funktion der betroffenen Proteine führen. Einige neurodegenerative Erkrankungen wie beispielsweise die Alzheimer- und die Parkinson-Krankheit werden mit der Akkumulation solcher geschädigter Proteine assoziiert (Choi et al., 2005). Oxidativ geschädigte Proteine können sich aber auch mit anderen zellulären Bestandteilen wie Lipiden zu unlöslichen Aggregaten zusammenlagern und, wie das so genannte Alterspigment Lipofuscin, im Laufe des Lebens in Lysosomen alternder postmitotischer Zellen akkumulieren (Terman & Brunk, 2004; Jung et al., 2007). Durch das speziesübergreifende Auftreten von Lipofuscin, bei relativ einfachen Organismen wie *C. elegans* ebenso wie bei Menschen, kann es einen universellen biologischen Marker für das Altern darstellen (Klass et al., 1977; Gerstbrein et al., 2005).

Einen weiteren Angriffspunkt für reaktive Sauerstoffspezies stellet die Lipiddoppelschicht von Zellorganellen und die Zellmembran selbst dar. Bei der Reaktion von ROS mit den Phospholipiden der Membranen können Lipidperoxide entstehen und eine radikalische Kettenreaktion, die Lipidperoxidation, in Gang gesetzt werden. Die oxidierten Lipide werden dadurch selbst zu Radikalen und sind wiederum in der Lage weitere Moleküle anzugreifen und zu schädigen. Die Lipidperoxidation kann zu einem Verlust der Membranintegrität oder zu einer veränderten Membranpermeabilität und letztendlich zum Tod der Zelle führen (Girotti, 1998). Obwohl Zellen während der Evolution diverse Mechanismen entwickelt haben, um einen ausgeglichenen Redox-Status aufrecht zu erhalten, kann es zu einer Verschiebung des Gleichgewichts zur pro-oxidativen Seite und damit zu oxidativem Stress kommen.

1.4.3 Mechanismen zum Schutz gegen oxidativen Stress

Zellen haben ausgeklügelte Verteidigungsstrategien entwickelt, um sich vor Schäden zu schützen, die durch ROS verursacht werden können. Es existieren prinzipiell zwei unterschiedliche zelluläre Schutzmechanismen, um die entstandenen ROS direkt abzufangen: Zum einen besitzen Zellen diverse Enzyme mit antioxidativer Wirkung, zum anderen fangen nicht-enzymatische Moleküle die entstandenen ROS ab. Enzyme mit antioxidativen Eigenschaften, welche die Reaktion der ROS zu weniger reaktiven Substanzen oder zu Wasser katalysieren, sind z.B. Superoxiddismutasen (SOD), Katalasen (CAT), oder Peroxidasen wie die Glutathion Peroxidasen (GPx). Superoxiddismutasen katalysieren die Disproportionierung der Superoxidradikalanionen zu Wasserstoffperoxid. Im Säuger sind drei Isoformen der SOD bekannt, die in unterschiedlichen Zellkompartimenten vorkommen und

verschiedene Metallionen als Kofaktoren verwenden: Eine cytosolische Kupfer/Zink–SOD (Cu/Zn-SOD), eine mitochondriale Mangan-SOD (Mn-SOD) und eine extrazelluläre Cu/Zn-SOD (EC-SOD) (Fridovich, 1998). Das durch die katalytische Reaktion der SOD entstandene Wasserstoffperoxid kann in hohen Konzentrationen ebenfalls eine toxische Wirkung auf Zellen ausüben. Um dem entgegen zu wirken, haben Zellen zwei unterschiedliche Systeme zum Abbau von Wasserstoffperoxid entwickelt. Zum einen übernehmen Katalasen eine weitere Disproportionierungsreaktion ($2H_2O_2 \rightarrow O_2 + 2H_2O$), zum anderen verwenden Peroxidasen diverse Reduktionsäquivalente wie Glutathion oder NADPH für die Reduktion von H_2O_2 zu $2H_2O$ (Fridovich, 1998).

Zusätzlich zu den antioxidativen Enzymen spielen nicht enzymatische Antioxidantien bei der Abwehr reaktiver Sauerstoffspezies eine wichtige Rolle. Neben Antioxidantien wie dem Tripeptid Glutathion (Glutamylcysteinylglycin), Cystein, Bilirubin oder Harnsäure, die Bestandteile des endogenen Stoffwechsels sind, werden viele weitere Antioxidantien über die Nahrung aufgenommen. Dazu zählen beispielsweise pflanzliche Sekundärmetabolite wie Polyphenole, ß-Carotin (Pro-Vitamin A), Ascorbinsäure (Vitamin C) oder Tocopherol (Vitamin E). Sowohl die zelleigenen als auch die durch die Nahrung aufgenommenen Antioxidantien sind aufgrund ihrer chemischen Eigenschaften in der Lage, reaktive Sauerstoffspezies abzufangen und zu eliminieren.

Trotz der antioxidativen Schutzmechanismen gelingt es nicht, alle schädlichen ROS abzufangen. Aus diesem Grund haben Zellen verschiedene Mechanismen entwickelt, um bereits entstandene oxidative Schäden zu erkennen und zu reparieren, oder wenn dies nicht mehr möglich ist, die geschädigten Moleküle oder Zellen zu beseitigen. Oxidativer Stress kann einen Zellzyklusarrest induzieren, um Schäden an der DNS im kondensierten Zustand vorzubeugen. Bereits aufgetretene DNS-Schäden können durch das Entfernen und Ersetzen modifizierter Basen oder Nukleotide und durch die Reparatur von Strangbrüchen behoben werden. Hitzeschock-Proteine (HSP) fungieren in der Zelle als molekulare Chaperone, d.h. sie besitzen die Fähigkeit nicht native Proteine selektiv zu erkennen und zu binden. Dadurch verhindern sie die irreversible Aggregation von Proteinen und katalysieren die Rückfaltung der Proteine in ihre native Form. Irreparabel modifizierte Proteine werden lysosomal oder proteasomal abgebaut. Sind Zellen zu stark geschädigt gehen sie in den programmierten Zelltod (Apoptose), um die umliegenden Zellen zu schützen (Lindquist & Craig, 1988; Buchner, 1996; Davies, 2000).

1.5 Alterung

Das Phänomen der Alterung von Lebewesen ist ein komplexer Prozess, der mit der Akkumulation von Schäden und dem Verlust physiologischer Funktionen einhergeht, welche die Anfälligkeit für Krankheiten erhöhen und schließlich zum Tod eines Organismus führen. Die Lebensspanne verschiedener tierischer Spezies reicht dabei von Tagen (*Saccharomyces cerevisiae*) und Wochen (*Caenorhabditis elegans*, *Drosophila melanogaster*) über Jahre (*Mus musculus*) bis hin zu vielen Jahrzenten (*Macaca mulatta*) (Taormina et al., 2019). Für den Menschen konnte ein nahezu linearer Anstieg der durchschnittlichen Lebenserwartung seit 1840 festgestellt werden, die von etwa 46 Jahren auf ca. 85 Jahre im Jahre 2007 angewachsen ist, wobei die Verringerung der Kindersterblichkeit und die Bekämpfung von Infektionskrankheiten einen wesentlichen Beitrag geleistet haben (Christensen et al., 2009). Da das Altern jedoch den größten Risikofaktor für altersassoziierte Krankheiten darstellt, wie z.B. Krebs, kardiovaskuläre und neurodegenerative Erkrankungen sowie Stoffwechselstörungen (Fernandes et al., 2016; Yang et al., 2020), ist es von großem Interesse, einerseits ein besseres Verständnis für die Prozesse, die dem Altern zugrunde liegen, zu erlangen (auf (epi)genetischer, molekularer und zellulärer Ebene) und andererseits Interventionen zu entwickeln, die eine gesunde Lebensspanne (die sog. *Healthspan*) ermöglichen. Hierzu zählen Maßnahmen, die den Lebensstil betreffen wie eine ausgewogene Ernährung, die reich an pflanzlichen Nahrungsmitteln ist, eine ausreichende körperliche Betätigung oder die Prävention von bekannten Risikofaktoren wie Rauchen oder übermäßigem Alkoholkonsum. Aber auch die Entwicklung von pharmakologisch wirksamen Produkten und das Verständnis ihrer Wirkmechanismen kann das Risiko an altersassoziierten Krankheiten zu sterben verringern und die *Healthspan* erhöhen.

1.5.1 Kennzeichen des Alterns

Nahezu alle höheren Organismen, vor allem aber die Klasse der Säugetiere, teilen dieselben Kennzeichen des Alterns. Hierbei werden neun grundlegende Kennzeichen des Alterns unterschieden (Übersicht in López-Otín et al. 2013): **Genomische Instabilität**: Schäden der DNS im Zellkern und den Mitochondrien, durch exo- und endogene Faktoren, akkumulieren über die Zeit und exzessive Schädigungen oder unzureichende Reparatur der DNS begünstigt den Alterungsprozess (Park & Larsson, 2011; Moskalev et al., 2013). **Verkürzung der Telomere**: Mit zunehmendem Alter erhöht sich die Anzahl der Zellteilungen, wobei die Enden der DNS (Telomere) nicht vollständig repliziert werden (Blackburn et al., 2006). **Epigenetische Veränderungen**: Veränderte DNS-Methylierung (Talens et al., 2012), Histonmodifikationen und Chromatinumstrukturierungen führen zu transkriptionellen Störungen, fehlerhafter DNS-

Reparatur und chromosomaler Instabilität (Mostoslavsky et al., 2006) und zu Fehlern der RNS-Prozessierung (Harries et al., 2011). **Verlust der Protein Homöostase**: Die Akkumulation von unlöslichen und nicht abbaubaren Proteinen steigt mit zunehmendem Alter und erzeugt proteotoxische Effekte (Powers et al., 2009). **Deregulierte Wahrnehmung von Nährstoffen**: Mit zunehmendem Alter verlieren Signalwege, die an der Wahrnehmung von Nährstoffen beteiligt sind ihre Funktionalität, wie der *Insulin/ IGF-1*-ähnliche Signalweg (Glukose), *mTOR* (Aminosäuren), *AMPK* (Energiestatus über *AMP*-Level) und *Sirtuine* (Energiestatus über NAD^+-Level) (Fontana et al., 2010). **Mitochondrielle Dysfunktion**: Die Effizienz der mitochondriellen Atmungskette nimmt mit dem Alter ab und es kommt vermehrt zu einem Ausstrom von Elektronen und einer reduzierten Produktion von ATP (Green et al., 2011). **Zelluläre Seneszenz**: Mit zunehmendem Alter verlieren Zellen ihre regenerativen Fähigkeiten, so dass Schäden akkumulieren, Entzündungen zunehmen und Gewebe und Organe ihre Funktionen verlieren (Campisi & d´Adda di Fagagna, 2007). **Verringerte Anzahl und Funktion von Stammzellen:** Im Laufe des Alterns nimmt die Fähigkeit zur Regeneration von hämatopoetischen, mesenchymalen und intestinalen Epithelstammzellen sowie von Sattelitenzellen ab (z.T. nimmt auch ihre Anzahl ab) und führt zu einem kontinuierlichen Funktionsverlust der entsprechenden Gewebe und Organe (Rando, 2006; Liu & Rando, 2011). **Veränderte Zell-Zell Kommunikation**: Über die Zeit verlieren Zellen die Fähigkeit der interzellulären Kommunikation und es tritt eine Störung der endokrinen, neuroendokrinen oder neuronalen Signalweiterleitung auf (Russel & Kahn, 2007; Zhang et al., 2013).

1.5.2 Theorien des Alterns

Es existieren mehr als 300 Theorien des Alterns, die allgemein in eine von zwei Kategorien eingeordnet werden: genetische Programmierung oder stochastische Akkumulation von Schäden (Medvedev, 1990; Lipsky & King, 2015). Theorien der genetischen Programmierung gehen davon aus, dass Organismen eine innere Uhr besitzen, welche das Altern bestimmt. Theorien der zufälligen Ansammlung von Schäden gehen davon aus, dass das zufällige Auftreten von Fehlern und Schäden, die im Laufe der Zeit angehäuft werden, ursächlich für das Altern sind. Letztere beinhalten "*wear and tear"*, "*error catastrophe"*, "*DNA damage hypothesis"*, "*loss of adaptive cellular mechanism"*, "*mitochondrial theory"* und die "*free radical theory"*. Eine weit verbreitete und akzeptierte Theorie des Alterns ist die „*free radical theory of ageing*" (FRTA), die bereits 1954 von Denham Harman eingeführt wurde (Harman, 2003). Sie besagt, dass das Altern auf der chemischen Natur und dem ubiquitären Vorkommen freier Radikale basiert, welche zu einer Akkumulation oxidativ geschädigter Moleküle, wie Nukleinsäuren, Proteine oder Lipide, über die Zeit führt.

1.6 *Caenorhabditis elegans* als Modellorganismus

Caenorhabditis elegans ist ein weltweit vorkommender, freilebender Nematode (Fadenwurm), der in den 1960er Jahren von Sydney Brenner als Modellorganismus zur Untersuchung von entwicklungs- und neurobiologischen Prozessen in die biomedizinische Forschung eingeführt wurde (Corsi et al., 2015). Seit dieser Zeit wurden einige wegweisende Entdeckungen durch Arbeiten mit *C. elegans* gemacht, wie z.B. Entdeckungen zur genetischen Regulierung der Organentwicklung und programmiertes Zellsterben (Verleihung des Nobelpreises des Jahres 2002 in Physiologie oder Medizin an Sydney Brenner, H. Robert Horvitz und John Sulston), die Entdeckung der RNA Interferenz- Genstummschaltung durch Doppelstrang-RNA (Verleihung des Nobelpreises des Jahres 2006 in Physiologie oder Medizin an Andrew Fire und Craig Mello) und die erstmalige Nutzung des grün fluoreszierenden Proteins, GFP, als molekularbiologisches Werkzeug *in vivo* (Verleihung des Nobelpreises des Jahres 2008 in Chemie an Osamu Shimomura, Martin Chalfie und Roger Tsien).

C. elegans hat sich als Modellorganismus in verschiedensten biomedizinischen Forschungsbereichen aufgrund zahlreicher positiver Eigenschaften etabliert, die hier kurz zusammengefasst werden. Die Tiere weisen nur eine geringe Körpergröße auf und sind als Adulte etwa 1 mm lang, wobei zwei Geschlechter auftreten: Hermaphroditen und Männchen (<0,2%). Die Nematoden weisen einen invariablen Zellstammbaum auf (Eutelie), d.h. das Schicksal jeder Zelle ist ab der Befruchtung bereits festgelegt, so dass Hermaphroditen aus 959 somatischen Zellen bestehen, von denen 302 Neuronen sind. Trotz dieser geringen Zahl an Zellen, aus denen die Tiere bestehen, besitzen sie spezialisierte Gewebe wie eine Cuticula, eine Epidermis, Muskeln, ein Nervensystem, einen Verdauungs- sowie einen Reproduktionsapparat. Der Reproduktionszyklus ist kurz und dauert vom befruchteten Ei bis zum Adultstadium ca. 3,5 Tage bei 20°C, wobei die geschlüpften Larven vier Larvalstadien durchlaufen (Abb. 1.7), die jeweils durch eine Häutung gekennzeichnet sind (mit einem alternativen dritten Larvalstadium (Dauerstadium), das bei adversen Bedingungen auftritt und bei dem die Larve bis zu vier Monate überdauern kann). Die Reproduktionsrate ist hoch und die in der Regel als Hermaphroditen auftretenden Nematoden zeugen durch Selbstbefruchtung bis zu 300 Nachkommen (Corsi et al., 2015). Die Lebensspanne der Tiere ist relativ kurz und beträgt nur wenige Wochen, wodurch sich *C. elegans* sehr gut für pharmakologische und genetische Studien zur Alterung eignet.

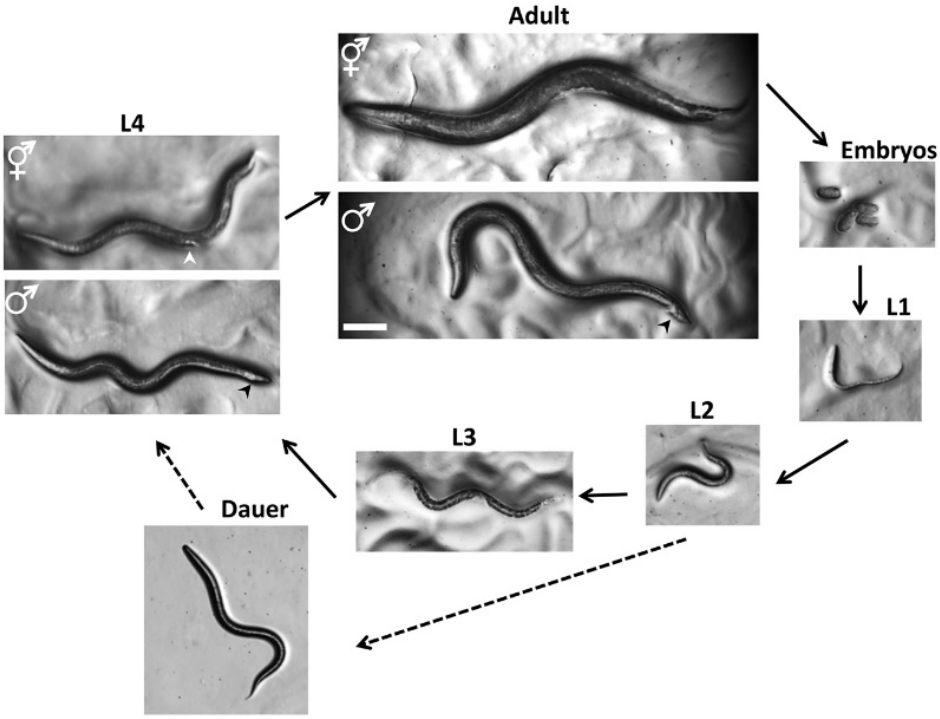

Abbildung. 1.7: Lebenszyklus von *C. elegans*. Dargestellt sind Adulte (oben: Hermaphrodit, unten Männchen), Embryonen, die Entwicklung über die vier Larvalstadien L1-L4, sowie das alternative Juvenilstadium der Dauerlarve. Der weiße Balken entspricht 0,1 mm. (Corsi et al., 2015)

C. elegans ist relativ einfach genetisch zu manipulieren, z.B. die Erzeugung von Mutanten (*loss-of-function* oder *gain-of-function*) durch chemische Mutagene wie Ethylmethansulfonat (EMS) (Brenner, 1974), das Erzeugen von transgenen Tieren durch das Einfügen von GFP-markierten Reportergenen (Chalfie et al., 1994) oder das Herunterregulieren der Translation von Zielgenen durch RNA vermittelte Interferenz (RNAi) (Fire et al., 1998; Timmons & Fire, 1998). Darüber hinaus sind fast alle je erzeugten mutierten oder transgenen Stämme einfach über das *Caenorhabditis* Genetics Center (University of Minnesota) zu beziehen und es steht eine genomweite RNAi Bibliothek zur Verfügung (Kamath & Ahringer, 2003). Unter Laborbedingungen ist *C. elegans* einfach zu halten, da er in einer großen Anzahl in Petrischalen und Mikrotiterplatten in flüssigen oder festen Nährmedien kultiviert werden kann und sich unter diesen kontrollierten Bedingungen von einer monoxenischen Diät aus *Escherichia coli* (*E. coli*) Bakterien ernährt. Aufgrund der geringen Größe und der Transparenz sind die Nematoden sehr einfach sowohl lichtmikroskopisch (Auflicht, Durchlicht, Fluoreszenz) als auch durch automatisierte fluoreszenzspektrophotometrische Verfahren zu untersuchen.

C. elegans war der erste multizelluläre Organismus, dessen Genom vollständig sequenziert wurde, es umfasst etwa 97 Megabasenpaare und enthält etwa 19000 Gene (The *C. elegans* Sequencing Consortium, 1998). Von den Protein kodierenden Genen in *C. elegans* besitzen mindestens 38% Orthologe im humanen Genom (Shaye & Greenwald, 2011), etwa 60-80% der menschlichen Gene besitzen Orthologe im Genom von *C. elegans* (Kaletta & Hengartner, 2006) und ca. 40% der Gene, die im Menschen mit Krankheiten assoziiert werden besitzen Orthologe in *C. elegans* (Culetto & Sattelle, 2000). Durch diese hohe evolutionäre Konservierung können Ergebnisse, die aus Studien mit dem Modellorganismus *C. elegans* stammen, einen bedeutenden Beitrag zum Verständnis sowohl menschlicher Krankheiten als auch der menschlichen Gesundheit im Alter leisten.

1.6.1 Der Insulin/IGF-1 ähnliche Signalweg in *C. elegans*

Grundlegende Entdeckungen bezüglich der molekularen Regulation der Prozesse des Alterns sind dem Modellorganismus *C. elegans* zu verdanken. Das erste entdeckte Gerontogen war ein Ortholog der katalytischen Untereinheit der Phosphatidylinositol-3-Kinase (PI3K), *age-1* (*age*ing alteration). *Age-1* Mutanten zeigen eine um 70% verlängerte mittlere und eine um 105% verlängerte maximale Lebensspanne gegenüber dem Wildtyp (Friedman & Johnson, 1988; Johnson et al., 2002). Gleichzeitig weisen *age-1* Mutanten eine erhöhte Resistenz gegen UV-Strahlung, Hitze sowie oxidativen Stress auf (Johnson et al., 2002). Im Folgenden wurden weitere Gerontogene entdeckt, wie z.B. *daf-2*, (abnormal *da*uer *f*ormation) das *C. elegans* Ortholog des Insulin/IGF-1 (*i*nsulin like *g*rowth *f*actor-1) ähnlichen Rezeptors der Säuger (einer nach Ligandenbindung autophosphorylierenden Rezeptortyrosinkinase), das bei einer Mutation mit Funktionsverlust zu einer bis auf das Vierfache gesteigerten Lebensspanne gegenüber dem Wildtyp führt und die Stressresistenz erhöht (Lithgow et al., 1995; Tissenbaum & Ruvkun, 1998). Neben weiteren Faktoren sind *daf-2* und *age-1* Komponenten des Insulin/IGF-1 ähnlichen Signalwegs (IIS), der in so unterschiedlichen Spezies wie *C. elegans*, *S. cerevisiae*, *D. melanogaster* und in Säugern evolutionär hoch konservierten ist (Cheng et al., 2005; Baumeister et al., 2006).

Die Faktoren des IIS, sowie die Mechanismen zur Regulation der Kinasekaskade werden nachfolgend kurz zusammengefasst (siehe Abb. 1.8): Nach Aktivierung des Insulin/IGF-1 ähnlichen Rezeptors DAF-2 findet eine Autophosphorylierung statt, in dessen Folge der Komplex der Phosphatidylinositol-3-Kinase zur Zellmembran rekrutiert wird wo die Reaktion der *second messenger* PIP_2 (Phosphatidylinositol-4,5-Bisphosphat) zu PIP_3 (Phosphatidylinositol-3,4,5-Trisphosphat) katalysiert wird.

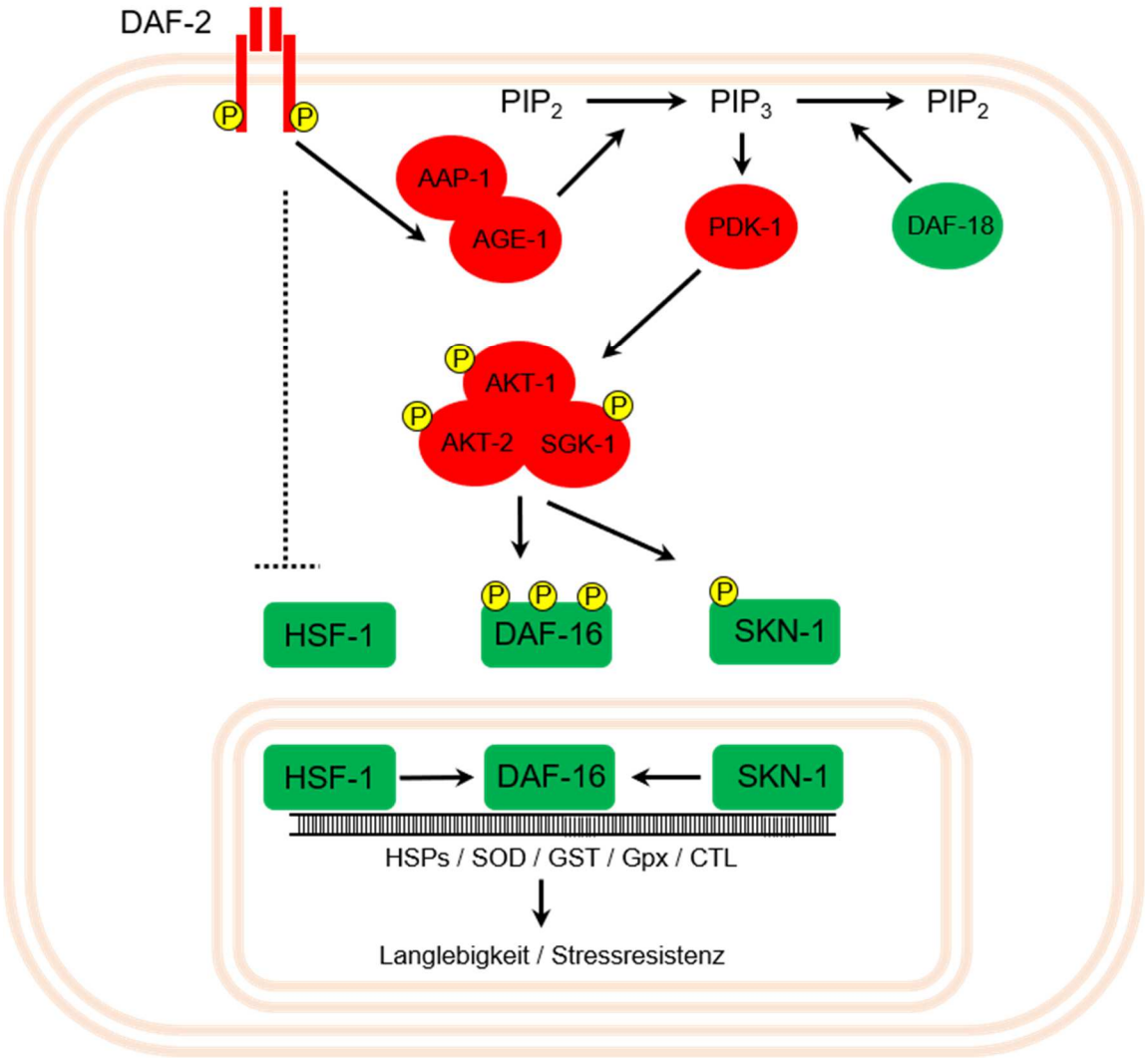

Abbildung. 1.8: Schematische Darstellung des IIS in *C. elegans*. Ein aktivierter IIS verhindert phosphorylierungsabhängig die Translokation der Transkriptionsfaktoren HSF-1, DAF-16 und SKN-1 aus dem Cytosol in den Zellkern und verhindert somit ihre transkriptionelle Aktivität. Faktoren, die eine agonistische Wirkung des IIS ausüben sind in Rot dargestellt, während antagonistische Faktoren in Grün gekennzeichnet sind (modifiziert nach Koch et al., 2014 und Murphy& Hu, 2013). Für Details siehe Text. Abkürzungen: DAF-2: Insulin/Insulinähnlicher Wachstumsfaktor Rezeptor; AAP-1: AGE-1 Adapter Protein; AGE-1: katalytische Untereinheit der Phosphatidyl-Inositol-3 Kinase; PIP_2: Phosphatidylinositol-4,5-Bisphosphat; PIP_3: Phosphatidylinositol-3,4,5-Trisphosphat; PDK-1: Phosphoinositid abhängige Proteinkinase; DAF-18: Phosphatase und Tensin Homolog (PTEN); AKT-1/AKT-2; Serin/Threonin Kinasen der AKT-Familie; SGK-1: Serum.Glukokortikoid-regulierte Kinase; HSF-1: Hitzeschock Transkriptionsfaktor; DAF-16: Forkheaed Box O (FoxO) Transkriptionsfaktor; SKN-1: Homolog des Nrf (Nuclear factor erythroid 2-related factor) Trankriptionsfaktor; HSPs: Hitzeschockproteine; SOD: Superoxiddismutase; GST: Glutathion-S-Transferase; Gpx: Glutathionperoxidase; CTL: Katalase.

Nachfolgend wird PDK-1 (Phosphoinositid abhängige Proteinkinase) aktiviert und phosporyliert den Komplex aus AKT-1/AKT-2 (Serin/Threonin Kinasen der AKT-Familie) und SGK-1 (Serum-Glukokortikoid-regulierte Kinase), jedoch steht hier ein zusätzlicher antagonistischer Mechanismus bereit, durch die Phosphatase DAF-18 (Phosphatase und Tensin Homolog (PTEN)). Der aktivierte Komplex aus AKT-1/AKT-2/SGK-1 übt seinerseits eine inhibitorische Phosphorylierung auf die Transkriptionsfaktoren DAF-16 (Forkheaed Box O (FoxO) Transkriptionsfaktor) und SKN-1 (Nuclear factor erythroid 2-related factor Homolog) aus, wodurch die Faktoren im Cytosol zurückgehalten werden. Über einen weiteren Komplex wird der Transkriptionsfaktor HSF-1 (Hitzeschock Transkriptionsfaktor) durch einen aktivierten IIS an der Kernlokalisation gehindert. Ist der IIS inaktiv, können die Transkriptionsfaktoren in den Zellkern wandern und dort die Transkription der Zielgene initiieren.

In *C. elegans* konnte gezeigt werden, dass der zur FoxO-Klasse zählende Transkriptionsfaktor DAF-16 innerhalb des IIS von zentraler Bedeutung ist. So führt eine Überexpression von DAF-16 zu einer verlängerten Lebensdauer wohingegen die lebensverlängernden und resistenzsteigernden Effekte von *daf-2* und *age-1* Mutationen durch *daf-16* Nullmutationen wieder aufgehoben werden (Kenyon et al., 1993; Ogg et al., 1997; Henderson et al., 2006). DAF-16 reguliert die Expression von Genen, die Entwicklung und Metabolismus steuern, aber auch an der Bekämpfung von oxidativem Stress und seinen Folgen beteiligt sind (Lee et al., 2003; Kenyon, 2005; Baumeister et al., 2006). So reguliert der Transkriptionsfaktor DAF-16 die Expression der antioxidativen Enzyme Katalase (CTL) sowie der mitochondrialen Mangan-Superoxiddismutase SOD-3, die zur Detoxifizierung von reaktiven Sauerstoffspezies beitragen (Honda & Honda, 1999). Die Expression von kleinen Hitzeschock-Proteinen wie HSP-16.2, welche die Bildung von geschädigten Proteinaggregaten verhindern, wird ebenfalls durch DAF-16 reguliert (Walker et al., 2001). Das DAF-16 Ortholog der Säuger, FoxO3a, wird ebenfalls durch den Insulin / IGF-1 ähnlichen Signalweg reguliert und es konnte eine verstärkte SOD-Expression nach oxidativem Stress in Abhängigkeit von FoxO3a gezeigt werden (Kops et al., 2002; Barthel et al., 2005).

Der IIS reguliert neben DAF-16 aber auch weitere Transkriptionsfaktoren, die an der Stressresistenz und der Lebensspanne beteiligt sind. Einerseits konnte nachgewiesen werden, dass HSF-1 eine wichtige Rolle im IIS spielt, da eine Überexpression von HSF-1, welcher die Expression von Hitzeschockproteinen reguliert, die Lebensspanne der Nematoden verlängert, RNAi gegen *daf-16* diesen Effekt wieder aufhebt und es wurde die Langlebigkeit von *daf-2* Mutanten durch Applikation von RNAi gegen *hsf-1* wieder aufgehoben (Hsu et al., 2003). Andererseits ist der Transkriptionsfaktor SKN-1 ebenfalls eine wichtige Komponente in der IIS vermittelten Stressresistenz und Lebensspanne. SKN-1 reguliert die Expression vieler Gene des Phase-II Fremdstoffmetabolismus, wie z.B. die γ-Glutamin-Cystein-Synthetase

(GCS(h)), die Glutathion Synthetase sowie Glutathion S-transferase, Superoxiddismutase und Katalase (An & Blackwell, 2003). Es konnte gezeigt werden, dass *skn-1 loss-of-function* Mutanten die Langlebigkeit und erhöhte Stresstoleranz bei reduzierter IIS Aktivität verringern und SKN-1 direkt durch einen aktivierten IIS inhibiert wird (Tullet et al., 2008).

2. Zielstellung

Ein weltweit stetig steigendes Alter der Bevölkerung und damit einhergehende Erkrankungen, stellen die Gesundheitssysteme vor große Herausforderungen. Es ist daher von besonderem Interesse Maßnahmen zu entwickeln, die ein gesundes Altern ermöglichen. Einen vielversprechenden Ansatz bieten bestimmte pflanzliche Polyphenole, die in *in vitro* Studien protektive Wirkungen im Zusammenhang mit dem Altern bzw. mit altersassoziierten Erkrankungen zeigen konnten, wie z.B. antioxidative, chemopräventive, neuroprotektive oder kardioprotektive Wirkungen. Doch lassen sich die *in vitro* gewonnenen Erkenntnisse oftmals nicht direkt auf die Situation *in vivo* übertragen und mechanistische Studien am Menschen sind selten. Daher war es das Ziel dieser Arbeit, *in vitro* protektiv wirkende Substanzen aus zwei Klassen der Polyphenole, Flavonoide und Stilbene, auf ihre Wirkung bezüglich ihrer antioxidativen Eigenschaften, der Lebensspanne sowie der Stressresistenz im Modellorganismus *Caenorhabditis elegans* zu untersuchen. Ein besonderes Augenmerk wurde dabei zum einen auf die molekularen Mechanismen, die den protektiven Wirkungen der Polyphenole zu Grunde liegen, gerichtet. Zum anderen wurde den Fragen nachgegangen, ob bestimmte funktionelle Gruppen der Moleküle für die Wirkungen der Substanzen verantwortlich sind, ob Modifikationen der Substituenten die protektiven Wirkungen modulieren können und welchen Einfluss die bakterielle Metabolisierung der Verbindungen ausübt.

Im ersten Teil der Arbeit war es das Ziel, ein besseres Verständnis der molekularen Wirkmechanismen der untersuchten Flavonoide zu erhalten, die zu einer verlängerten Lebensspanne in *C. elegans* führen können. Hierzu wurde die antioxidative Wirkung *in vivo* unter physiologischen Bedingungen und bei thermalem Stress bestimmt, da die durch ROS verursachten zellulären Schäden für den Alterungsprozess mitverantwortlich gemacht werden. Ebenso wurde die Resistenz gegen thermalen Stress untersucht, da eine Verlängerung der Lebensspanne in *C. elegans* häufig mit einer gesteigerten Stressresistenz korreliert. Weiterhin wurde untersucht, ob eine Beteiligung der Transkriptionsfaktoren DAF-16 und SKN-1 vorliegt. DAF-16 und SKN-1 sind Faktoren, die durch den evolutionär hoch konservierten Insulin/IGF-1 ähnlichen Signalweg (IIS) reguliert werden, welcher eine entscheidende Rolle in der Stressresistenz und Langlebigkeit spielt. Ein weiterführender Teil der Arbeit bestand darin, Struktur-Wirkungsbeziehungen der Flavonoide aufzuklären. Durch die Untersuchung der methylierten Myricetinderivate Laricitrin, Syringetin und Myricetintrimethylether wurde die Fragestellung bearbeitet, ob die Hydroxylgruppen im B-Ring des Myricetin für seine protektiven Wirkungen notwendig sind. Des Weiteren wurde untersucht, wie zusätzliche funktionelle Gruppen die protektiven Wirkungen in *C. elegans* beeinflussen, wozu das methylierte und prenylierte Flavanon Isoxanthohumol eingesetzt wurde.

Im zweiten Teil der Arbeit war es das Ziel, protektive Wirkungen von Stilbenen und die zugrunde liegenden molekularen Mechanismen *in vivo* zu untersuchen, insbesondere im Hinblick auf die Änderung der biologischen Aktivität durch Modifikation der funktionellen Gruppen oder durch bakterielle Metabolisierung der Stilbene. Hierzu wurden einerseits Resveratrol und das glykosylierte Derivat TSG verglichen, andererseits wurden neu synthetisierte Resveratrolderivate, die von der Arbeitsgruppe von Prof. Dr. Csuk hergestellt und zur Verfügung gestellt wurden, auf ihre Wirksamkeit *in vivo* im Vergleich mit Resveratrol untersucht. Protektive Eigenschaften wurden über die Resistenz gegen thermalen Stress, die antioxidative Wirkung sowie die Lebensspanne ermittelt. Die mechanistischen Untersuchungen zur Beteiligung des IIS erfolgten zum Teil durch die Bestimmung der zellulären Lokalisation der Transkriptionsfaktoren DAF-16 und SKN-1, durch *loss-of-function* Mutanten für DAF-16, SKN-1 und SIR-2.1 oder über die Expression der antioxidativen Enzyme SOD-3 und GST-4. Die Verwendung von metabolisch aktiven bzw. inaktiven Bakterien lässt Rückschlüsse darüber zu, inwiefern die Stilbene selbst oder ihre Metabolite protektive Wirkungen vermitteln.

3. Originalarbeiten

3.1 Studie 1: Myricetin-Mediated Lifespan Extension in *Caenorhabditis elegans* Is Modulated by DAF-16

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Article

Myricetin-Mediated Lifespan Extension in *Caenorhabditis elegans* Is Modulated by DAF-16

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Abstract: Myricetin is a naturally occurring flavonol found in many plant based food sources. It increases the lifespan of *Caenorhabditis elegans*, but the molecular mechanisms are not yet fully understood. We have investigated the impact of this flavonoid on the transcription factors DAF-16 (*C. elegans* FoxO homologue) and SKN-1 (Nrf2 homologue), which have crucial functions in the regulation of ageing. Myricetin is rapidly assimilated by the nematode, causes a nuclear translocation of DAF-16 but not of SKN-1, and finally prolongs the mean adult lifespan of *C. elegans* by 32.9%. The lifespan prolongation was associated with a decrease in the accumulation of reactive oxygen species (ROS) detected by DCF. Myricetin also decreases the formation of lipofuscin, a pigment consisting of highly oxidized and cross-linked proteins that is considered as a biomarker of ageing in diverse species. The lifespan extension was completely abolished in a *daf-16* loss-of-function mutant strain (CF1038). Consistently with this result, myricetin was also not able to diminish stress-induced ROS accumulation in the mutant. These results strongly

indicate that the pro-longevity effect of myricetin is dependent on DAF-16 and not on direct anti-oxidative effects of the flavonoid.

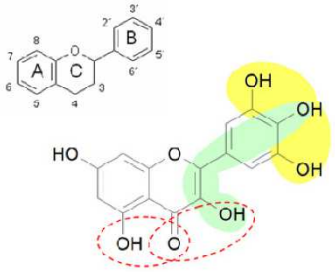
Keywords: *C. elegans*; *daf-16*; flavonoid; lifespan; myricetin; insulin-like signalling; oxidative stress; SKN-1

1. Introduction

Flavonoids are phenolic plant metabolites that occur ubiquitously in fruit, vegetables, grains, nuts, tea and wine [1–3]. Up to now, over 6000 different compounds have been identified. Myricetin (3,5,7,3',4',5'-hexahydroxyflavone) is a major flavonol that is widely distributed in berries, fruit, vegetables, and medicinal herbs.

Myricetin (Figure 1) possesses strong anti-oxidative properties due to three OH-groups in ring B (3',4',5'-position): Therefore, the compound can scavenge reactive oxygen species (ROS) by oxidation of these hydroxyl groups. Further, direct anti-oxidative effects may also be caused by chelation of redox-active metal ions, e.g., Fe^{2+}/Fe^{3+}, thereby inhibiting the formation of fenton reaction products like hydroxyl radicals [4].

Figure 1. Structure of myricetin. The structural elements of myricetin mediating a direct antioxidative effect are: (i) the catechol groups in ring B (3'OH/4'OH and 4'OH/5'OH) forming a semiquinone radical or ortho quinines after oxidation; (ii) the 4'OH group of ring B in combination with the 2,3 double bond and the 3-OH group forming a quinine methide after oxidation; (iii) the ketogroup (position 4) in combination with 3-OH or 5-OH group chelating redox-active metal ions.



Flavonoids show both anti-oxidative as well as pro-oxidative properties depending e.g., on intracellular concentration. Pro-oxidative properties of myricetin are caused mutually by the anti-oxidative action of the substance, e.g., reduction of molecular oxygen to superoxide anions (O_2^-) or Fe^{3+} to Fe^{2+}. Using a deoxyribose degradation assay system, Chobot & Hadacek [4] were able to show that in the presence of ascorbic acid, myricetin exhibited anti-oxidant properties (especially in a complex with iron), while in an ascorbic acid-free system, the pro-oxidant activity prevailed (enhanced if iron was complexed with EDTA). Since pro-oxidant effects of myricetin are known, it is not

surprising, that the flavonoid also activates redox-active signalling pathways within the cell: Using a microarray-based pathway analysis, Qin *et al.* [5] showed that activation of the antioxidant response element (ARE) is involved in myricetin-induced modulation of gene expression in human HepG2 hepatoma cells. They propose that myricetin activates this pathway by inhibiting Nuclear factor erythroid 2-related factor 2 (Nrf2) ubiquitination and protein turnover, stimulating Nrf2 expression and kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1) modification. Myricetin induces pancreatic cancer cell death via inhibition of the phosphatidylinositol 3-kinase (PI3K) signalling pathway [6].

Myricetin is being discussed as an important chemopreventive compound: Physiological concentrations of myricetin significantly inhibited DNA strand breakage induced by both peroxynitrite and its generator 3-morpholinosydnonimine [7]. Myricetin acts as a promising agent for the chemoprevention of skin cancer: Kang *et al.* [8] described that myricetin attenuated the ultraviolet B (UVB)-induced COX-2 expression and skin tumor formation in a mouse skin model by regulating the receptor associated tyrosine kinase Fyn. Furthermore, myricetin was found to inhibit UVB-induced angiogenesis by targeting PI3K in an SKH-1 hairless mouse skin tumorigenesis model. However, it has to be taken into account that the effects of myricetin depend on the bioavailability of the compound: Duthie and Morrice [9] demonstrated that there is an important distinction between the good *in vitro* anti-oxidant effectivity of myricetin and the ability to suppress the oxidation of lipids in hepatic microsomes *in vivo*. In contrast to good anti-oxidative effects *in vitro*, the compound did not significantly affect lipid peroxidation and tissue damage *in vivo*.

Myricetin also influences the glucose metabolism of mammals: Treatment of insulin-resistant rats with this flavonoid affected the phosphorylation of the insulin receptor, insulin receptor substrate-1, Akt and Akt substrate, with subsequent effects on glucose-transporter subtype 4 translocation [10].

Several polyphenols have been reported to increase the lifespan of *Caenorhabditis elegans*, including quercetin, fisetin, resveratrol, catechin, epigallocatechin-gallate or a polyphenolic fraction rich in proanthocyanidines [11–19]. It was also demonstrated that myricetin increases the lifespan of *C. elegans* [20]. Grünz *et al.* observed that myricetin, but no other flavonoid analysed, elongated the lifespan of *mev-1* (*kn1*) mutant animals, suggesting that an anti-oxidant function of a compound is not sufficient for longevity. As a molecular mechanism for the lifespan extension, they suggested a modulation of the insulin/IGF-like signalling pathway. They investigated the activation of the transcription factor DAF-16 (*C. elegans* orthologue of mammalian FoxO transcription factors): Myricetin activates this pathway as detected by an enhanced nuclear localization of DAF-16, thereby increasing the promoter activity of superoxide dismutase 3 (*sod-3*). However, in spite of an increased promoter activity of the anti-oxidative enzyme *sod-3*, no correlation of *daf-16* and the myricetin-induced mitochondrial ROS accumulation and life prolongation was detected [20]. From their experiments, they concluded that myricetin-induced activation of the FoxO orthologue DAF-16 is not the cause of lifespan extension.

Aim of the study

Since there are several questions remaining concerning the anti-oxidative and lifespan-prolonging effects of myricetin in *C. elegans*, we investigated the kinetics of myricetin uptake in *C. elegans*, the

modulation of heat induced intracellular ROS in general (fluorescent probe: DCF) and the effect of myricetin on lipofuscin accumulation, an autofluorescent pigment that is used as a biomarker of ageing. Furthermore, we analysed its effects on DAF-16 and SKN-1 and correlated the modulation of DAF-16 with anti-oxidative effects and ageing. To exclude the possibility that the effects of myricetin were mediated by caloric restriction, we finally analysed the effect of the flavonoid on food uptake by investigating pharyngeal pumping activity and body size.

2. Results and Discussion

The flavonoid myricetin increases lifespan of *C. elegans*, but the molecular mechanisms are not fully understood. It has been suggested that the anti-oxidant action of this compound alone is not sufficient to mediate longevity, but that an interaction with distinct intracellular pathways is required. An activation of DAF-16 was described, however no influence of *daf-16* on the life prolonging action induced by myricetin was reported [20]. In contrast to this report, we observed that for the enhancement of lifespan by myricetin the presence of DAF-16 is required.

2.1. Uptake of Myricetin by C. elegans

Visualization of myricetin uptake in living *C. elegans* by use of the fluorescence enhancer 2-aminoethyl diphenylborinate (Naturstoffreagent A, NSRA) has already been reported and a concentration dependent increase in fluorescence was shown [20]. Here we were able to demonstrate a time dependent increase in myricetin fluorescence in *C. elegans*. Pharyngeal and intestinal fluorescence were clearly visible already after 30 min of myricetin treatment (Figure 2). An increase in fluorescence over time could be observed, with the brightest fluorescence 24 h after myricetin treatment (100 µM). These results indicate that myricetin is rapidly taken up by *C. elegans* and accumulates in the intestine of the nematode in a time dependent manner.

Figure 2. Myricetin is rapidly taken up by *C. elegans*. As early as 30 min after incubation with myricetin (100 µM) followed by two hours of post-treatment with the fluorescence enhancer NSRA (0.2%), a bright fluorescence was detectable in the pharynx and intestine. Representative images of fluorescence (**right**) and brightfield (**left**) micrographs are shown.

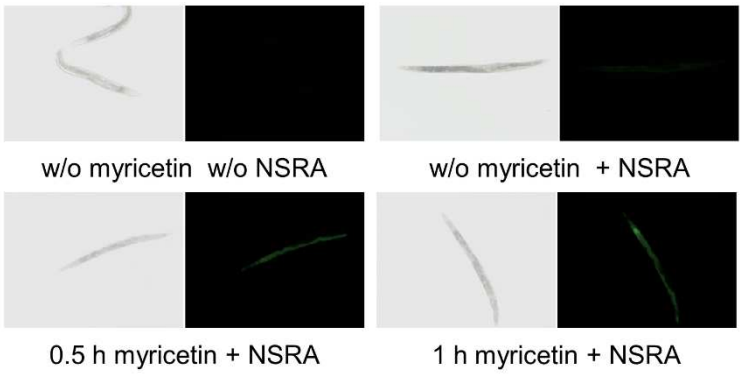
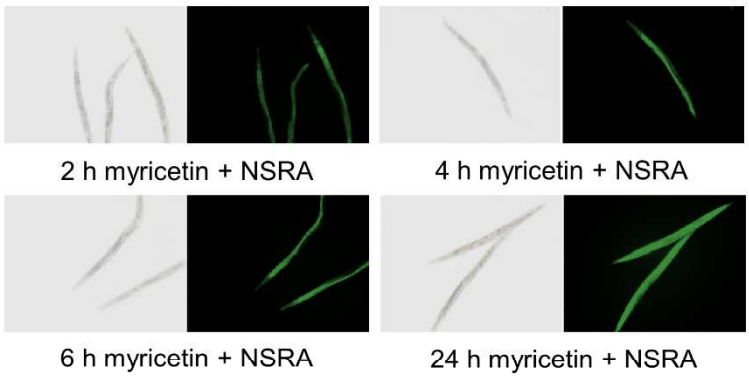


Figure 2. *Cont.*



2.2. Anti-Oxidative Effects of Myricetin

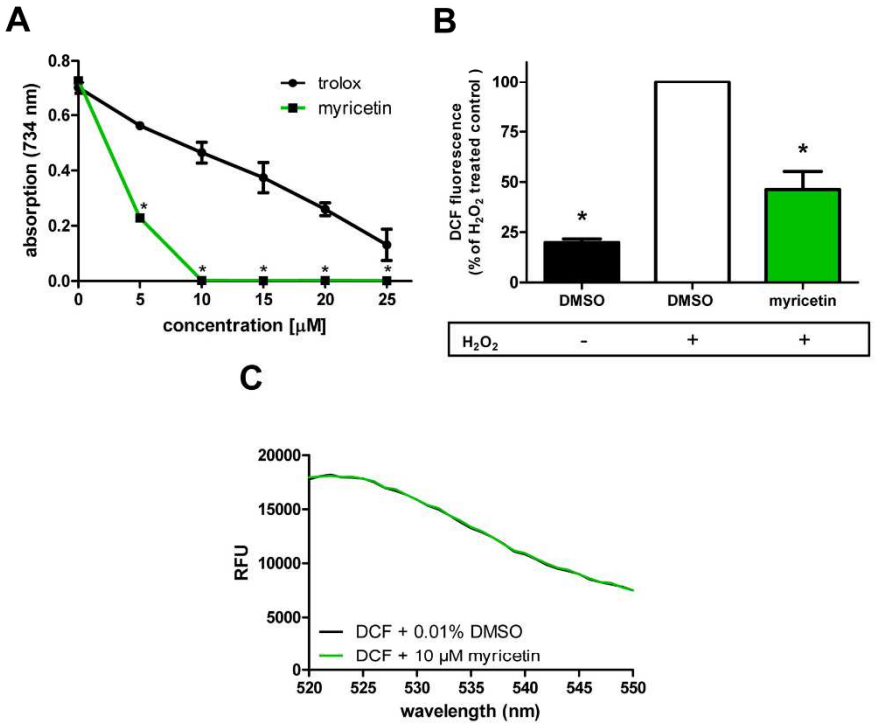
It is well-known that myricetin exerts anti-oxidative effects in various model systems [21]. To evaluate the anti-oxidative potency of myricetin, we first analysed the radical-scavenging capacity of this flavonoid using the cell free TEAC assay and, furthermore investigated anti-oxidative effects in a cellular system (Hct116 human colon carcinoma cells).

2.2.1. Anti-Oxidative Effects of Myricetin in Cell-Free System/in Hct116 Cells

Using the Trolox Equivalent Anti-oxidative Capacity (TEAC) assay, we were able to show that even low concentrations of myricetin possess a radical scavenging capacity (Figure 3A): 5 µM myricetin reduced the absorption of the ABTS radical solution by 68.6%. At a concentration of 10 µM the maximum of radical-scavenging was exceeded. We found that the radical scavenging capacity of myricetin is much stronger than that of the reference compound trolox, a synthetic vitamin E derivative: 5 µM trolox reduced the ABTS radical only by 19.7%; the maximum radical scavenging effect was not reached even at the highest concentration used (25 µM).

Since the anti-oxidative capacity of a compound may differ between cell-free assays and cellular assays, we investigated the anti-oxidative effects of myricetin in Hct116 human colon carcinoma cells: We used DCF, a fluorescent probe for the detection of the overall intracellular ROS content. A pre-incubation with myricetin (50 µM) strongly reduced intracellular ROS accumulation in Hct116 cells after application of oxidative stress (500 µM H_2O_2 for 1 h) to 46.1% ± 9.1% of the corresponding control value (DMSO + 500 µM H_2O_2) (Figure 3B). An effect of myricetin on basal ROS levels was not detectable in this experimental system (data not shown). The experiment was performed with a concentration of 50 µM myricetin, since in a previous investigation a slight toxicity of this flavonoid was observed at the concentration of 100 µM. The toxicity of myricetin in Hct116 cells (approximate 20% lower ability to reduce MTT compared to control cells) occurred only after 24 h of incubation and not during the short time incubation of the DCF assay, but we decided to work in our experimental systems in a non-toxic range.

Figure 3. (**A**) Myricetin is a strong radical scavenger in the cell-free TEAC assay; mean values ± SD, $n = 3$, *: $p < 0.05$ *vs.* corresponding trolox value; one way ANOVA; (**B**) Myricetin treatment (50 µM) also reduced intracellular ROS accumulation in Hct116 human colon carcinoma cells after application of 500 µM H_2O_2 for 1 h, mean values ± SD (DMSO + H_2O_2 set as 100%, the rfu-value of DMSO control before standardization to H_2O_2 control was 8447 ± 2121), $n = 3$, *: $p < 0.05$ *vs.* corresponding DMSO + H_2O_2 value; one way ANOVA; (**C**) Myricetin (10 µM) did not change the emission spectrum and intensity of DCF fluorescence (measurement without cells to exclude that quenching effects are caused by this flavonoid).



To exclude that the effects of myricetin were mediated by physical quenching and not anti-oxidative activity, we analysed the influence of myricetin on the fluorescence intensities of the probe DCF using a monochromator-based fluorescence detector: No interference of myrcetin with DCF was detected (Figure 3C). We could show that myricetin possesses a high direct anti-oxidative capacity both in a cell-free system and in a cellular system.

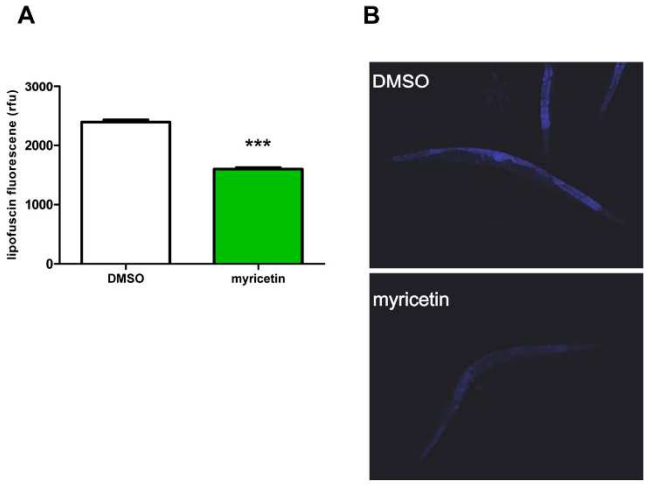
These results concerning radical-scavenging effects of myricetin in cell-free assay systems are in line with a study by Wang *et al.* [22]. Our results concerning anti-oxidative effects of myricetin in cellular systems are in accordance with studies by Khang *et al.* [23] and Wang *et al.* [22].

2.2.2. Effect of Myricetin on Lipofuscin Accumulation in *C. elegans*

The ageing process is associated with an increased accumulation of highly oxidized and cross-linked proteins called lipofuscin. These modified molecules form insoluble aggregates, which are not degradable by the proteasomal or lysosomal systems. Lipofuscin is a so called "age pigment" and considered as a hallmark of ageing. It is well established that the amount of lipofuscin increases with age, and that the rate of lipfuscin accumulation correlates with the rate of ageing. Moreover, lipofuscin serves as a marker of oxidative stress. Aggregates have been found in various cell types and are associated with the age of these cells [24]. It was shown that these aggregates also accumulate in *C. elegans* with increasing age [25–27]. Lipofuscin is autofluorescent and can be visualized by fluorescence microscopy. We investigated the effect of myricetin on this phenomenon to confirm (i) the anti-oxidative effects of myricetin and (ii) the influence of this flavonoid on the ageing process.

Treatment with myricetin reduced the intestinal lipofuscin accumulation in *C. elegans* by 33% ± 1% as compared to the solvent control (DMSO: 2396 ± 36 rfu; myricetin 1602 ± 23 rfu) (Figure 4). A decrease in lipofuscin accumulation was previously shown for various flavonoids, such as quercetin, fisetin and kaempferol [28–30]. In most cases, the reduction of lipofuscin was associated with an increase in lifespan of *C. elegans*. Therefore, the observed reduction of lipofuscin indicates not only a decrease in oxidatively damaged macromolecules; rather it can also point to a decelerated ageing process in myricetin treated nematodes.

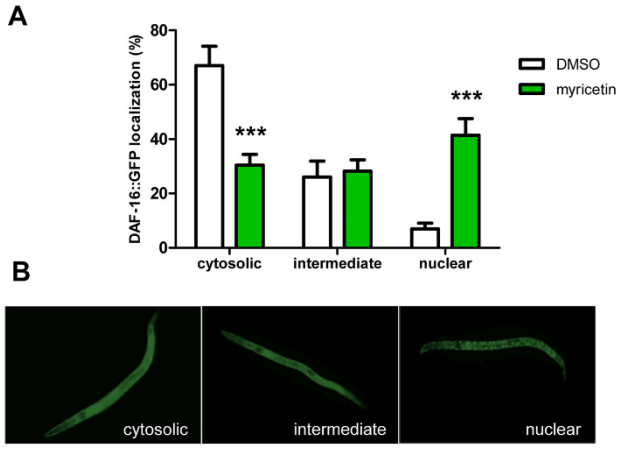
Figure 4. (**A**) Treatment of *C. elegans* (L4 larvae) with myricetin (100 µM) for three days attenuates lipofuscin accumulation; data are mean values ± SEM; $n \geq 90$ in 4 independent experiments; *** $p < 0.001$; unpaired Student's *t*-test. Fluorescence intensities of whole animals (except for head and tail regions) were determined by densitometric analyses; (**B**) Representative images of lipofuscin fluorescence in DMSO-treated nematodes and myricetin-treated nematodes are shown.



2.2.3. Effect of Myricetin on Translocation of DAF-16 and SKN-1

Certain flavonoids protect against oxidative stress either by direct radical scavenging or indirectly by increasing the stress resistance of the organism for example by induction of anti-oxidative enzymes. In mammalian cells, this indirect effect is often mediated via the insulin-like signalling pathway or the redox-active Nrf2/ARE signalling pathway. To analyse the effect of myricetin on these signalling pathways, we investigated if this flavonoid induces a nuclear translocation of the corresponding transcription factors DAF-16 (homologue to the mammalian transcription factor FoxO) and SKN-1 (homologue to the mammalian transcription factor Nrf2) by using transgenic strains expressing the fusion proteins DAF-16::GFP and SKN1::GFP, respectively. When GFP fluorescence was detected mainly in the nuclei (in case of SKN-1 exclusively in intestinal nuclei), the signalling pathway of the nematode was classified as active (see Figure 5C). Regarding the insulin-like signalling pathway, myricetin induced an increase in nuclear DAF-16::GFP translocation (relative DAF-16::GFP localization: DMSO: 6% ± 2%; myricetin: 41% ± 7%) and a reduction in the cytosolic DAF-16::GFP fraction (DMSO: 65% ± 8%; myricetin: 30% ± 4%), respectively (Figure 5A). A nuclear translocation of DAF-16 or target gene expression was reported for different flavonoids, e.g., quercetin [28], apigenin [31], fisetin [29], kaempferol [29] and epigallocatechin-gallate [16]. A shift from cytosolic to nuclear localization of DAF-16 by myricetin (20% cytosolic compared to 70% cytosolic in control nematodes) was previously reported [20].

Figure 5. Myricetin induced nuclear translocation of DAF-16::GFP, but not SKN-1::GFP. Nematodes were treated with DMSO or myricetin (100 µM) for 72 h (DAF-16::GFP) or 24 h (SKN-1::GFP), followed by determination of the GFP-localization phenotype. (**A**) Activation of DAF-16 nuclear translocation by myricetin; mean ± SEM, $n > 200$ in four independent experiments; *** $p < 0.001$ *vs.* corresponding DMSO value; two-way ANOVA; (**B**) Representative images of transgenic strain TJ356 with cytosolic (**left**) intermediate (**center**) and nuclear (**right**) DAF-16::GFP localization; (**C**) Myricetin showed no effect on the SKN-1 signalling pathway; mean ± SEM, $n > 90$ in four independent experiments, $p > 0.05$ *vs.* corresponding DMSO value; two-way ANOVA.



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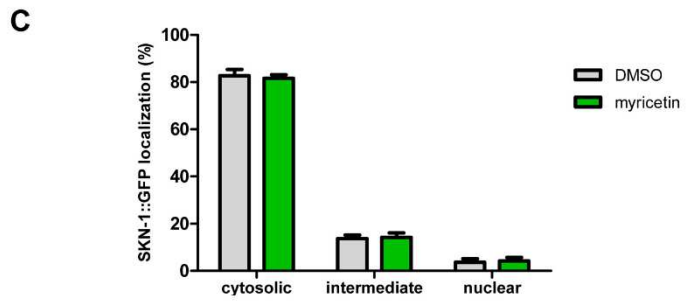


Furthermore, we investigated the effect of myricetin on the Nrf2/ARE signalling pathway in *C. elegans* (=SKN-1). Nrf2 is a redox-active transcription factor that binds to the antioxidant responsive element (ARE), a distinct DNA motif in the promoter region of various antioxidant and phase II genes. Due to induction of antioxidant enzymes, this signalling pathway is a key cellular defense mechanism against oxidative stress and hence activation of this pathway is associated with beneficial effects. The Nrf2 signalling pathway builds up a prolonged defense system compared to the uptake of compounds that act only by scavenging radicals. An activation of the SKN-1 signalling pathway by flavonoids was reported previously for baicalein [32] and epigallocatechin-gallate [33]. However, treatment of *C. elegans* with myricetin (100 µM) did not change the localization of the transcription factor showing that (i) SKN-1 is not activated by this flavonoid (Figure 5C) and (ii) flavonoids need to possess distinct structural features to activate this signalling pathway.

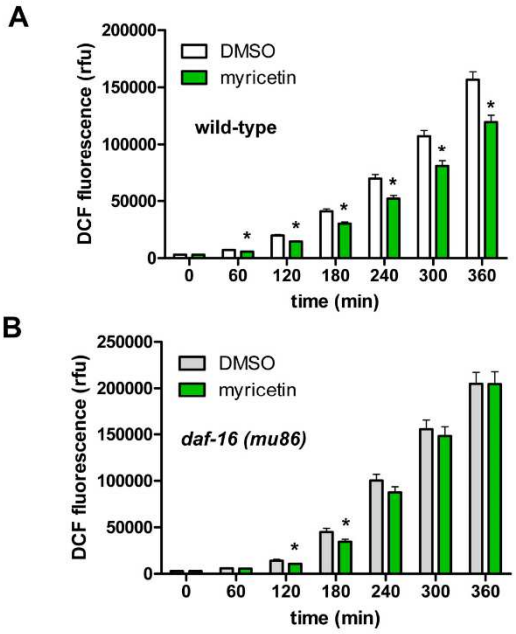
2.2.4. Requirement of DAF-16 for the Anti-oxidative Effects of Myricetin in *C. elegans*

Flavonoids can either modulate oxidative stress directly by radical scavenging or indirectly by increasing the stress resistance of the organism e.g., by induction of anti-oxidative enzymes via activation of protective signalling pathways such as SKN-1 or DAF-16. We showed that myricetin strongly induced the nuclear translocation of DAF-16, but had no influence on SKN-1. For this reason we investigated the effect of myricetin on stress-induced ROS accumulation in wild-type (N2) as well as in *daf-16* (*mu86*) mutant nematodes. In this experimental setting, an increased formation of ROS in the nematodes was provoked (measurement at 37 °C) as detected by the fluorescent probe DCF.

In analogy to the results obtained in the mammalian cell system, incubation with myricetin strongly diminished ROS accumulation in wild-type nematodes (Figure 6A). This result is comparable to data of Grünz *et al.* [20] who investigated the effect of myricetin on the formation of mitochondrial ROS under basal conditions in *C. elegans*. In contrast to this group, we used H_2DCF-DA in order to show alterations in ROS concentration in general during the application of heat stress.

Since we have shown that myricetin stimulated an enhanced nuclear translocation of DAF-16, we further investigated whether the presence of DAF-16 is necessary for the anti-oxidative effects of myricetin in *C. elegans*. To this end, we analysed stress induced ROS accumulation in *daf-16* (*mu86*) mutant nematodes. Loss of function of the FoxO transcription factor DAF-16 nearly completely abolished the protective effect of myricetin (Figure 6B).

Figure 6. (**A**) Myricetin treatment (100 µM) reduced ROS accumulation in wild-type (N2) nematodes at 37 °C. The fluorescence intensity of DCF (rfu) was used as a marker for intracellular ROS; data are mean values ± SEM, $n = 64$ in four independent experiments; * $p < 0.05$, unpaired Student's *t*-test; (**B**) The reduction in ROS accumulation was abolished in *daf-16* (*mu86*) mutant animals with the exception of 120 min and 180 min (**B**); data are mean values ± SEM, $n = 64$ in four independent experiments; * $p < 0.05$ *vs.* corresponding DMSO control, unpaired Student's *t*-test.



The slight reduction of oxidative stress which was detectable only at the early time points (120 + 180 min) may be due to a DAF-16-independent antioxidative effect of myricetin, e.g., radical scavenging. This result clearly shows that functional DAF-16 is necessary for a prolonged anti-oxidative effect of myricetin; the radical scavenging activity of the flavonoid alone seems not to be sufficient to decrease the formation of ROS in our experimental system efficiently for a prolonged time span.

2.3. DAF-16 Is Required for Myricetin-Induced Prolongation of Lifespan

Next, we investigated if myricetin modulates the lifespan of *C. elegans*: Wild-type nematodes were treated with myricetin (100 µM) during their complete adult lifetime. As shown in Figure 7A and Table 1, myricetin extended the mean adult lifespan by 32.9%: lifespan of DMSO-treated nematodes was 18.7 ± 0.9 days, while myricetin-treated *C. elegans* had a mean lifespan of 24.8 ± 0.9 days. These data are in line with observations showing that myricetin prolongs lifespan in wild-type nematodes by 18% [20]. Several anti-oxidative polyphenols have already been identified to extend the lifespan of *C. elegans*, e.g., curcurmin [34], quercetin [11] and baicalein [32].

However, the myricetin-mediated prolongation of lifespan in wild-type *C. elegans* was completely abolished in *daf-16* (*mu86*) mutant animals (Figure 7B and Table 1). This effect indicates that the transcription factor DAF-16 is responsible for myricetin mediated lifespan extension in wild-type nematodes, probably due to a modulation of antioxidant and stress responsive genes. Moreover, the data also indicate that lifespan extension by myricetin is largely independent of its radical scavenging properties. In contrast to these results, Grünz *et al.* [20] reported that myricetin mediates a lifespan extension independent of *daf-16*. This discrepancy could be explained by different experimental settings, which may alter the outcome of the experiment. In our experiments we used liquid culture medium and a temperature of 25 °C for the treatment, while Grünz *et al.* used agar plates and 20 °C for their experimental setup.

Figure 7. (**A**) Myricetin treatment (100 µM) during the whole adult lifespan extended mean lifespan of wild-type (N2) *C. elegans*; (**B**) Lifespan extension was abolished in a strain bearing the *daf-16* (*mu86*) loss of function mutation. Lifespan data are shown in Table 1; Kaplan-Meier survival analysis.

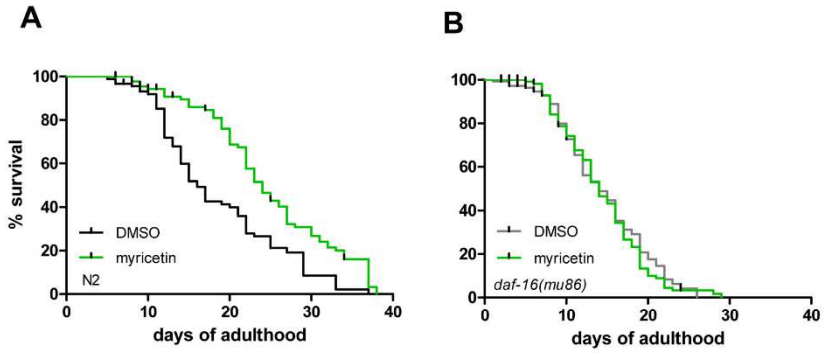


Table 1. Summary of the lifespan data depicted in Figure 7.

Strain	Exp.	Treatment	Mean lifespan (days)	SE	*n* (censored)	% Difference	*p* value
wild-type	3	DMSO	18.7	0.9	90 (20)	32.9	<0.001
wild-type	3	myricetin	24.8	0.9	90 (18)		
daf-16 (mu86)	3	DMSO	15.1	0.5	153 (46)	0.8	0.788
daf-16 (mu86)	3	myricetin	15.2	0.5	150 (46)		

2.4. Myricetin Has No Effect on Resistance against Thermal Stress

Since myricetin reduces oxidative stress and increases lifespan in *C. elegans*, we investigated if this flavonoid also mediates resistance to thermal stress. Thermal stress (37 °C) is known to be lethal in *C. elegans*. We analysed the effect of myricetin on the tolerance of *C. elegans* against thermal stress using the semi-automated SYTOX Green assay: 50% of the nematodes were counted dead after 3.75 h; after 5.25 h at 37 °C, no animal was alive (Figure 8A). In spite of the protective effects of myricetin against ROS accumulation, the flavonoid failed to elicit any protective effects against the lethal heat stress: Mean survival time of DMSO treated wild-type individuals was 3.94 ± 0.09 h compared to

4.01 ± 0.12 h survival time of myricetin-treated nematodes (Table 2). Using the *daf-16* (*mu86*) mutant strain, similar results were obtained: myricetin again failed to protect against thermal stress (Figure 8B). Mean survival time of DMSO treated *daf-16* (*mu86*) nematodes was 3.84 ± 0.12 h compared to 3.76 ± 0.09 h survival of animals treated with myricetin (Table 2). Taken together, in contrast to lifespan extending effects of myricetin (depending at least in part on DAF-16) this flavonoid failed to protect against heat stress in both wild-type and *daf-16* (*mu86*) nematodes. These results show that an increase in lifespan and protection from ROS are not necessarily associated with a protection from heat stress in spite of the thermal-mediated generation of ROS.

Figure 8. Myricetin treatment (100 µM) had no effect on the resistance to thermal stress of wild-type (**A**) and *daf-16* (*mu86*) nematodes (**B**). The corresponding survival data are summarized in table 2; Kaplan-Meier survival analysis.

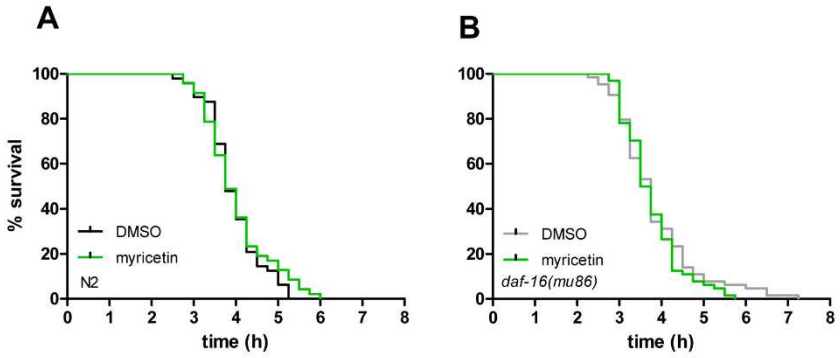


Table 2. Myricetin treatment (100 µM) had no effect on the resistance to thermal stress of wild-type (A) and *daf-16* (*mu86*) nematodes: Summary of the heat stress survival data depicted in Figure 8.

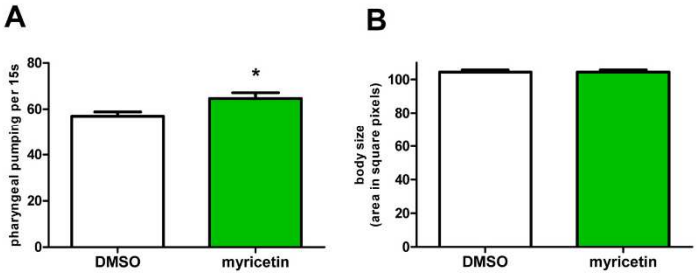
Strain	Exp.	Treatment	Mean lifespan (hours)	SE	*n*	% Difference	*p* value
wild-type	3	DMSO	3.94	0.09	48	1.8	0.473
wild-type	3	myricetin	4.01	0.12	47		
daf-16 (mu86)	4	DMSO	3.84	0.12	64	2.1	0.403
daf-16 (mu86)	4	myricetin	3.76	0.09	64		

2.5. Effects of Myricetin Are Not Mediated by Caloric Restriction

It is well-known, that caloric restriction results in a prolongation of lifespan [35,36]. To exclude that the effects of myricetin on lifespan of *C. elegans* were mediated by a restriction in food uptake due to e.g., bitter taste of the compound, we determined food uptake of the nematodes by analysing the pharyngeal pumping activity. Since myricetin-treated nematodes showed no reduction in pharyngeal pumping activity compared to control nematodes (Figure 9A), we conclude that the myricetin induced effects were not due to caloric restriction. Further, this hypothesis is supported by an analysis of the body size of the nematodes, which is another indicator of caloric restriction. Myricetin had also no

effect on the body size of *C. elegans* (Figure 9B). Therefore, we conclude that myricetin exerts its prolonging effect on lifespan independent of caloric restriction.

Figure 9. Effects of myricetin are not mediated by caloric restriction: Myricetin treatment (100 µM) for 2 days (**A**) and 4 days (**B**) did not reduce food uptake (**A**) and body size (**B**) of the nematodes; (**A**) data are mean values ± SEM, $n = 15$ in 3 independent experiments; * $p < 0.05$; unpaired Student's *t*-test; (**B**) data are mean values ± SEM, $n \geq 101$ in 3 independent experiments; $p > 0.05$; unpaired Student's *t*-test.



3. Experimental Section

3.1. C. elegans *Strains and Maintenance*

The strains used in this study were N2 var. Bristol, CF1038 *[daf-16(mu86) I.]*, TJ356 *[zIs356 IV (pdaf-16-daf-16::gfp; rol-6)]* and LD001 *[Is007 (skn-1::gfp; rol-6)]*. Some strains were provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Strains were maintained on nematode growth medium (NGM) agar plates at 20 °C containing a lawn of Escherichia coli var. OP50 (provided by the CGC) as the food source, as described elsewhere [37]. Treatment of *C. elegans* with the test compound was performed in 2 mL of liquid NGM containing 1% (*w*/*v*) bovine serum albumin (Sigma, Deisenhofen, Germany), 50 µg/mL streptomycin (Sigma) and 1×10^9 OP50-1/mL (provided by the CGC) in 35 mm petri dishes (Greiner Bio-One, Frickenhausen, Germany). Myricetin (Extrasynthese, Genay, France) stock solution was prepared with the solvent DMSO (Merck) in a concentration of 100 mM. In all assays, myricetin was used in a final concentration of 100 µM and 0.1% (*v*/*v*) DMSO was used as the solvent control. Age synchronous animals were obtained by bleaching of gravid adults. Briefly, gravid adults were rinsed off NGM agar plates with liquid NGM, collected in 0.5 mL liquid NGM and mixed with 0.5 mL bleaching solution (50% 5 M NaOH/50% NaClO). Worms were then incubated at room temperature for three minutes, occasionally vortexed, pelleted by centrifugation (5000 rpm/4 °C/1 min) and the supernatant was discarded. The worm pellet was washed three times with liquid NGM and transferred to fresh NGM agar plates (containing OP50 lawn) and maintained for three days at 20 °C to obtain an age synchronous population of mainly L4 larvae.

3.2. In Vivo *Visualization of Myricetin: Fluorescent Staining with 2-Aminoethyl Diphenylborinate*

In vivo visualization of myricetin in *C. elegans* was performed with slight modifications as described elsewhere [20]. Briefly, randomly picked 4 d old young adult animals were placed in liquid NGM ± 100 µM myricetin and 2% heat killed *E. coli* OP50 for the indicated time (0 min, 5 min, 30 min, 1 h, 2 h, 4 h, 6 h and 24 h) at 20 °C. Then, worms were transferred into liquid NGM containing 2% heat killed *E. coli* OP50 and 0.2% (*w*/*v*) 2-aminoethyl diphenylborinate (Naturstoffreagent A, NSRA) (Sigma, Deisenhofen, Germany) for 2 h. The enhanced fluorescence of myricetin in *C. elegans* was detected by fluorescence microscopy (excitation wavelength 460–495 nm; emission wavelength 510–550 nm; Olympus BX43). Experiments were repeated two times.

3.3. Determination of Lipofuscin Accumulation

Over the lifetime of *C. elegans*, the autofluorescent pigment lipofuscin accumulates in gut granules and serves as an established marker of ageing [26]. Randomly picked L4 larvae were placed in liquid NGM ± 100 µM myricetin as described above and incubated for 72 h at 20 °C, followed by 24 h of incubation in compound free medium. During the incubation period, worms were transferred to fresh culture media daily. The lipofuscin fluorescence of seven days old worms was detected by fluorescence microscopy (excitation wavelength 360–370 nm; emission wavelength 420–460 nm; Olympus BX43, Olympus, Hamburg, Germany) and analysed densitometrically (ImageJ, National Institutes of Health, Bethesda, MD, USA). Therefore, the fluorescence of the whole body of each animal (except for head and tail regions) was determined and the background fluorescence was subtracted. The experiment was repeated four times.

3.4. Intracellular Localization of DAF-16::GFP and SKN-1::GFP

Transgenic strain TJ356 *[zIs356 IV (pdaf-16-daf-16::gfp; rol-6)]* was used to detect the intracellular localization of GFP tagged DAF-16 protein. Therefore, embryos of this strain were placed in liquid NGM ± 100 µM myricetin directly after the synchronization procedure and incubated for 72 h at 20 °C. Subsequently, three days old larvae were placed on microscope slides, covered with cover slips and the cellular localization of DAF-16::GFP was detected by fluorescence microscopy (excitation wavelength 460–495 nm; emission wavelength 510–550 nm; Olympus BX43). The experiment was repeated four times. Strain LD001 *[Is007 (skn-1::gfp; rol-6)]* was used to observe intestinal nuclear localization of SKN-1::GFP. Three days old larvae and young adult animals were placed in liquid NGM ± 100 µM myricetin as described above for 24 h at 20 °C. Thereafter, animals were transferred on microscope slides, covered with cover slips and the cellular localization of SKN-1::GFP was detected by fluorescence microscopy (excitation wavelength 460–495 nm; emission wavelength 510–550 nm; Olympus BX43). Experiments were repeated four times.

3.5. Lifespan Assays

Lifespan analyses were performed with N2 and CF1038 *[daf-16(mu86) I]*. About 30–50 L4 larvae per group and experiment were placed in liquid NGM ± 100 µM myricetin as described above and incubated at 25 °C. The starting day in liquid culture was considered as day 0 of the lifespan.

Nematodes were transferred daily to new culture dishes during their fertile period to prevent overcrowding and to discriminate the test nematodes from their progeny. After the fertile period, worms were transferred to fresh medium every other day. Worms were scored as dead when they did not respond to gentle prodding with a flexible glass rod and when they showed no pharyngeal pumping movement. Lost worms and animals containing hatched larvae were censored. Experiments were repeated three times and Kaplan-Meier survival analysis was used to detect statistical differences (IBM SPSS 19).

3.6. Measurement of Intracellular ROS Accumulation in C. elegans

The fluorescent probe H_2DCF-DA (2',7'- dichlorodihydrofluorescein–diacetate; Sigma) was used to detect the intracellular ROS level in living individual nematodes. H_2DCF-DA is able to freely cross cell membranes, however, after entering the cell, non-fluorescent H_2DCF-DA becomes deacetylated to form the non-fluorescent derivative H_2DCF that is trapped within the cell. Then H_2DCF can quickly be oxidized by intracellular ROS to form fluorescent DCF that can be measured in a fluorescence spectrophotometer (excitation wavelength 485 nm; emission wavelength 535 nm). The fluorescence intensity correlates with the intracellular amount of ROS. The experiment was performed as described elsewhere [28]. Briefly, L4 larvae were incubated in liquid NGM ± 100 µM myricetin or 0.1% DMSO for 48 h at 20 °C. During the incubation period, worms were transferred to fresh culture media daily. After 48 h, all animals were transferred into PBST (PBS with 0.1% Tween 20) for one hour. Then single worms were transferred individually in 1 µL PBST into each well of a 384-well plate (384-well µClear® plate, Greiner Bio-One, Frickenhausen, Germany) containing 7 µL PBS. For measurement of the background fluorescence (without nematodes), 8 µL PBS were added into one column of the 384-well plate (16 wells). Subsequently, when all animals were transferred, 2 µL H_2DCF-DA (250 µM in PBS) were added into each well to obtain a final concentration of 50 µM H_2DCF-DA. A black backing tape (Perkin Elmer) was applied to the top of the plate to avoid evaporation. ROS accumulation was induced by thermal stress at 37 °C and recorded every 15 min for a period of 12 h in a fluorescence spectrophotometer (Wallac Victor^2 1420 Multilabel-Counter, Perkin Elmer, Wellesley, MA, USA). The experiment was repeated four times.

3.7. Cell Culture and Measurement of ROS Accumulation in Hct116 Cells

Human colon carcinoma cell line Hct116 was maintained in DMEM high glucose (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat inactivated FCS and 100 U/mL penicillin/100 µg/mL streptomycin (Gibco) at 37 °C and 5% CO_2 in a humidified incubator (Binder, Tuttlingen, Germany).

The fluorescent probe H_2DCF-DA (2',7'- dichlorodihydrofluorescein–diacetate; Sigma), was used to detect intracellular accumulation of ROS. Briefly, Hct116 cells were seeded into 6-well plates with a density of 5×10^5 cells/well and allowed to attach for 24 h. Subsequently, Hct116 cells were treated with 50 µM myricetin or DMSO as solvent control in serum-free DMEM for 4 h. Then, cells were washed with medium, incubated with 10 µM H_2DCF-DA for 15 min, washed again and treated with 500 µM H_2O_2 to induce the generation of ROS. Afterwards, cells were washed with phosphate buffered saline (PBS) and the DCF fluorescence was determined by flow cytometry (Accuri C6, Accuri Cytometers, St. Ives, Cambs, UK).

3.8. Measurement of Fluorescence Emission Spectra

Emission spectra of oxidized DCF (dichlorofluorescein; Sigma) in the presence of myricetin (10 µM) or the equal volumen DMSO (0.01%) as solvent control were determined in a monochromator-based microplate reader (Synergy Mx; BioTek, Bad Friedrichshall, Germany) to exclude quenching effects of the flavonoid in the DCF assay. A solution of oxidized DCF in PBS was prepared, then myricetin or DMSO were added to yield an end-concentration of 10 µM myricetin and 0.01% DMSO, respectively. Samples (100 µL) were transferred into black 96 well plates (Nalge Nunc International, Thermo Fischer Scientific Inc., Waltham, MA, USA) and the fluorescence emission spectra were recorded from 520 to 550 nm (1 nm intervals) with an excitation wavelength of 475 nm ($n = 2$).

3.9. Trolox Equivalent Anti-Oxidative Capacity (TEAC) Assay

The TEAC assay is a cell-free method for the measurement of radical scavenging properties of compounds [38]. The principle of this reaction is the reductive conversion of a stable, blue-green radical by an antioxidant. The solution becomes decolorized when an antioxidant is added and can be quantified photometrically. Substances without antioxidant activities show no decolorization of the radical solution. Thus, the decolorization of the radical solution indicates the anti-oxidative capacity of a compound which is compared to the potency of the reference substance Trolox (Calbiochem, Merck, Darmstadt, Germany), which is a synthetic vitamin E derivative. The radical solution was prepared the day before use and consists of a 1:1 mixture of 4.9 mM APS and 14 mM ABTS (Sigma, Deisenhofen, Germany) and is stored in the dark at room temperature. The absorption of this solution (1.4 at a wavelength of 734 nm) was adjusted by dilution with 80% (*v*/*v*) ethanol. The reference- and test-substances were measured in a concentration range from 0 to 25 µM by mixing 500 µL of the radical solution with 500 µL of the test solution. The radical scavenging activity was measured after two minutes at 734 nm with a spectrophotometer (Lambda 25 UV/VIS Spectrometer, Perkin Elmer, Wellesley, MA, USA). Three independent trials were performed.

3.10. Thermotolerance Assay

Survival of individual nematodes at 37 °C was monitored with an assay described previously [39,40] with slight modifications. After treating L4 larvae for 48 h with 100 µM myricetin or 0.1% DMSO (daily transfer of the animals into fresh culture medium), worms were washed in PBST for 1 h and then individually transferred in 1 µL PBST into the wells of a 384-well plate (384-well µClear® plate, Greiner Bio-One, Frickenhausen, Germany) containing 9 µL PBS. Following the complete transfer of the nematodes, 10 µL of 2 µM SYTOX® Green Nucleic Acid Stain (Molecular Probes Inc., Leiden, The Netherlands) in PBS were added to each well and the plate was sealed using black backing tape (Perkin Elmer, Wellesley, MA, USA) to avoid evaporation. SYTOX® Green Nucleic Acid Stain is unable to pass the membranes of intact cells. However, thermal stress causes an impairment of the cellular membrane, thereby enabling the dye to enter the cells. There the dye binds to DNA and exerts a bright fluorescence that can be used as a marker for cellular damage and thus for the viability of individual nematodes [39]. The fluorescence intensity was determined with a fluorescence spectrophotometer (Wallac Victor^2 1420 Multilabel-Counter, Perkin Elmer Wellesley, MA, USA) and was recorded every

15 min for 12 h (excitation wavelength 485 nm; emission wavelength 535 nm). The fluorescence curve of each nematode was calculated and the individual cut off value was determined by multiplying the average fluorescence of the first four measurements by the factor 3. The time point when the fluorescence exceeded the cut off value for each well was defined as the point of death of the respective nematode. The factor 3 in the calculation of the cut off value was previously shown to be adequate [39]. Survival curves and mean survival times were determined from these individual times of death (Kaplan-Meier survival analysis, IBM SPSS 19). Experiments were repeated at least three times.

3.11. Pharyngeal Pumping Assay

For the uptake of food from the surrounding environment, *C. elegans* permanently shows pharyngeal pumping movements to filter bacteria and discard the remaining fluid. Therefore, the frequency of pharyngeal movements serves as a indicator of the feeding status of *C. elegans*; e.g., *eat-2* mutants suffer from caloric restriction due to a reduced pharyngeal pumping activity [41]. For testing an influence of myricetin on the feeding behavior of the nematodes, wild-type L4 larvae were treated with 100 µM myricetin or 0.1% DMSO in liquid medium for 48 h at 20 °C. During the incubation period, worms were transferred to fresh culture media daily. Subsequent to the treatment, pharyngeal movement of worms was counted for 15 s and repeated three times with the corresponding worm, using a stereo microscope (Stemi 2000-C, Zeiss, Göttingen, Germany). Each experiment was performed with 5 nematodes per group and the experiment was repeated three times.

3.12. Determination of Body Size

About 30 wild-type L4 larvae per experiment and group were randomly selected, transferred into liquid NGM ± 100 µM myricetin and incubated for 96 h at 20 °C. During the incubation period, worms were transferred to fresh culture media daily. Images of individual nematodes were prepared (Olympus BX43, Olympus, Hamburg, Germany) and the body size was determined by measuring the area of each worm (ImageJ, National Institutes of Health, Bethesda, MD, USA). The experiment was repeated three times.

3.13. Statistical Analysis

Statistical analysis was performed with SPSS 19 (IBM) and Prism 5 (GraphPad) software and the results are presented as mean ± SD (*in vitro* experiments) and mean ± SEM (*in vivo* experiments). Statistical significance was determined by Student's *t*-test, one-way ANOVA or two-way ANOVA with Bonferroni post-test. Lifespan analysis was performed using Kaplan-Meier survival analysis; animals that were lost, killed or showed internal hatching were censored. The minimum level of significance was set to $p < 0.05$.

4. Conclusions

The lifespan prolongation in *C. elegans* caused by myricetin is associated with anti-oxidative effects, including reduced accumulation of ROS and a decrease in lipofuscin aggregates but not with a reduction in food uptake (caloric restriction). The effect of myricetin on lifespan was completely abolished in a *daf-16* loss-of-function mutant strain. Consistently, the effect of myricetin on

stress-induced ROS accumulation was also largely blocked in this mutant strain. These results strongly indicate that the effect of myricetin on lifespan in *C. elegans* is dependent on DAF-16 and not mediated by the direct anti-oxidative effects of this flavonoid.

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Conflict of Interest

The authors declare no conflict of interest.

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3.2 Studie 2: Methylated derivatives of myricetin enhance life span in *Caenorhabditis elegans* dependent on the transcription factor DAF-16

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Methylated derivatives of myricetin enhance life span in *Caenorhabditis elegans* dependent on the transcription factor DAF-16†

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Only certain flavonoids have been shown to enhance life span. This was pointed out for *e.g.* myricetin in the nematode *Caenorhabditis elegans*. However, the structural requirements responsible for this effect are not known. We used methylated derivatives of myricetin (laricitrin, syringetin, myricetintrimethylether) to investigate if free OH moieties in the B-ring are necessary for the life span extending effect. In analogy to myricetin, all derivatives increased the life span, decreased oxidative stress (DCF) and decreased the accumulation of lipofuscin. In contrast to myricetin, the methylated compounds strongly enhanced the resistance against thermal stress. Furthermore, treatment with the derivatives induced a much stronger nuclear localization of the DAF-16 transcription factor (FoxO homologue). Additionally, no antioxidant effects and only minor effects on life span prolongation and stress resistance were detectable for the methylated compounds in a DAF-16 deficient nematode strain. Comparable to the dietary flavonoid myricetin, the methylated myricetin derivatives laricitrin, syringetin and myricetintrimethylether strongly enhance the life span of *C. elegans*. Therefore, OH groups of ring B are not necessary for this effect. Only the methylated compounds increase the stress resistance of the nematode which was dependent on DAF-16. These findings suggest that methylation of myricetin increases the biofunctionality.

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1. Introduction

Flavonoids are polyphenolic compounds that occur ubiquitously in food of plant origin. This class of compounds has become increasingly popular in terms of health protection because of their remarkable spectrum of biochemical and pharmacological activities. Flavonoids affect basic physiological functions in mammalian cells such as cell growth, differentiation and apoptosis. Epidemiological studies suggest that dietary flavonoid consumption could protect against various stages of the carcinogenesis process and is associated with a reduced incidence of coronary heart diseases (reviewed by Beecher[1] and Wätjen *et al.*[2]).

Myricetin is a widely distributed flavonol that is found in various plants, including tea, berries, fruit, vegetables, and medicinal herbs. It has been reported that this compound has a chemopreventive effect on skin carcinogenesis[3] and protective effects against UVB-induced photodamage. Myricetin is suggested to possess anti-aging properties that might be helpful for skin care due to inhibition of UVB-induced intracellular hydrogen peroxide production, lipid peroxidation and JNK activation.[4] Ono *et al.*[5] postulated that myricetin also exhibits neuroprotective properties since it inhibits amyloid β (Aβ) aggregation and blocks Aβ oligomerization.

In a previous study we have shown that the dietary flavonoid myricetin increases the mean life span of *C. elegans* by up to 33%,[6] similar results were reported by Grünz *et al.*[7] Cheng *et al.*[8] postulated anti-aging effects of an extract from *Chamaecyparis obtusa* var. *formosana* leaves which contain the myricetin derivative myricetin-3-*O*-α-rhamnopyranoside: the extract caused protection against oxidative stress in *C. elegans* enhancing the survival rates of the nematodes from 48.3% (DMSO) to 70.1%.

Out of the large group of flavonoids, consisting of more than 6000 different structures,[1] only distinct compounds have been shown to enhance the life span of *C. elegans* (reviewed by Koch *et al.*[9]). Therefore, we used methylated derivatives of myricetin to determine which structural features are responsible for the life prolonging effect: laricitrin, syringetin and myricetintrimethylether are used to investigate if free hydroxyl moieties in the B-ring are necessary for this effect.

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These three compounds were chosen since (i) it was hypothesized that the 2′,3′,4′-OH groups in ring B are primarily relevant for the life span extension induced by myricetin and (ii) due to the relevance of these compounds as ingredients of pharmacologically active herbs which are used in traditionally medicine: syringetin is a component of the leaves of *Zanthoxylum bungeanum Maxim.*[10] laricitrin is found in *Moldenhawera nutans*[11] and myricetrintrimethylether is a constituent of *Bridelia ferruginea* stem bark.[12]

In addition to life span analyses, we also investigated the effect of the methylated derivatives on surrogate markers in the nematode, *e.g.* resistance against thermal stress. Furthermore, antioxidative potential *in vivo*, lipofuscin accumulation, fertility and cellular localization of the DAF-16 transcription factor were analyzed to determine the relevance of free hydroxyl moieties in the B-ring for mediating physiological effects in the nematode.

2. Experimental

2.1 Reagents

Myricetin, syringetin, laricitrin and myricetintrimethylether were purchased from Extrasynthese (Genay, France), SYTOX® Green Nucleic Acid Stain was obtained from Molecular Probes Inc. (Leiden, The Netherlands). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich (Seelze, Germany) unless stated otherwise.

2.2 Cultivation of *C. elegans*

In this study, the following nematode strains were used: N2, CF1038 [*daf-16(mu86) I.*] and TJ356 [*zIs356 IV (pdaf-16-daf-16::gfp; rol-6)*]. N2 was used as wild type strain throughout this publication. Nematodes were maintained at 20 °C on NGM agar plates containing a lawn of *E. coli* var. OP50 according to standard protocols described elsewhere.[13] Flavonoid treatment was performed at 20 °C in 2 ml of liquid NGM, 1% (w/v) bovine serum albumin, 50 μg ml^{-1} streptomycin, 10^9 per ml *E. coli* var. OP50-1 and 100 μM compound or 0.1% DMSO as the solvent control, unless stated otherwise. The nematode and bacterial strains used in this work were provided by the *Caenorhabditis* Genetics Centre, which is funded by the NIH National Centre for Research Resources (NCRR).

2.3 Determination of body size

The body size of randomly selected seven days old nematodes, which have been treated with the phytochemicals (100 μM) in 2 ml of liquid NGM, 1% (w/v) bovine serum albumin, 50 μg ml^{-1} streptomycin, 10^9 per ml *E. coli* var. OP50-1 for 96 h from the L4 larval stage (daily media change), was determined by preparing images of individual nematodes (Olympus BX 43; Olympus, Hamburg, Germany) and measuring the area of each animal (ImageJ, National Institutes of Health, Bethesda, MD, USA).

2.4 Intracellular flavonoid content

(a) Fluorescent staining with 2-aminoethyl diphenylborinate. 2-Aminoethyl diphenylborinate is a chelating agent that is able to enhance the weak autofluorescence of certain flavonoid compounds. *In vivo* visualization of myricetin and its methylated derivatives was performed as described by Grünz *et al.*[7] with slight modifications described in Büchter *et al.*[6] Briefly, randomly collected 4 d old young adult nematodes were transferred in liquid NGM containing 1% (w/v) bovine serum albumin and phytochemicals (100 μM) or 0.1% DMSO and 2% heat killed *E. coli* var. OP50-1 for 30 min at 20 °C. Then, the animals were transferred into liquid NGM containing 2% heat inactivated *E. coli* var. OP50 and 0.2% (w/v) 2-aminoethyl diphenylborinate (Naturstoffreagent A, NSRA) (Sigma) for 2 h. The enhanced fluorescence of the phytochemicals in *C. elegans* was detected by fluorescence microscopy (excitation wavelength 460–495 nm; emission wavelength 510–550 nm; Olympus BX43).

(b) HPLC analysis. Wild type nematodes (N2) were age synchronized by hypochlorite/NaOH treatment of gravid adults. The resulting nematode embryos were evenly dispensed on NGM-agar plates containing a lawn of *E. coli* OP50 bacteria and maintained at 20 °C for three days. Prior to the treatment with 0.1% DMSO or 100 μM myricetin, L4 larvae and young adults were washed off the plates with liquid NGM, collected in 15 ml tubes (Greiner Bio-One, Frickenhausen, Germany) and were left for a couple of minutes to settle to the bottom of the tube. The supernatant was then discarded and the nematodes were transferred in 500 μl of liquid NGM into 200 ml Erlenmeyer flasks containing 10 ml of the incubation media (liquid NGM, 100 μM myricetin or 0.1% DMSO, 1% (w/v) bovine serum albumin, 50 μg ml^{-1} streptomycin, 10^9 per ml *E. coli* var. OP50-1). The treatment was conducted at 20 °C for 1 h, 6 h or 24 h, respectively.

After the incubation period, animals were collected in 15 ml tubes to settle and a 500 μl aliquot containing the nematodes was transferred into 2 ml cups. Then, the animals were washed as follows: the first step was performed with dH_2O, followed by two steps with PBST (phosphate buffered saline/0.01% Tween 20), one step with PBS and lastly with dH_2O. Subsequently, the supernatant was aspirated and 500 μl of worm sample was mixed with 1.5 ml of methanol and directly stored at −20 °C overnight.

Afterwards, nematodes were transferred into homogenisation tubes containing 0.5 mm glass beads (Precellys, Bertin Technologies, France) and homogenized for 1 min at maximum force (Minilys, Bertin Technologies, France). The samples were transferred into 2 ml reaction cups and centrifuged at 13 000*g* for 10 min at 4 °C. Supernatants were collected and the remaining worm debris in the homogenisation tubes was extracted three more times with methanol, followed by centrifugation. All methanolic extracts were combined and stored at −20 °C. One 2 ml sample of worms that was previously not treated with myricetin was spiked with 100 μM myricetin after the washing steps, but prior to the homogenisation procedure as a control for accuracy of sample preparation.

Prior to HPLC analysis, extracts were concentrated, freeze dried and solved in 0.5 ml or 1 ml MeOH. 5 μl of each sample was injected into a 1260 Infinity Quarternary LC system

(Agilent Technologies, USA) with 150 × 4 mm ID reversed phase column (YMC-Pack ODS-A, YMC Europe GmbH, Germany) using a flow rate of 0.5 ml min^{-1} beginning with 95% solvent A (water/0.1% formic acid) and 5% solvent B (acetonitrile/0.1% formic acid), gradually increasing the concentration of solvent B to 100%. Flavonoid detection was performed at 275 nm.

2.5 Pharyngeal pumping assay

Under laboratory conditions, *C. elegans* is cultured on a monoxenic bacterial diet and the pharynx of the nematodes is permanently pumping to filter bacteria from the surrounding medium. Therefore, the pharyngeal pumping activity is used as a marker of the feeding status of the nematodes. Wild type L4 larvae were treated with the phytochemicals (100 μM) or 0.1% DMSO for two days at 20 °C in 2 ml of liquid NGM, 1% (w/v) bovine serum albumin, 50 μg ml^{-1} streptomycin, 10^9 per ml *E. coli* var. OP50-1 and the culture media were exchanged daily. Afterwards, pharyngeal pumping activity of individual nematodes was counted for 15 seconds and repeated three times for each nematode (Stemi 2000-C; Zeiss, Göttingen, Germany).

2.6 Fertility assay

For the assessment of fertility, 10 wild type L4 larvae per group were transferred into 35 mm petri dishes containing 1.5 ml of liquid NGM, 1% (w/v) bovine serum albumin, 50 μg per ml streptomycin, 10^9 per ml *E. coli* var. OP50-1 and phytochemicals (100 μM) or 0.1% DMSO. Then, nematodes were allowed to lay eggs for seven consecutive days at 20 °C and every 24 h the adult animals were transferred into fresh culture medium. The hatched larvae were counted 48 h post egg laying with a dissecting microscope (Stemi 2000-C; Zeiss, Göttingen, Germany) and the total as well as daily progeny per adult nematode were calculated.

2.7 Trolox equivalent antioxidative capacity (TEAC) assay

An established method for the *in vitro* determination of radical scavenging properties of substances is the TEAC assay. Antioxidants decolorize the blue-green radical solution (equal volumes of 4.9 mM ammoniumperoxodisulfate (Merck, Darmstadt, Germany) and 14 mM ABTS (2,2′-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid)) and this effect is quantified spectrophotometrically at a wavelength of 734 nm two minutes after starting the reaction (Lambda 25 UV/VIS Spectrometer, Perkin Elmer). The synthetic vitamin E derivative trolox (Calbiochem) was used as reference compound.

2.8 *In vivo* determination of thermal-induced ROS accumulation

Measurement of intracellular ROS levels in N2 and CF1038 [*daf-16(mu86) I.*] strains was accomplished by application of the fluorescent probe H_2DCF-DA (2′,7′-dichloro-dihydrofluorescein-diacetate). The increase in DCF fluorescence over time correlates with the intracellular formation of ROS. L4 larvae of the respective strain were treated with the phytochemicals (100 μM) or 0.1% DMSO in 2 ml of liquid NGM, 1% (w/v) bovine serum albumin, 50 μg ml^{-1} streptomycin, 10^9 per ml *E. coli* var. OP50-1 for 2 days (daily exchange of culture media), followed by a washing step in PBST (PBS with 0.1% Tween 20) for one hour. Individual animals were transferred in 1 μl of PBST into the cavities of a 384-well plate (384-well μClear® plate, Greiner Bio-One, Frickenhausen, Germany) containing 7 μl of PBS. Thereafter, H_2DCF-DA (250 μM in PBS) was added to each well, to reach a final concentration of 50 μM and a black backing tape was applied to the top of the plate to avoid evaporation. The generation of ROS was induced by thermal stress (37 °C), the DCF fluorescence was determined every 15 min for 12 as described previously.[6]

2.9 Measurement of lipofuscin accumulation *in vivo*

The autofluorescent pigment lipofuscin is a well-established biomarker of aging across phyla which accumulates in intestinal gut granules of *C. elegans* during aging.[14] For the determination of lipofuscin in *C. elegans*, wild type L4 larvae were treated for 72 h at 20 °C with the phytochemicals (100 μM) or 0.1% DMSO in liquid NGM containing 1% (w/v) bovine serum albumin, 50 μg per ml streptomycin and 10^9 per ml *E. coli* var. OP50-1 (culture media were exchanged daily), followed by 24 h treatment in compound free medium. Fluorescence of seven days old nematodes was determined microscopically (excitation: 360–370 nm; emission: 420–460 nm; Olympus BX43; Olympus, Hamburg, Germany) and images were analyzed densitometrically (ImageJ, National Institutes of Health, Bethesda, MD, USA).

2.10 Thermotolerance assay

Lethal thermal stress (37 °C) was applied to determine the survival of individual nematodes in a semi-automated assay according to Gill *et al.*[15] with slight modifications described in Büchter *et al.*[6] Briefly, L4 larvae/young adults (raised at 20 °C) of the respective strain (N2 and CF1038 [*daf-16(mu86) I.*]) were collected from NGM agar plates containing a lawn of *E. coli* var. OP50 and transferred into petri dishes (35 mm diameter) containing 2 ml of liquid NGM, 1% (w/v) bovine serum albumin, 50 μg per ml streptomycin, 10^9 per ml *E. coli* var. OP50-1 and the phytochemicals (100 μM) or 0.1% DMSO as the solvent control, respectively. The nematodes were treated with the compounds for 2 d at 20 °C and the culture medium was changed daily. Thereafter, nematodes were washed in 2 ml of PBST (PBS containing 0.1% Tween 20) for one hour and individually transferred in 1 μl of PBST into the cavities of a 384-well plate (384-well μClear® plate, Greiner Bio-One, Frickenhausen, Germany) containing 9 μl of PBS. Finally, 10 μl of 2 μM SYTOX® Green Nucleic Acid Stain (Molecular Probes Inc.) were added to each well and the top of the plate was sealed with black backing tape (Perkin Elmer) to avoid evaporation. SYTOX® Green Nucleic Acid Stain (Molecular Probes Inc.) is a dye that is only able to enter cells with impaired cell membranes, due to *e.g.* thermal stress. Within these damaged cells, the dye intercalates into DNA and exerts a bright fluorescence that is recorded every 15 minutes for a period of 12 h. The fluorescence curve of each nematode was calculated and

the individual cut off value was determined by multiplying the average fluorescence of the first four measurements by the factor 3. The time point when the fluorescence exceeded the cut off value for each well was defined as the point of death of the respective nematode.

2.11 Life span analyses

For the life span analyses the strains N2 and CF1038 [*daf-16 (mu86) I.*] were used. Life span analyses were performed at a temperature of 25 °C. L4 larvae and young adult nematodes were randomly picked from NGM agar plates containing a lawn of *E. coli* var. OP50 and transferred into petri dishes (35 mm diameter) containing 2 ml of liquid NGM, 1% (w/v) bovine serum albumin, 50 μg per ml streptomycin, 10^9 per ml *E. coli* var. OP50-1 and the phytochemicals (100 μM) or 0.1% DMSO as the solvent control. The starting day in liquid culture was considered day 0 of the life span. During the fertile period, nematodes were transferred into fresh medium daily, to prevent overcrowding and thereafter animals were transferred every other day. Live and dead animals were assessed by touch provoked movement. Lost nematodes or animals that displayed internal hatching of larvae/protruding internal organs were censored.

2.12 Cellular localization of DAF-16::GFP

Cellular localization of DAF-16 was determined by using the transgenic strain TJ356 [*zIs356 IV. (pdaf-16-daf-16::gfp; rol-6)*]. Therefore, L4 larvae and young adult animals raised at 20 °C on NGM agar plates as previously described, were treated with the phytochemicals (100 μM) or 0.1% DMSO in liquid NGM containing 1% (w/v) bovine serum albumin, 50 μg ml^{-1} streptomycin and 10^9 per ml *E. coli* var. OP50-1 for one hour. Subsequently, the cellular localization of the fusion protein was determined by fluorescence microscopy using a GFP-filter set (Axioskop; Zeiss, Göttingen, Germany).

2.13 Statistical analysis

Statistical analysis of the experiments was accomplished with SPSS 19 (IBM, Armonk, NY, USA) and GraphPad Prism 6.03 (Graphpad Software Inc., La Jolla, USA). The results are presented as mean ± SEM unless stated otherwise. Statistical significances were determined by one-way ANOVA or two-way ANOVA with Bonferroni post-tests unless stated otherwise. Life span and thermotolerance analyses were performed using Kaplan–Meier survival analyses. The minimum level of significance was assumed at $p < 0.05$. All experiments were repeated at least three times.

3. Results

3.1 Prolongation of life span by methylated derivatives of myricetin

To identify structural elements responsible for myricetin-induced life span prolongation, we analyzed if this effect is dependent on the presence of free OH groups in the B-ring:

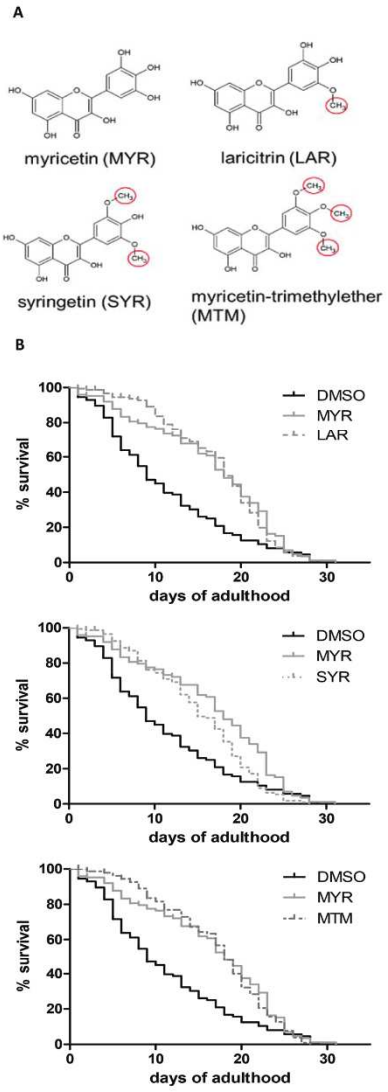

Fig. 1 Prolongation of life span by methylated myricetin derivatives. A: Chemical structures of myricetin, laricitrin, syringetin and myricetintrimethylether. B: Life span analysis of wild type nematodes treated with DMSO (0.1%) or myricetin, laricitrin, syringetin or myricetintrimethylether (100 μM), respectively. The survival was monitored at 25 °C. Kaplan Meier survival analysis with Log Rank test (Mantel–Cox); 165–183 individuals per treatment group in three independent experiments; corresponding data are summarized in Table 1. Nematodes were transferred into fresh medium daily, to prevent overcrowding and thereafter animals were transferred every other day. Live and dead animals were assessed by touch provoked movement. Lost nematodes or animals that displayed internal hatching of larvae/protruding internal organs were censored.

compared to the DMSO-treated nematodes (mean life span 11.2 ± 0.7 days), an incubation with myricetin increased the life span by 48.2% (mean life span 16.6 ± 0.7 days). Treatment of the nematodes with the methylated derivatives resulted in a comparable extension of life span. While syringetin was less potent (mean life span: 15.2 ± 0.56 days = prolongation by 35.7%), the effect of laricitrin (mean life span: 17.3 ± 0.53 days = prolongation by 54.5%) and myricetintrimethylether (mean life span: 17.2 ± 0.59 days = prolongation by 53.6%) was significantly higher than the effect of myricetin (Fig. 1B, Table 1).

The longevity effect was not due to caloric restriction caused by the secondary plant compounds, since the pharyngeal pumping rate as well as the body size were not changed by the compounds (Fig. 2A and B). The extended life span induced by phytochemical treatment was probably also independent of an altered germline signaling, because we were not able to observe a reduced or delayed reproduction (Fig. 2C and D). A HPLC analysis of *C. elegans* that were in contact with the different compounds had been performed but was finally not sufficient to document the *in vivo* modifications and the bioavailability of myricetin and its methylated derivatives.

3.2 Modulation of stress resistance by methylated derivatives of myricetin

Since the methylated derivatives of myricetin showed life span extending effects, we analyzed if these compounds may also modulate the resistance of the nematodes against lethal thermal stress. In accordance with previous studies,[6] the parent compound myricetin had no effect on the stress resistance (mean survival time of DMSO and myricetin-treated nematodes: 3.94 ± 0.09 and 4.01 ± 0.12 h, respectively).

In contrast to the non-methylated compound, all methylated derivatives increased the stress resistance of the nematodes: the mean survival times were 4.51 ± 0.13 h, 4.63 ± 0.13 h and 4.71 ± 0.14 h for laricitrin, syringetin and myricetintrimethylether, respectively (Fig. 3, Table 2).

3.3 Modulation of oxidative stress by methylated derivatives of myricetin

To elucidate molecular mechanisms responsible for the protective effects of the methylated derivatives of myricetin, we

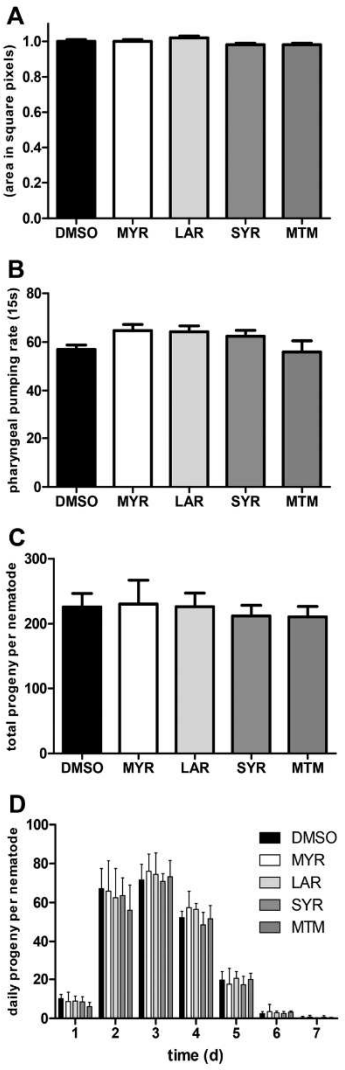

Fig. 2 Food uptake, body size and fertility are not reduced by methylated myricetin derivatives. Effects of myricetin, laricitrin, syringetin and myricetintrimethylether are not mediated by caloric restriction: Treatment with the compounds (100 μM) for 3 days (A) and 2 days (B) did not reduce food uptake (A) and body size (B) of the nematodes. A: data are mean values ± SEM, 101–120 individuals per treatment group in 3 independent experiments; one-way ANOVA with Bonferroni post test; B: data are mean values ± SEM, 15 individuals per treatment group in 3 independent experiments; one-way ANOVA with Bonferroni post test. Compound treatment had no effect on fertility. Total fertility (C) and daily offspring (D) were not changed by compound treatment. Data are mean values ± SD calculated per nematode. 10 adult animals per group in three independent experiments; one-way ANOVA with Dunnett's post test.

Table 1 Life span analyses (wild type nematodes)

N2 (wild type)	Adult survival (d)			
Treatment	Mean (±SEM)	Median (±SEM)	*n* (censored)	*p* value *vs.* DMSO (log-rank)
DMSO	11.2 ± 0.70	9.0 ± 0.87	183 (75)	
MYR	16.6 ± 0.72	18.0 ± 0.95	172 (77)	<0.001
LAR	17.3 ± 0.56	18.0 ± 0.60	165 (56)	<0.001
SYR	15.2 ± 0.56	15.0 ± 0.92	165 (50)	0.002
MTM	17.2 ± 0.59	18.0 ± 0.57	168 (63)	<0.001

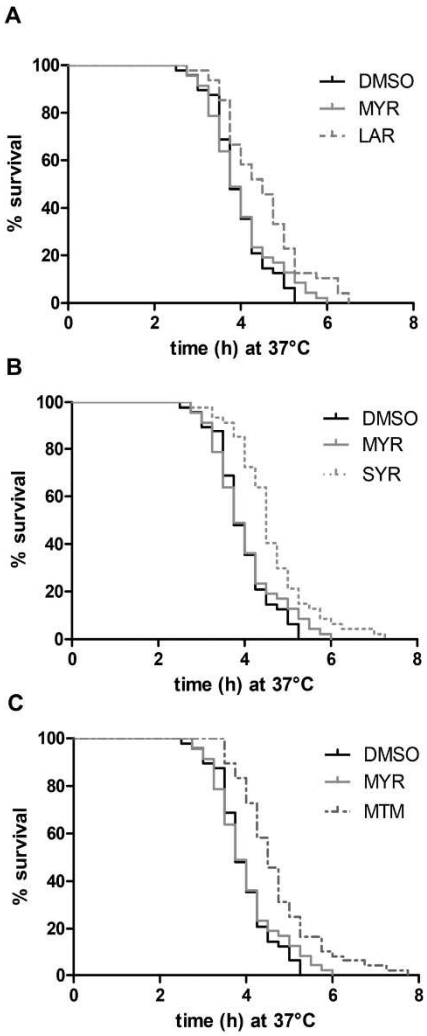

Fig. 3 Modulation of stress resistance by methylated myricetin derivatives. Wild type nematodes were treated for 2 d with myricetin, laricitrin (A), syringetin (B) or myricetintrimethylether (C) (100 μM) in liquid NGM starting at the L4 larval stage. Subsequently, single nematodes were transferred into the wells of a 384 well plate containing 1 μM SYTOX® Green nucleic acid stain and the survival at 37 °C was monitored by an increase in fluorescence. Kaplan Meier survival analysis with Log Rank test (Mantel–Cox); 47–48 individuals per treatment group in three independent experiments. The corresponding data are summarized in Table 2.

analyzed their effects on the amount of intracellular ROS formation since antioxidative effects are suggested to be a key mechanism of flavonoid action.

Table 2 Resistance against thermal stress (wild type nematodes)

N2 (wild type)	Survival at 37 °C (h)			
Treatment	Mean (±SEM)	Median (±SEM)	*n* (censored)	*p* value *vs.* DMSO (log-rank)
DMSO	3.9 ± 0.09	3.75 ± 0.11	48 (0)	
MYR	4.0 ± 0.12	3.75 ± 0.13	47 (0)	0.473
LAR	4.5 ± 0.13	4.5 ± 0.22	48 (0)	0.001
SYR	4.6 ± 0.13	4.5 ± 0.08	47 (0)	<0.001
MTM	4.7 ± 0.14	4.5 ± 0.13	48 (0)	<0.001

We first analyzed the effect of the methylated derivatives on the accumulation of lipofuscin, an auto-fluorescent aging pigment consisting of molecular aggregates of highly oxidized proteins and lipids. It is known that antioxidative compounds are capable of reducing the formation of these toxic aggregates. This is true for all compounds analyzed: myricetin reduced the lipofuscin formation by 33.1% (Fig. 4A), laricitrin has a similar effect (35.4%). Syringetin and myricetintrimethylether caused an even stronger reduction of the lipofuscin fluorescence than myricetin (46.1% and 55.6%). We further analyzed the antioxidative effects of the compounds more directly in the DCF assay: thermal stress increased ROS formation in the nematodes dramatically: a 50-fold increase in DCF fluorescence after 6 h was detectable compared to the DCF fluorescence at t = 0 h. A pre-incubation with myricetin reduced the amount of DCF fluorescence by 24.2% (3 h value: 30 287 ± 1507 rfu compared to 41 048 ± 2032 rfu of the control value) (Fig. 4B). This reduction of intracellular ROS is comparable to the results obtained with the methylated derivatives syringetin and myricetintrimethylether (3 h values: 30 040 ± 2201 rfu and 32 924 ± 2196 rfu, respectively). However, laricitrin showed a significantly higher antioxidative capacity compared to the non-methylated flavonoid myricetin (3 h value: 24 228 ± 801 rfu).

To investigate the molecular mechanism of the antioxidative effects, we analyzed the radical scavenging capacity in a cell-free assay (Fig. 4C): compared to the synthetic vitamin E derivative trolox which was used as a positive control (IC_{50} value 16 μM), myricetin exhibited a much higher antioxidative capacity (IC_{50} value: 3.8 μM). Also laricitrin and syringetin showed potent antioxidative capacities in this experimental system (IC_{50} values of 6.5 and 12.3 μM, respectively), only in case of myricetintrimethylether a slightly lower antioxidative capacity was detectable (IC_{50} value 23.2 μM).

3.4 Modulation of DAF-16 translocation by methylated derivatives of myricetin

Since the direct antioxidative capacities of the flavonoids detected in the TEAC assay only poorly correlate with the results observed in the DCF assay and the lipofuscin accumulation, we analyzed if the antioxidative effects of the flavonoids are mediated indirectly by a modulation of redox-active signal transduction pathways. We analyzed the effects of the com-

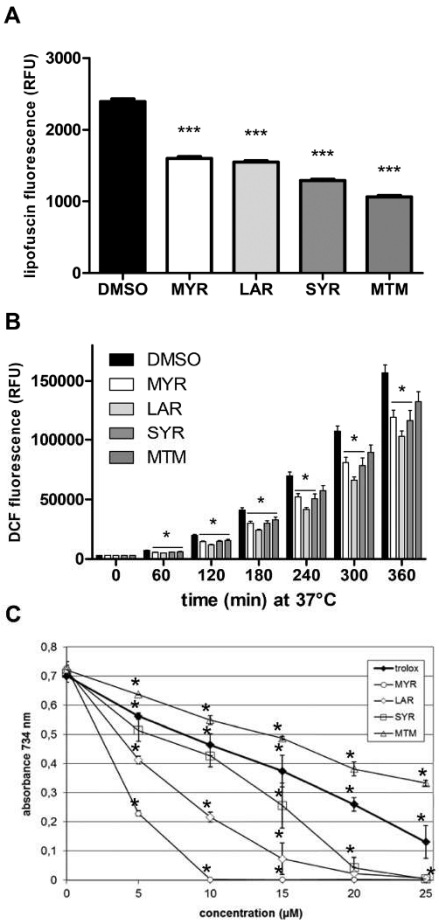

Fig. 4 Antioxidative effects of methylated myricetin derivatives. Treatment of wild type *C. elegans* with myricetin, laricitrin, syringetin or myricetintrimethylether (100 µM) for three days (lipofuscin accumulation) and two days (ROS accumulation), respectively. A: Myricetin and the methylated derivatives attenuate lipofuscin accumulation; data are mean values ± SEM; 90–109 individuals per treatment group in 4 independent experiments; ***: $p < 0.0001$; one-way ANOVA, Bonferroni post test. B: Modulation of ROS accumulation at 37 °C; the fluorescence intensity of DCF (rfu) was taken as a marker for intracellular ROS; data are mean values ± SEM, 64 individuals per treatment group in 4 independent experiments; *: $p < 0.05$, one-way ANOVA with Bonferroni post test. C: Radical-scavenging potential of the myricetin derivatives was analyzed in the TEAC assay using the vitamin E derivative trolox as a reference compound. The reduction of absorption correlates with radical scavenging activity. Data are mean ± SD, $n = 3$, *: $p < 0.05$; one-way ANOVA, Bonferroni post test.

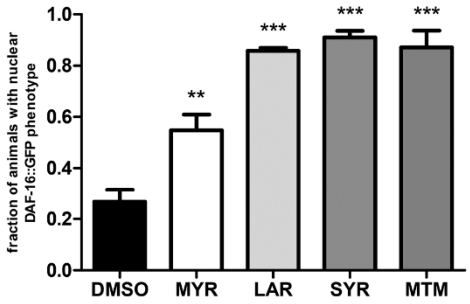

Fig. 5 Modulation of DAF-16 translocation by methylated myricetin derivatives. Methylated myricetin derivatives induced nuclear translocation of DAF-16::GFP. Animals (strain TJ356) were treated with DMSO (0.1%) or myricetin, laricitrin, syringetin or myricetintrimethylether (100 µM) for one hour. Subsequently, determination of the GFP-localization phenotype (nuclear/cytosolic) was performed by fluorescence microscopy. Data are mean ± SEM, 135–154 individuals per treatment group in 3 independent experiments; ***, $p < 0.0001$ *vs.* corresponding DMSO value; one-way ANOVA with Bonferroni post test.

pounds on the insulin-like signaling pathway in *C. elegans* because this pathway is strongly involved in the antioxidative defense of the nematode, *e.g.* by induction of antioxidative gene products like SOD-3. In *C. elegans*, the transcription factor DAF-16 is the only FOXO homolog and the main effector of the insulin-like signaling pathway. We used transgenic nematodes to analyze the effect of the compounds on the cellular localization of this transcription factor (Fig. 5). In DMSO-treated animals, DAF-16 was located mainly cytosolic; an incubation with myricetin led to a twofold increase in the amount of nuclear localized DAF-16 (55 ± 8% *vs.* 27 ± 7%), which is a prerequisite for DAF-16 transcriptional activity. However, treatment with the methylated derivatives further enhanced the translocation of this transcription factor: treatment of the nematodes with syringetin, laricitrin and myricetintrimethylether resulted in a nuclear localization of DAF-16 by 91 ± 3%, 86 ± 3% and 87 ± 9%, respectively. Since these effects were significantly higher compared to the effects of myricetin, we assume that the methylated derivatives exert, at least in parts, a different molecular mechanism.

3.5 Involvement of DAF-16 in the physiological effects of the methylated derivatives of myricetin

Since the methylated derivatives resulted in a very prominent nuclear localization of the transcription factor DAF-16, we used a DAF-16 deficient strain to analyze if the molecular effects caused by these compounds are dependent on this factor. The protective effects against thermal stress (SYTOX assay) induced by the methylated compounds laricitrin and syringetin are completely abolished in the DAF-16 mutated strain (Fig. 6A and B, Table 3). Compared to the DMSO-treated control (mean survival time: 3.8 ± 0.12 h), myricetin, laricitrin and syringetin caused no prolongation of the survival time (3.7 ± 0.09,

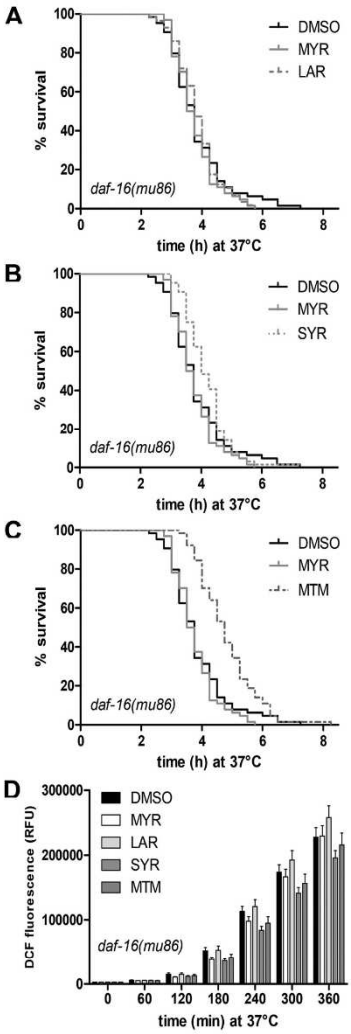

Fig. 6 Effects of methylated myricetin derivatives on stress resistance and antioxidative effects in a DAF-16 deficient strain. A, B, C: Age-synchronized *daf-16(mu86)* L4 larvae were incubated for two days with 100 μM of the compounds in liquid medium. After a washing step in compound-free medium, single nematodes were placed in the wells of a 384 well plate containing 1 μM SYTOX® Green nucleic acid stain, temperature was increased to 37 °C and the time points of death were calculated by the increase in fluorescence. Kaplan Meier survival analysis with Log Rank test (Mantel–Cox); 57–64 individuals per treatment group in 4 independent experiments. Corresponding data are summarized in Table 3. D: Treatment of a *C. elegans* strain bearing the *daf-16(mu86)* loss of function mutation with myricetin, laricitrin, syringetin or myricetintrimethylether (100 μM) for two days modulates ROS accumulation at 37 °C; the fluorescence intensity of DCF (rfu) was taken as a marker for intracellular ROS; data are mean values ± SEM, 48 individuals per treatment group in 3 independent experiments; one-way ANOVA with Bonferroni post test.

Table 3 Life span analyses and resistance against thermal stress (DAF-16 deficient nematodes)

CF1038 (*daf-16(mu86)*)	Adult survival (d)			
Treatment	Mean (±SEM)	Median (±SEM)	*n* (censored)	*p* value *vs.* DMSO (log-rank)
DMSO	10.7 ± 0.34	11.0 ± 0.32	161 (31)	
MYR	11.0 ± 0.39	12.0 ± 0.21	158 (35)	0.248
LAR	11.0 ± 0.38	11.0 ± 0.51	155 (13)	0.294
SYR	12.3 ± 0.33	12.0 ± 0.41	156 (26)	0.001
MTM	10.9 ± 0.45	11.0 ± 0.53	162 (11)	0.131
CF1038 (*daf-16(mu86)*)	Survival at 37 °C (h)			
Treatment	Mean (±SEM)	Median (±SEM)	*n* (censored)	*p* value *vs.* DMSO (log-rank)
DMSO	3.8 ± 0.12	3.75 ± 0.11	64 (0)	
MYR	3.8 ± 0.09	3.5 ± 0.09	64 (0)	0.403
LAR	3.9 ± 0.10	3.75 ± 0.11	57 (0)	0.901
SYR	4.2 ± 0.09	4.0 ± 0.14	64 (0)	0.066
MTM	4.8 ± 0.12	4.75 ± 0.14	64 (0)	<0.001

3.8 ± 0.1 and 4.18 ± 0.09 h, respectively). Myricetintrimethylether is the only compound that still prolongs the survival time (mean survival time: 4.81 ± 0.12 h) (Fig. 6C). The necessity of this transcription factor is also obvious for the antioxidative effects *in vivo*: none of the compounds was able to reduce the DCF fluorescence significantly 3 h after application of thermal stress in *daf-16(mu86)* (Fig. 6D).

3.6 Involvement of DAF-16 in the life span extending effects of the methylated derivatives of myricetin

In analogy to the results obtained in the SYTOX and DCF assay, we further performed a life span analysis of the compounds in the DAF-16 deficient strain. In contrast to the results obtained with the wild type nematodes, myricetin, laricitrin (Fig. 7A) and myricetintrimethylether (Fig. 7C) did not increase the life span. Only in case of syringetin (Fig. 7B) a slightly enhanced life span was still detectable (12.3 ± 0.33 days *vs.* 10.7 ± 0.34 days of the DMSO-treated control). However, compared to the increase in life span in wild type nematodes (35.7% ± 0.55%), the increase in the DAF-16 mutated strain was only 14.9% (Table 3).

4. Discussion

Since the population in Western countries is getting more susceptible for age-related diseases, there is an urgent need for anti-aging compounds in terms of health preservation. Life prolongation in *C. elegans* was shown for various secondary plant compounds, *e.g.* 10-*O-trans-p*-coumaroylcatalpol,[16] quercetin,[17] caffeine,[18] baicalein[19] and monascin.[20]

Because these compounds show a great structural diversity, we used *C. elegans* as a simple model organism to determine molecular parameters which are at least in parts responsible

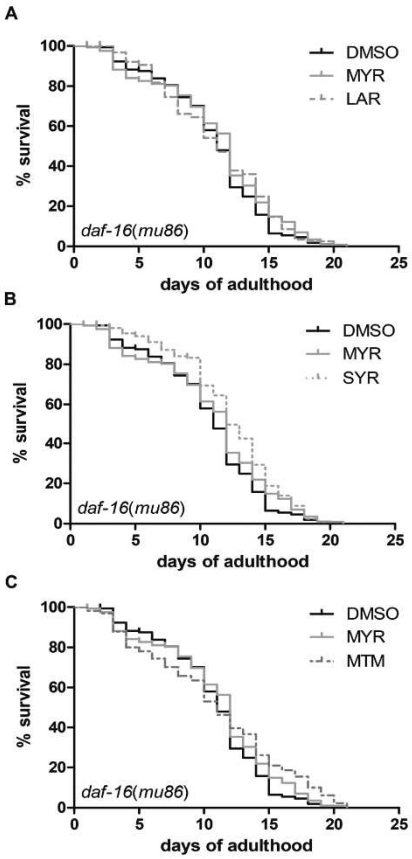

Fig. 7 Effects of methylated myricetin derivatives on life span in a DAF-16 deficient strain. L4 larvae of the strain CF1038 *(daf-16(mu86))* were transferred into liquid NGM containing DMSO (0.1%) or 100 μM of myricetin, laricitrin (A), syringetin (B) or myricetintrimethylether (C). The survival was monitored at 25 °C. Kaplan Meier survival analysis with Log Rank test (Mantel–Cox); 157–165 individuals per treatment group in three independent experiments; the corresponding data are summarized in Table 3.

for the prolongation of life span. This structure–activity relationship analysis was performed with myricetin, a flavonoid which was previously reported to cause a significant extension of life span of this model system.[6,7]

In this study, the prolongation of life span mediated by myricetin is in a range comparable to previously reported effects.

We now analysed the effect of methylated derivatives of myricetin (laricetrin, syringetin and myricetintrimethylether) in the nematode. In general, methylated flavonoids have promising physiological properties compared to the non-methylated flavonoids: methylation of free phenolic hydroxyl groups leads to derivatives not susceptible to glucuronic acid or sulfate conjugation, resulting in increased metabolic stability.[21] Methylation also leads to greatly improved transport through biological membranes, such as in intestinal absorption, and much increased oral bioavailability.[21] However, methylation of flavonoids also results in derivatives with increased biological activity, *e.g.* they possess an intrinsic ability to inhibit cancer cell proliferation.[22] Furthermore, Walle *et al.* (2007)[22] demonstrated that fully methylated flavones can be more potent inhibitors of cancer cell proliferation than their corresponding nonmethylated analogs. They concluded that the effects of methylated flavonoids appear to be selective for cancer *versus* noncancer cells and their mode of inhibition appears to be distinctly different from non-methylated flavonoids.[22]

We were able to show that the methylated derivatives extend life span similar to the non-methylated parent compound. The lack of physiological relevance of the free OH groups was surprising since it was hypothesized that the OH groups at position 2′,3′,4′ are at least in parts responsible for the life span prolonging effect of myricetin.[9]

Moreover, the effects of the methylated derivatives were even more pronounced compared to myricetin: laricitrin showed a higher antioxidative potential in the DCF assay, myricetintrimethylether was more potent preventing the lipofuscin accumulation and all methylated compounds resulted in a higher amount of nuclear DAF-16 compared to myricetin. In the case of resistance against thermal stress, all methylated compounds caused a significant protection while myricetin had no protective effect.

These results clearly show that the methylated derivatives had a similar or even higher potency to protect the nematodes. This may be explained with the importance of bulky groups (OCH_3 > OH) in position 2′, 3′and 4′of the B-ring of the flavonoid structure.[21] It has to be suggested, that the methylated compounds modulate physiological parameters, *e.g.* stress resistance and DAF-16 translocation, by a different mechanism than the non-methylated parent compound myricetin.

Since treatment with the methylated compounds results in a high percentage of nuclear DAF-16, which is a prerequisite of DAF-16 activity, we analyzed if the effect of the derivatives is dependent on this transcription factor. However, experiments with a DAF-16 deficient strain show that DAF-16 is not generally responsible for the effects induced by the methylated derivatives. While all methylated derivatives of myricetin induce a reduction in the amount of ROS that depends on DAF-16, the increased thermotolerance induced by myricetintrimethylether as well as the prolonged life span induced by syringetin seem to be independent of DAF-16. These findings suggest, that the methylated derivatives may modulate other aging- or stress-related pathways in the nematode, too.

Since the methylated derivatives of myricetin show more potent effects *e.g.* on DAF-16 translocation, it may be suggested that this is caused by an increased uptake of the methylated derivatives in the nematode followed by a demethylation. However, due to a previous study of Surco-Laos *et al.*[23] this

seems to be unlikely. Surco-Laos *et al.*[23] compared the uptake of quercetin in comparison to its methylated derivatives isorhamnetin (quercetin 3′-*O*-methylether) and tamarixetin (quercetin 4′-*O*-methylether) in *C. elegans*. They showed that after treatment of the nematodes with the methylated derivatives (200 μM), only small amounts of quercetin were detectable. On the other hand, glycosylation and methylation processes occur in the nematode: after quercetin treatment, high amounts of conjugated metabolites were detectable.

Quantification of the levels of quercetin, isorhamnetin and tamarixetin, as well as their detected metabolites indicated a greater accumulation of quercetin (approximately 50%) than its methylated derivatives by the nematode.

Since the fluorescence signal of the flavonoid-NSRA complex allows qualitative rather than quantitative assumptions of flavonoid uptake, it cannot be concluded that the bright fluorescence of the myricetin-NSRA complex shown in ESI Fig. S1,† is correlated with the highest uptake of the compound by the nematodes. A quantification of the flavonoid content *via* HPLC was not successful due to degradation processes of the myricetin/myricetin derivatives during the extraction of the nematodes.

5. Conclusions

We conclude that the methylated myricetin derivatives positively modulate *C. elegans*' health span and these effects are in parts even more prominent than the effects induced by myricetin. However, the molecular mechanisms that are responsible for and transduce the observed effects of the methylated compounds seem to be different in comparison to the parent compound. This means that methylation of distinct OH groups, *e.g.* by the catechol-*O*-methyltransferase (COMT) alters the biofunctionality of the phytochemical. In contrast to various reports demonstrating a reduction or inactivation of biofunctionality (*e.g.* in case of antioxidative capacity, toxicity) by methylation, we clearly show an increase in stress resistance by the methylated compounds.

Acknowledgements

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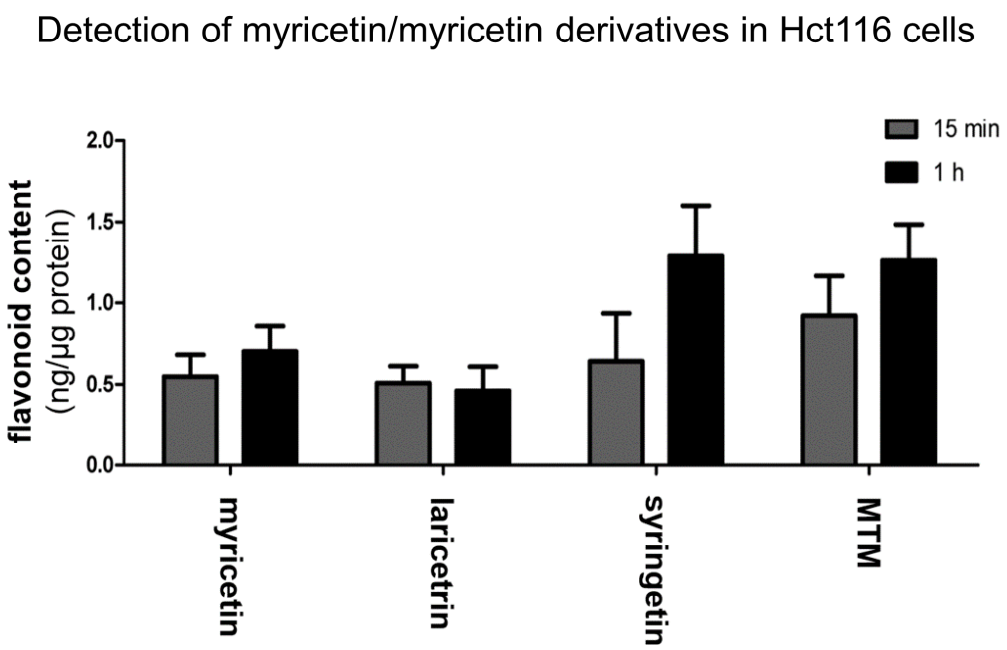
Electronic Supplementary Material (ESI) for Food & Function.

Supplementary material for reviewer 1

a) Detection of myricetin/myricetin derivatives in cells



Quantification of myricetin and its metabolites in Hct116 human colon carcinoma cells.

$1x10^6$ Hct116 cells were plated in 60 mm cell culture dishes and allowed to attach for 48 h. Subsequently medium was changed and cells were allowed to grow for further 24 h. Then 50 µM myricetin, laringetin, syrengetin, MTM were added. After 15 min/1 h of incubation time, cell culture medium was removed and the cells were washed three times with PBS containing 1% bovine serum albumin. The cell pellet was dissolved in 300 µl methanol and lysed by sonification. Afterwards, samples were centrifuged and 20 µl of the supernatant was injected into a HPLC system coupled to a UV detector. The flavonoid content was normalized to the protein amount of the cell pellet (Lowry).

b) Detection of myricetin/myricetin derivatives in *C. elegans*

Detection of myricetin/myricetin derivatives in C. elegans (representative chromatograms)

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                  für XAN & AG Wätjen Biozentrum

Additional Info : Peak(s) manually integrated
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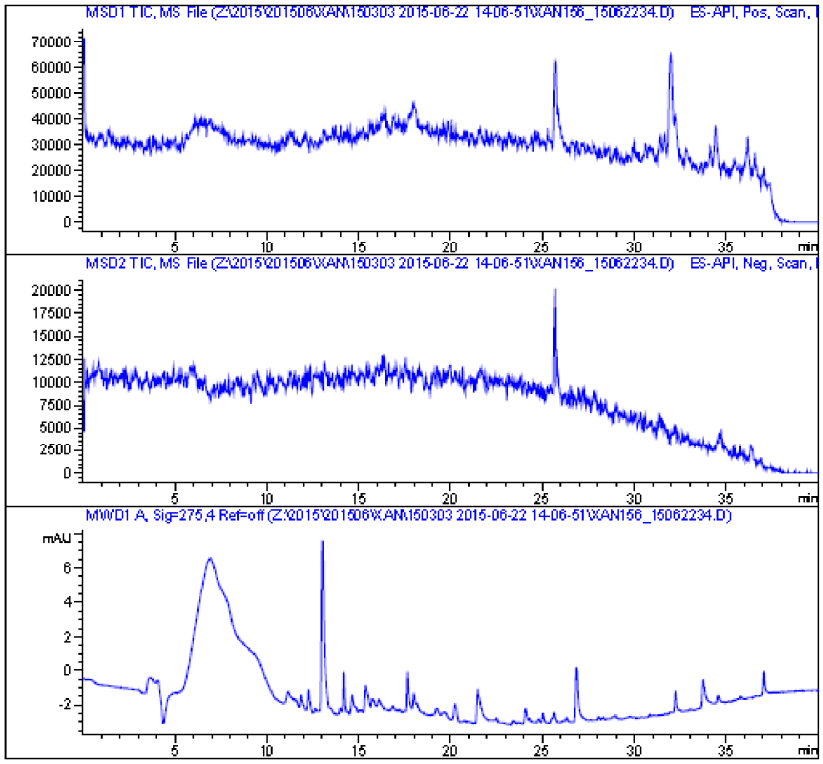


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                      für XAN & AG Wätjen Biozentrum

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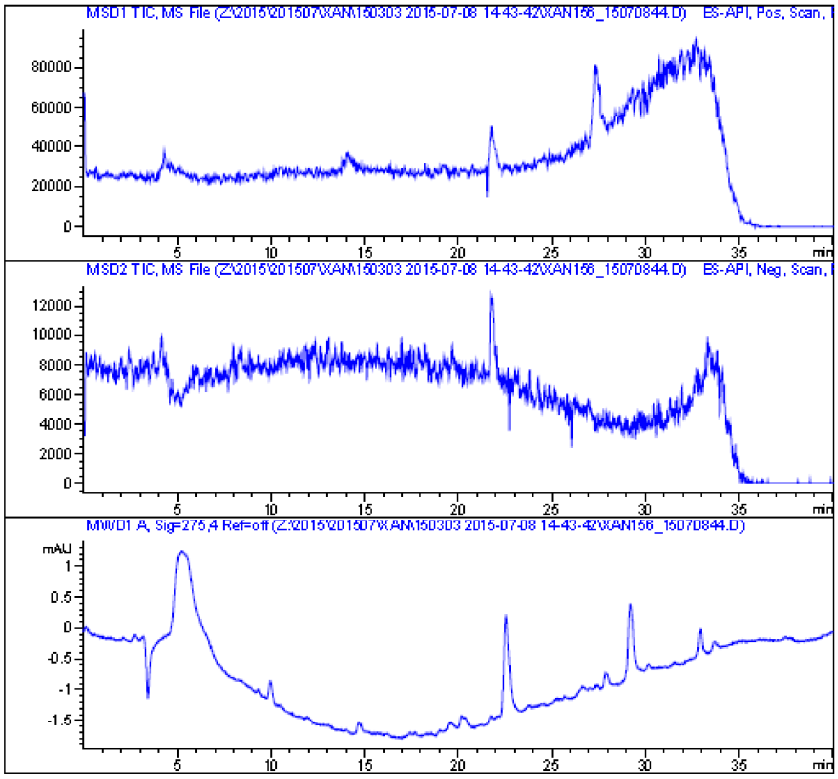


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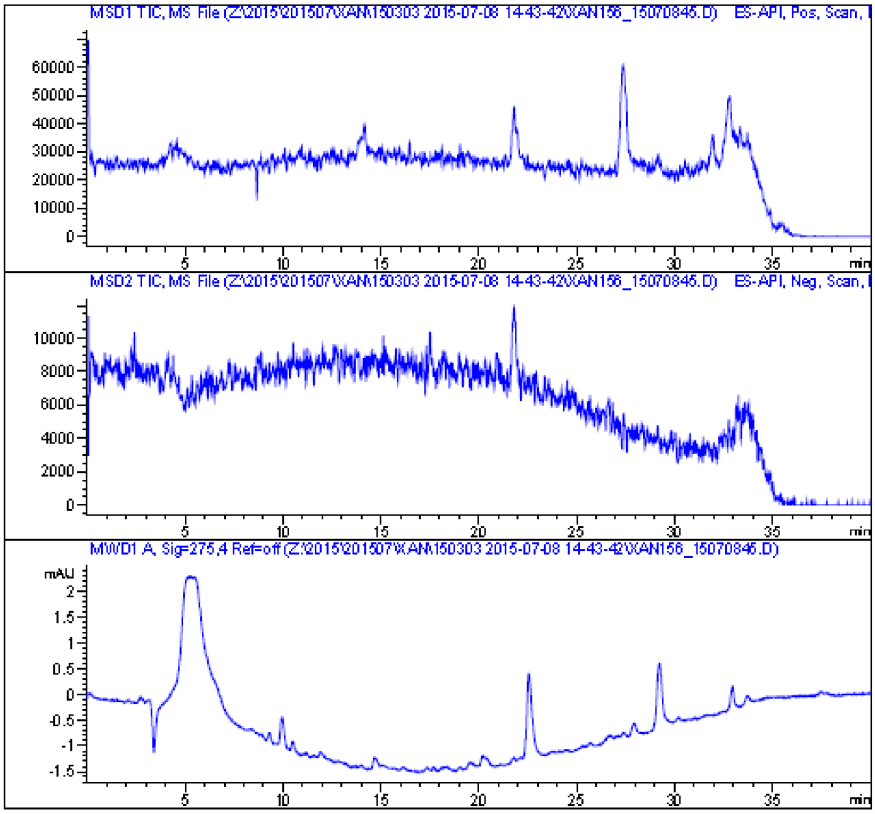
Additional Info : Peak(s) manually integrated



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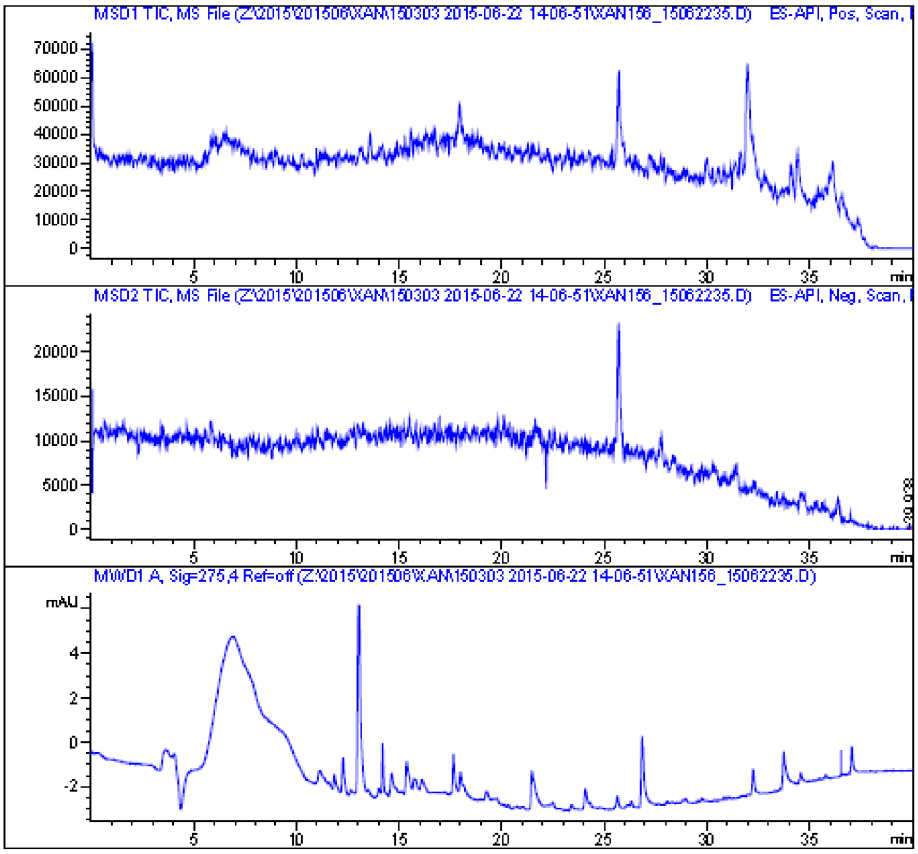


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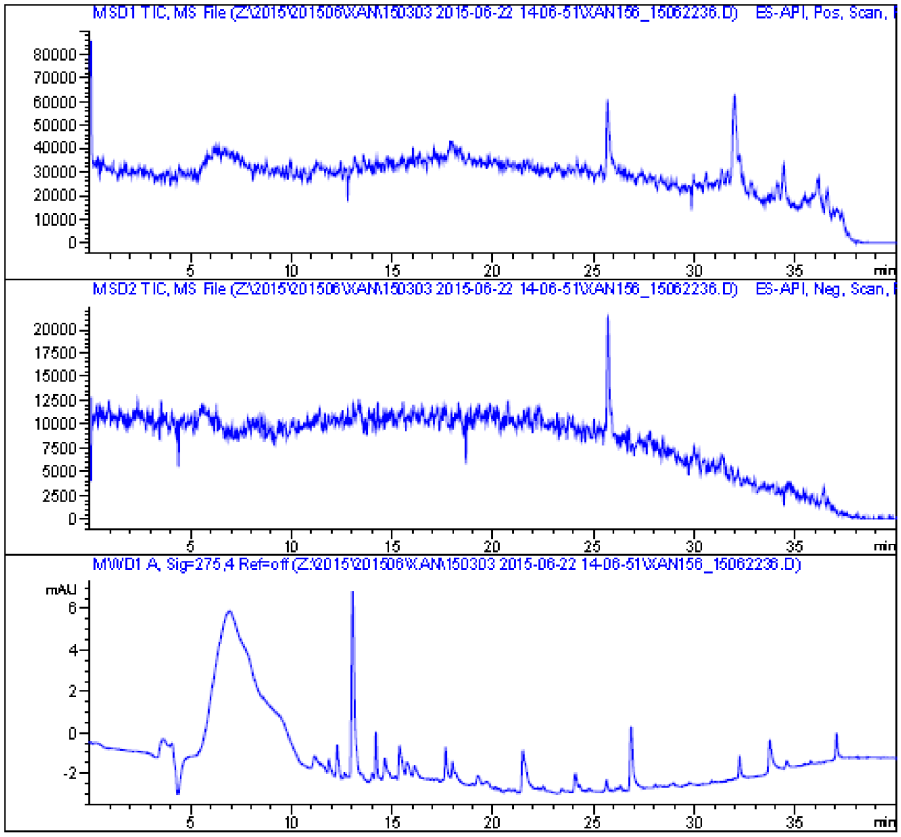


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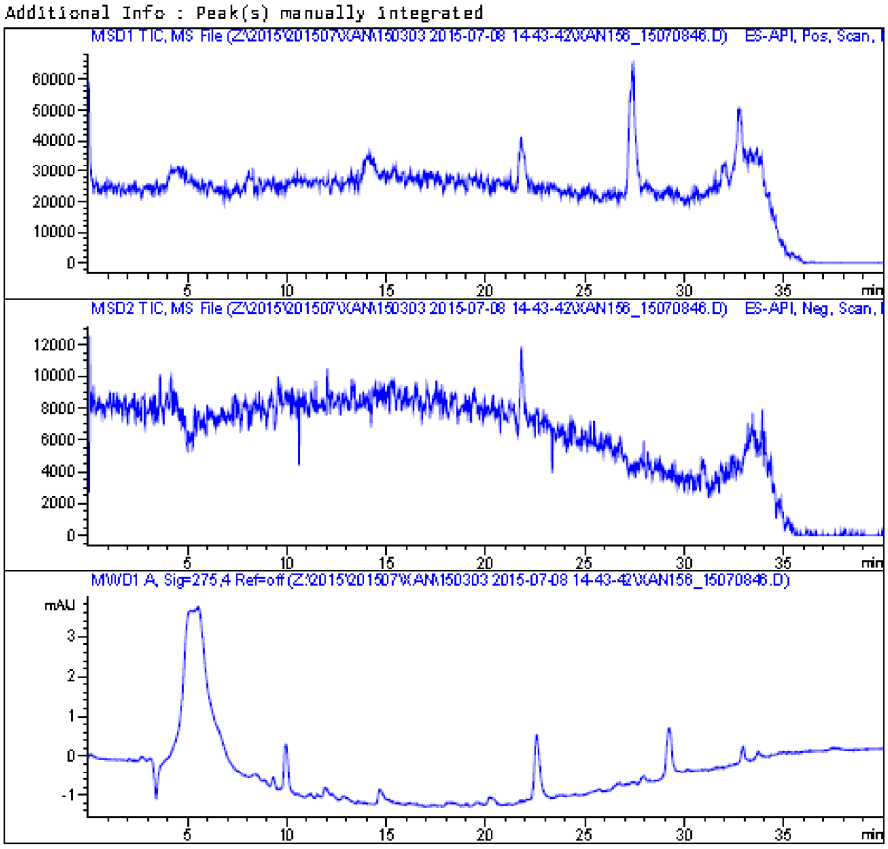


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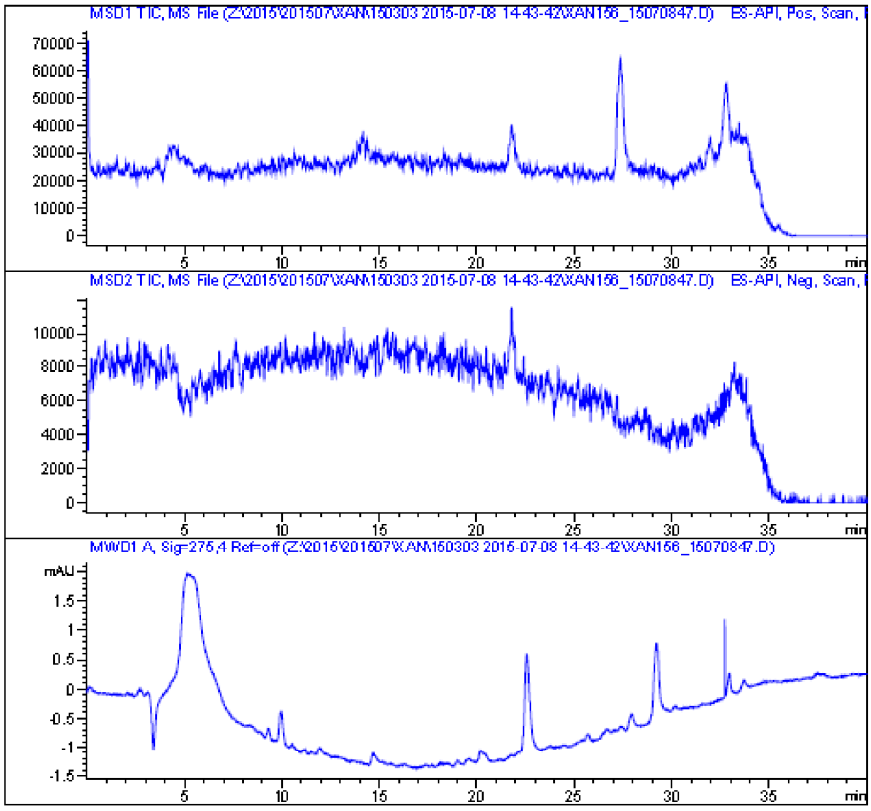


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für XAN & AG Wätjen Biozentrum

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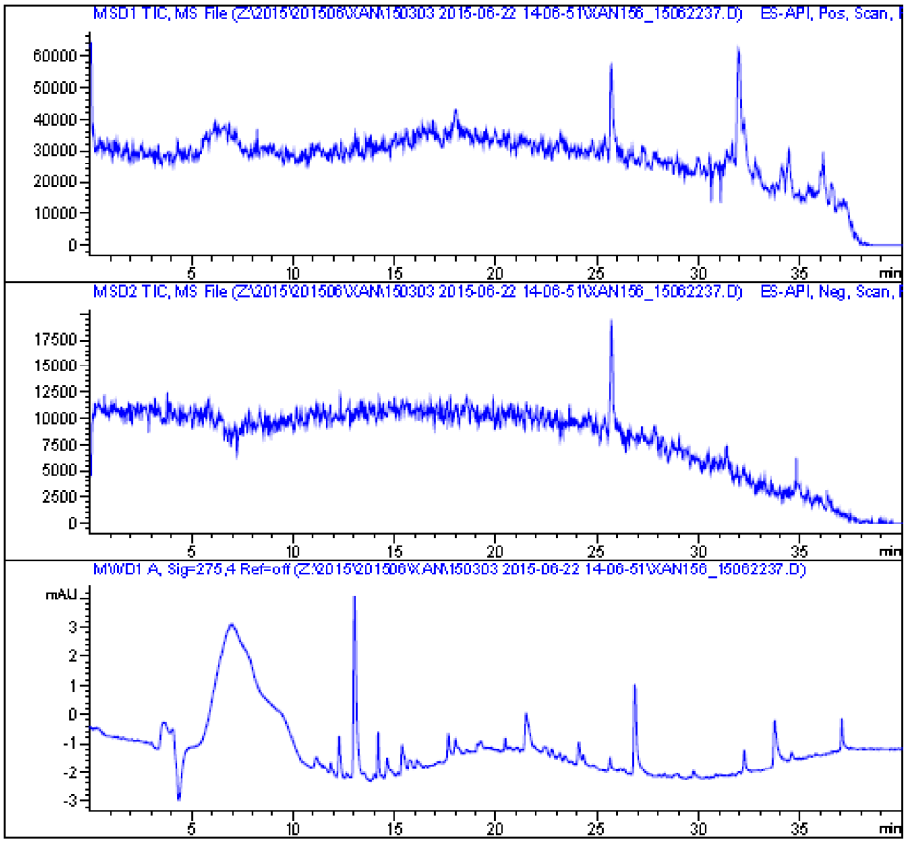

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für XAN & AG Wätjen Biozentrum

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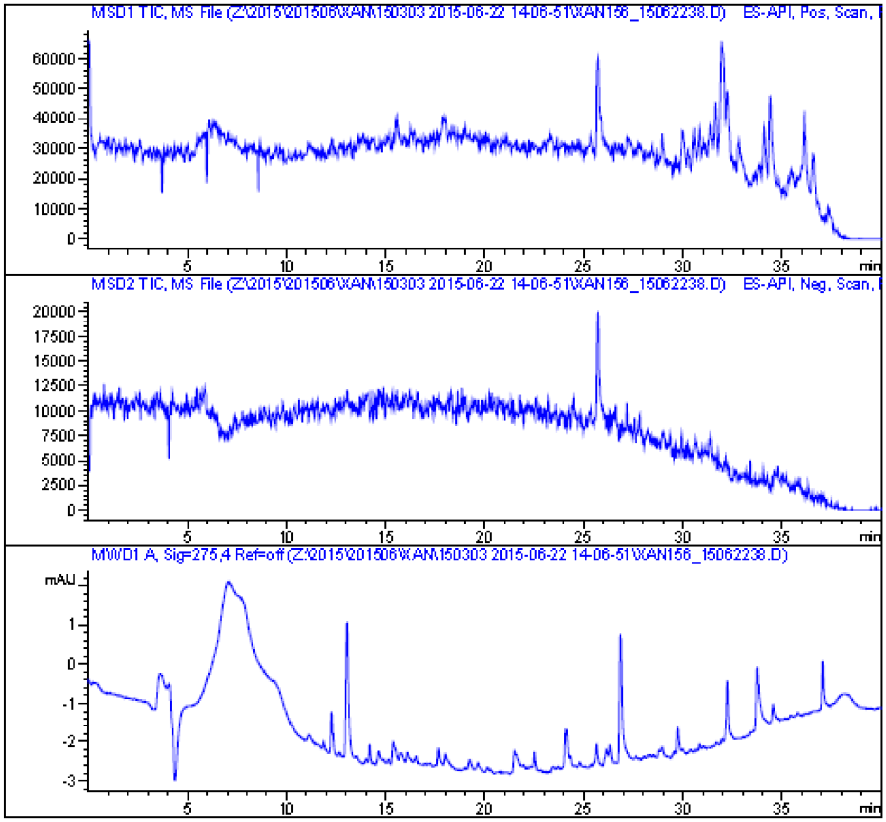

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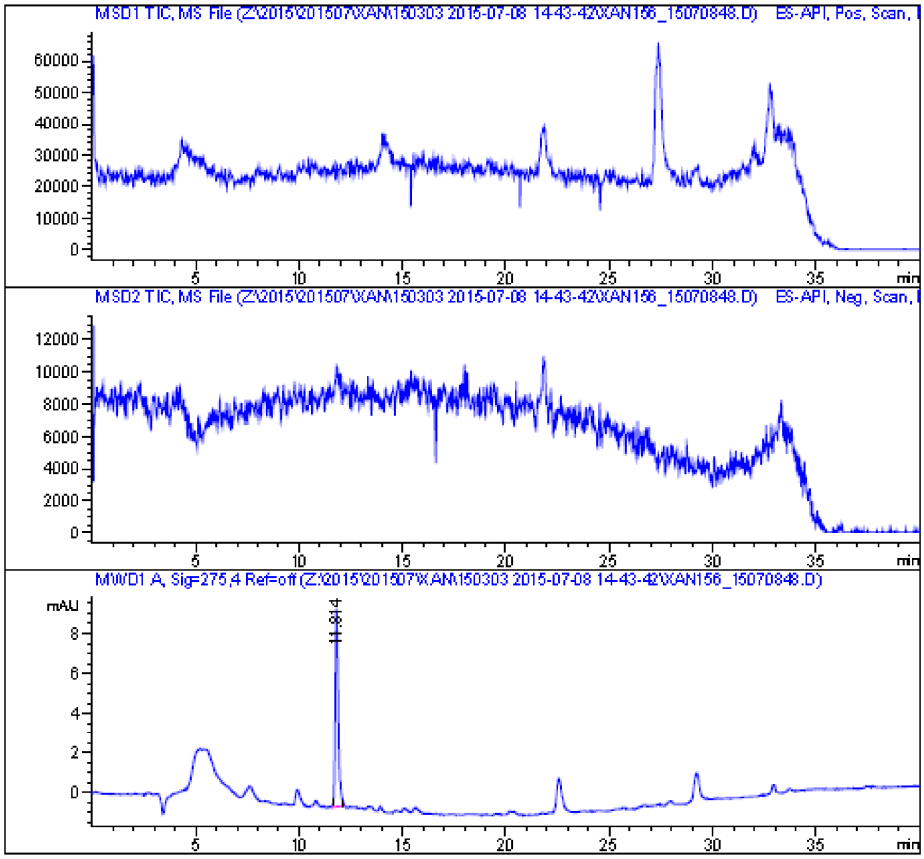


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3.3 Studie 3: Isoxanthohumol, a constituent of hop (*Homulus lupulus* L.) increases stress resistance in *Caenorhabditis elegans* dependent on the transcription factor DAF-16

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ORIGINAL CONTRIBUTION

Isoxanthohumol, a constituent of hop (*Humulus lupulus* L.), increases stress resistance in *Caenorhabditis elegans* dependent on the transcription factor DAF-16

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Abstract
Purpose The flavanone isoxanthohumol (IX) has gained attention as antioxidative and chemopreventive agent, but the molecular mechanism of action remains unclear. We investigated effects of this secondary plant compound in vivo using the model organism *Caenorhabditis elegans*.
Methods Adult *C. elegans* nematodes were incubated with IX, and then, the stress resistance was analysed in the SYTOX assay; lifespan was monitored by touch-provoked movement method, the amount of reactive oxygen species (ROS) was measured in the DCF assay, and the nuclear localisation of the transcription factor DAF-16 was analysed by using a transgenic strain. By the use of a DAF-16 *loss-of-function* strain, we analysed whether the effects are dependent on DAF-16.
Results IX increases the resistance of the nematode against thermal stress. Additionally, a reduction in ROS in vivo was caused by IX. Since the flavanone only has a marginal radical-scavenging capacity (TEAC assay), we suggest that IX mediates its antioxidative effects indirectly via activation of DAF-16 (homologue to mammalian FOXO proteins). The nuclear translocation of this transcription factor is increased by IX. In the DAF-16-mutated strain, the IX-mediated increase in stress resistance was completely abolished; furthermore, an increased formation of ROS and a reduced lifespan was mediated by IX.
Conclusion IX or a bacterial metabolite of IX causes antioxidative effects as well as an increased stress resistance in *C. elegans* via activation of DAF-16. The homologous pathway may have implications in the molecular mechanism of IX in mammals.

Keywords Ageing · Beer · DAF-16 · Nutrition · Oxidative stress · Secondary plant compounds

Electronic supplementary material The online version of this article (doi:10.1007/s00394-015-0843-z) contains supplementary material, which is available to authorized users.

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Introduction

The female inflorescences or cones of the hop plant (*Humulus lupulus*) are a source of the prenylated flavanone isoxanthohumol (IX, Fig. 1a). Since this compound is formed from the isomeric chalcone xanthohumol (XH, 3′-[3,3-dimethyl allyl]-2′,4′,4-trihydroxy-6′-methoxychalcone) during the brewing process, the consumption of beer contributes largely to the dietary exposure to IX. Recently, IX gained attention as cancer chemopreventive agent, as antioxidant as well as an anti-inflammatory compound (reviewed by [1, 2]) (Table 1).

IX possesses a weakly oestrogenic activity, but is converted to the more oestrogenic 8-prenylnaringenin by the microbiota of the colon [3]. Due to the content of, for example, XH and IX, hop supplements are used as alternatives for the management of menopausal symptoms [4]. These "phytoestrogens" are suggested to act as selective oestrogen receptor modulators (SERMs): Monteiro et al. [5] showed that IX inhibits aromatase activity and thus oestrogen formation in a Sk-Br-3 breast cancer cell line. Izzo et al. [6] demonstrated that IX exerts maturation-dependent effects on Leydig cell steroidogenesis by inhibition of the hCG-stimulated androgen production. However, in a dietary intervention study, Bolca et al. [7] were able to show that the plasma concentration of IX ranges

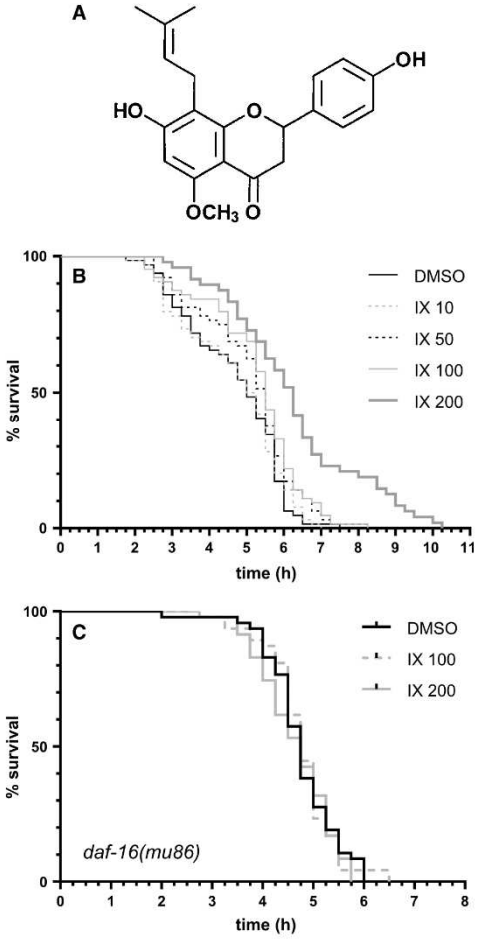

Fig. 1 Isoxanthohumol enhances the heat-stress resistance of *C. elegans* depending on DAF-16. **a** Chemical structure of isoxanthohumol; **b**, **c** modulation of stress resistance by IX: 3 days after hatching age-synchronised, wildtype nematodes (**b**) or *daf-16(mu86)* nematodes (**c**) are incubated with IX or 0.2 % DMSO in liquid S-medium for 48 h. After a washing step, single nematodes are placed in the wells of a 384-well plate containing 2 µM SYTOX Green. The microplate is kept at 37 °C, and the deaths of the nematodes are measured via the increase in fluorescence. The *graph* shows the percentage of viable nematodes. Corresponding data are summarised in Table 1; Kaplan–Meier survival analysis with log-rank test (Mantel–Cox)

only between 3.3 and 31.5 nmol/l after administration of IX-containing hop supplements. They suggest that these low doses of prenylflavonoids are unlikely to elicit oestrogenic responses in breast tissue. Negrão et al. [8] reported that IX decreases inflammatory signals, e.g. TNF-α and NFκB, in human aortic smooth muscle cells and human umbilical vein endothelial cells. Since IX also seems to modulate vascular proliferation and stabilisation, they suggest IX as a potential agent for diseases in relation to angiogenesis and inflammation. Furthermore, Serwe et al. [9] reported that IX antagonises the cellular effects of TGF-β in HepG2 cells and also blocks IFN-γ-, IL-4- and IL-6-dependent Jak/Stat signalling. IX was reported to be toxic to different cancer cell lines, e.g. to MCF-7 cells (EC_{50} value 26.5 ± 12.6 µM), PC-3 cells (EC_{50} value 71.3 ± 19.5 µM), HT-29 cells (EC_{50} value 88.8 ± 4.1 µM) [10] and DU145 cells (EC_{50} value 47.4 ± 1.1 µM) [11]. Allsopp et al. [12] reported anticancer effects of IX on in vitro models of key stages of colon tumorigenesis. Monteiro et al. [5] suggest that hop flavonoids have anti-breast cancer effects through their ability to decrease oestrogen levels by inhibition of aromatase activity. Furthermore, antioxidative properties of IX are described in the literature: Tronina et al. [10] reported a DPPH radical-scavenging ability of IX with an IC_{50} value of 35.42 ± 0.11 µM (concentration $\mu M_{antiox}/\mu M_{DPPH}$ for a 50 % inhibition). Potaniec et al. [13] reported a 250-fold lower antioxidative capacity in the DPPH assay, and the EC_{50} value was determined as 8.38 ± 0.796 mM. On the other hand, Yang et al. [14] reported that IX increases the amount of reactive oxygen species (ROS) production in mature adipocytes which can be prevented by the antioxidants ascorbic acid and 2-mercaptoethanol. In correlation with the antioxidative effects, hop extracts have been reported to be potent anti-ageing agents, especially against ageing of the skin [15].

However, in spite of the frequent use of dietary hop-based supplements as "phytoestrogens" or, more recently, as anti-ageing products (e.g. "anti-ageing-beer"), the knowledge about the relevant secondary plant compounds is limited and, at least to some extent, controversial. Most of the data about IX are generated by in vitro studies, and the knowledge of in vivo effects is relatively scarce. Therefore, we have used the nematode model organism *Caenorhabditis elegans* to analyse antioxidative, stress protective as well as ageing modulating effects of IX in vivo.

Materials and methods

Materials

All chemicals were of analytical grade and were obtained from Sigma (Deisenhofen, Germany), with the exceptions of isoxanthohumol (purity ≥99 %; Carl Roth; Karlsruhe, Germany), DMSO (Merck; Darmstadt, Germany), ammonium peroxydisulphate (Merck, Darmstadt, Germany), Trolox (Calbiochem, Darmstadt, Germany) and SYTOX® Green nucleic acid stain (Molecular Probes Inc.; Leiden, the Netherlands).

Table 1 Summary of the heat-stress resistance data of wildtype (N2) and *daf-16(mu86)* (CF1038) nematodes after IX treatment (depicted in Fig. 1)

Treatment	Adult survival at 37 °C (h)			*p* value versus DMSO (log-rank)
	Mean (±SEM)	Median (±SEM)	*n*	
N2 (wildtype)				
DMSO	4.6 ± 0.16	5.0 ± 0.23	64	
IX 10 µM	4.6 ± 0.17	5.0 ± 0.25	64	0.874
IX 50 µM	5.0 ± 0.16	5.5 ± 0.12	64	0.081
IX 100 µM	5.2 ± 0.16	5.5 ± 0.11	64	0.014
IX 200 µM	6.2 ± 0.26	6.2 ± 0.21	48	<0.001
CF1038 (*daf-16(mu86)*)				
DMSO	4.7 ± 0.10	4.7 ± 0.09	47	
IX 100 µM	4.7 ± 0.10	4.7 ± 0.10	47	0.875
IX 200 µM	4.6 ± 0.10	4.7 ± 0.04	47	0.414

Caenorhabditis elegans strains and maintenance

Strains that were used in this study are wildtype N2 (var. Bristol), CF1038 [*daf-16(mu86) I.*] and TJ356 [*zIs356 IV (pdaf-16::daf-16-gfp; rol-6)*]. All strains were provided by the *Caenorhabditis Genetics Center* (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Strains were maintained by the following standard procedures, as described previously [16]. Treatment of nematodes with IX was performed in 2 ml of liquid S-medium containing 1 % (w/v) bovine serum albumin, 50 µg/ml streptomycin and 10^9 OP50-1/ml in 35-mm petri dishes. Stock solutions of IX (100 mM) were prepared in DMSO. Age synchronisation of the nematodes was performed by hypochlorite treatment of gravid adults.

Stress resistance

Survival of individual nematodes at the temperature of 37 °C was monitored with a semi-automated assay according to Gill et al. [17] with slight modifications as described elsewhere [18]. The fluorescence intensity was determined using a fluorescence spectrophotometer (Synergy MX, BioTek; Bad Friedrichshall, Germany). Experiments were repeated at least three times.

Measurement of intracellular ROS accumulation in vivo

To detect the in vivo ROS levels in individual nematodes, the fluorescent probe H_2DCF-DA (2′,7′-dichlorodihydrofluorescein–diacetate) was used. The assay was performed as described previously [18], with following modifications: briefly, L4 larvae/young adults were treated in liquid S-medium containing IX or 0.2 % DMSO for 48 h at 20 °C. During the treatment, nematodes were transferred to fresh culture media daily. After 48 h, all animals were transferred into M9T (M9 buffer containing 0.1 % Tween 20) for 1 h at 20 °C to wash off bacteria and residual compounds. Then ten nematodes per group (triplicates) were transferred in 25 µl M9T into each well of a 384-well plate (384-well µClear® large volume plate, Greiner Bio-One; Frickenhausen, Germany). Subsequently, when all animals were transferred, 25 µl of H_2DCF-DA (100 µM in M9T) was added into each well to obtain a final concentration of 50 µM H_2DCF-DA. A black backing tape (Perkin Elmer; Wellesley, MA, USA) was applied to the top of the plate to avoid evaporation. ROS accumulation was induced by thermal stress at 37 °C and recorded using a fluorescence spectrophotometer (Synergy HT, BioTek; Bad Friedrichshall, Germany). The experiment was repeated at least three times.

Trolox equivalent antioxidative capacity (TEAC) assay

Equal volumes of 14 mM 2,2′-azinobis-(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS) and 4.9 mM ammonium peroxydisulphate were mixed and allowed to form stable ABTS radicals. The radical solution was diluted (70 % ethanol) to an absorption of about 1.4 at a wavelength of 734 nm. Equal volumes of compound solutions and radical solutions were mixed, and the absorption was measured spectrophotometrically 2 min after starting the reaction (Synergy MX, BioTek; Bad Friedrichshall, Germany). The synthetic vitamin E derivative Trolox was used as the positive control.

Intracellular Localisation of DAF-16-GFP

For the detection of the intracellular DAF-16 localisation, the strain TJ356 [*zIs356 IV (pdaf-16::daf-16-gfp; rol-6)*] was used. Three days old L4 larvae and young adult animals of this strain were placed in liquid S-medium ± IX and were treated for 1 h at 20 °C. Subsequently, an aliquot

of 10 µl medium containing the nematodes was placed on a microscope slide, mixed with 10 µl levamisole (10 mM) and covered with a cover slip, and the cellular localisation of DAF-16-GFP was detected by fluorescence microscopy using a Zeiss Axioskop and a GFP filter set (Zeiss; Göttingen, Germany). The experiment was repeated four times.

Lifespan assays

Lifespan analyses were performed with N2 and CF1038 [*daf-16(mu86)*]. Age-synchronised animals (L4 larvae/young adults) were placed in liquid S-medium ± IX and kept at 20 °C. Nematodes were transferred to new culture dishes every 2–3 days. The starting day in liquid culture was considered as day 0 of the lifespan. During the fertile period of the animals (the first 13 days of treatment), the medium was supplemented with 120 µM 5′fluorodeoxyuridine (FUDR) to prevent viable progeny. This compound does not influence the experimental results by, for example, masking possible beneficial lifespan effects of IX (see supplementary figure 3). Nematodes were scored as dead when they did not respond to gentle prodding and when they showed no pharyngeal pumping activity. Lost nematodes and animals showing prolapsed internal organs were censored. The experiments were repeated three times.

Statistics

Data are given as mean ± SD (or mean ± SEM, as indicated) of at least three independent experiments. Statistical analyses were performed with IBM SPSS 19 (Armonk, NY, USA) and GraphPad Prism 6 software (La Jolla, USA). Statistical significance was determined by one-way ANOVA with Dunnett's post-test and two-way ANOVA with Dunnett's post-test, respectively. Lifespan and stress resistance analyses were performed using Kaplan–Meier survival analyses; animals that were lost, killed or showed other abnormalities were censored. Differences were considered to be significant at $p < 0.05$.

Results

Modulation of stress resistance by isoxanthohumol

We first investigated whether a pre-incubation with the flavanone IX exerts protective effects in our experimental model system: to determine the resistance of *C. elegans* against lethal thermal stress, we used the SYTOX assay measuring the uptake of a membrane-impermeable fluorescent probe into the nematode to calculate "virtual death points". Thermal stress is lethal for the nematode as depicted in Fig. 1b: the viability of the adult nematodes at 37 °C revealed a mean survival time of 4.68 ± 0.16 h. A pre-incubation with 100 or 200 µM IX increases the survival time by 11.2 and 33.4 %, respectively (5.24 ± 0.16 and 6.25 ± 0.26 h).

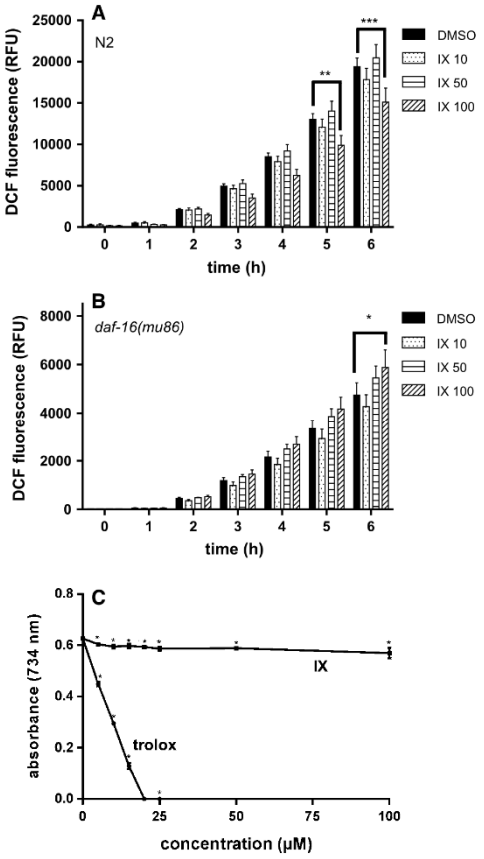

Fig. 2 Isoxanthohumol exerts antioxidative properties in *C. elegans* depending on DAF-16. Three days after hatching age-synchronised, wildtype nematodes (**a**) or *daf-16(mu86)* nematodes (**b**) are incubated with IX or 0.2 % DMSO in liquid S-medium for 48 h. After a washing step, ten nematodes are placed in each well of a 384-well plate containing 50 µM H_2DCF-DA, kept at 37 °C, and the ROS accumulation is measured by an increase in DCF fluorescence. Mean ± SEM; four experiments with 30 individuals per group; $^{**}p < 0.01$; $^{***}p < 0.001$ versus corresponding DMSO-treated control value; two-way ANOVA with Dunnett's post hoc test. **c** The radical-scavenging properties (=antioxidative capacity) of IX are measured by the TEAC assay in vitro. The decolourisation of the radical solution is detected spectrophotometrically at 734 nm (mean values ± SD, $n = 3$, $^{*}p < 0.05$ vs. control value; one-way ANOVA with Dunnett's post hoc test)

Antioxidative potential of isoxanthohumol

An improvement of the stress resistance in *C. elegans* by secondary plant compounds is often mediated via antioxidative effects. Therefore, we have investigated the antioxidative potential of IX in *C. elegans* using DCF, a fluorescent probe for the detection of reactive oxygen species. The generation of oxidative stress was provoked by an increase in temperature (thermal stress): the DCF fluorescence as a measure of reactive oxygen species highly increased from 260 ± 140 rfu (0 min; ±SEM) to 19,393 ± 1,716 rfu (6 h; ±SEM) in wildtype nematodes (Fig. 2a). A pre-treatment with 100 µM IX clearly reduces the DCF fluorescence at the late time points: we clearly see a decrease in ROS to 76.1 % of the DMSO value after 5 h (13,022 ± 1,152–9,911 ± 1,921 rfu; ±SEM) and 77.9 % after 6 h (19,393 ± 1,716–15,112 ± 2,805 rfu; ±SEM), respectively. However, the DCF fluorescence was not significantly influenced by a pre-treatment with 10 or 50 µM IX (Fig. 2a). Using the TEAC assay, we further analysed whether the antioxidative effect of IX was mediated by direct antioxidative radical-scavenging effects of the compound: we could show that IX (up to 100 µM) has only a marginal radical-scavenging effect: while an incubation with the water-soluble synthetic vitamin E derivative Trolox reveals a dose-dependent reduction in the ABTS radical with 100 % antioxidative capacity at 20 µM, IX only shows a slight reduction in the ABTS radical of approximately 5 % at 100 µM (Fig. 2c). This suggests that the decrease in DCF fluorescence caused by IX is not due to a direct radical-scavenging effect of this compound, but due to a modulation of the antioxidant defence system of the nematode.

Effect of isoxanthohumol on DAF-16 translocation

In *C. elegans*, the insulin/IGF-1 signalling pathway controls many biological processes such as lifespan, fat storage, dauer diapause, reproduction and stress response. This pathway is comprised of many genes including the insulin/

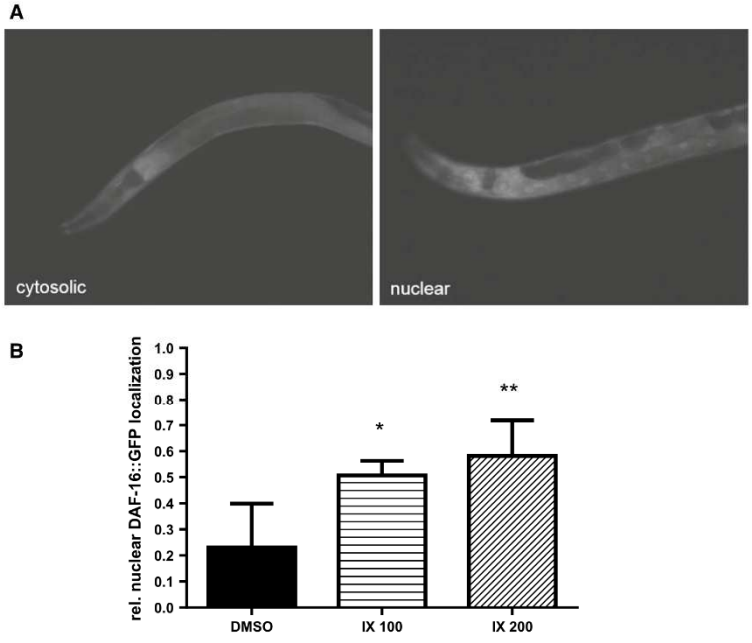

Fig. 3 Isoxanthohumol induces nuclear translocation of DAF-16. Transgenic nematodes (DAF-16-GFP; strain TJ356) are used to detect the localisation of the transcription factor DAF-16. **a** The nuclear localisation of DAF-16-GFP is visible by distinct fluorescent dots in the nematode (*right*) in contrast to the diffuse fluorescence of DMSO-treated nematodes (*left*). **b** Young adult nematodes (DAF-16-GFP; strain TJ356) are treated with IX or 0.2 % DMSO for 1 h (S-medium) followed by microscopic determination of the GFP localisation phenotype. Mean ± SD, four independent experiments with a total amount of 208 (DMSO), 240 (100 µM IX) and 264 (200 µM IX) individuals, $*p < 0.05$; $**p < 0.01$ versus corresponding DMSO value; one-way ANOVA with Dunnett's post hoc test

IGF-1 receptor (DAF-2) that signals through a conserved PI 3-kinase/AKT pathway and ultimately down-regulates DAF-16, a forkhead transcription factor (FOXO) [19]. Since insulin/IGF-1 signalling pathway is pivotal in the modulation of stress response in the nematode, we have analysed whether this molecular signalling pathway is modulated by IX. We have used the transgenic strain TJ356 (DAF-16-GFP) to detect the translocation of the DAF-16 transcription factor from the cytosol to the nucleus (Fig. 3). We clearly see an induction of the nuclear translocation of DAF-16 by 100 and 200 µM IX (51 ± 5 and 58 ± 12 % compared to the control value of 23 ± 15 %).

Effect of isoxanthohumol on lifespan

Since antioxidative effects DAF-16 activation and increased stress resistance often are associated with an extension of lifespan, we have investigated the effect of different concentrations of IX on the lifespan of *C. elegans*. However, no modulation of lifespan was detectable after incubation with 100 or 200 µM IX (Fig. 4a; Table 2): the mean lifespan of DMSO-treated nematodes was 28.8 ± 0.7 days. IX (100, 200 µM) changed the lifespan non-significantly to 29.5 ± 0.6 and 28.2 ± 0.5 days, respectively. It cannot be excluded, that at lower concentrations, protective effects may occur, due to hormetic effects of isoxanthohumol, but due to the previous results (SYTOX, DCF), lower concentrations were not used in this study.

Involvement of DAF-16 in the isoxanthohumol-mediated effects in *C. elegans*

Since we have shown a nuclear translocation of DAF-16 induced by IX, we have used DAF-16 *loss-of-function* mutant nematodes (*daf-16(mu86)*) to analyse whether this transcription factor is necessary for the maintenance of the biological effects of IX in *C. elegans*, i.e. whether the effects of this flavanone are mediated via an activation of DAF-16.

First, we analysed to which extend IX prolongs the resistance of *C. elegans* against thermal stress (SYTOX assay) in the DAF-16 mutant strain: in the DAF-16 *loss-of-function* strain, no prolongation of the survival rate was detectable after incubation with IX; the protective effect was completely abolished (Fig. 1c). In wildtype animals, 200 µM of the compound extended the survival time from 4.68 ± 0.16 to 6.25 ± 0.26 h; in the DAF-16 mutant strain, no change was detectable (4.74 ± 0.1–4.64 ± 0.1 h). Next, we analysed the effect of DAF-16 on the antioxidative properties of IX in *C. elegans* (DCF assay). The antioxidative effects detected for IX were completely abolished in the DAF-16 *loss-of-function* strain: Moreover, the compound increases the DCF fluorescence at 100 µM by 23.8 % (Fig. 2b). The results obtained with isoxanthohumol in the N2 and CF1038.

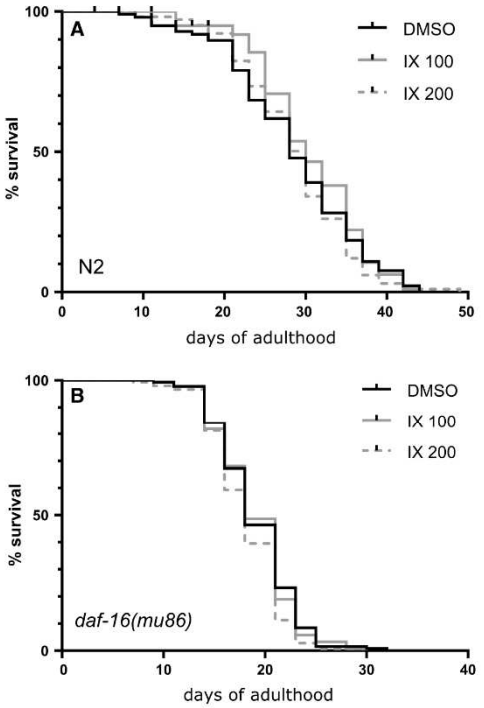

Fig. 4 Effect of isoxanthohumol on adult lifespan of *C. elegans*. Three days after hatching age-synchronised animals are transferred into liquid S-medium containing IX or 0.2 % DMSO. The survival at 20 °C is monitored three times per week and living nematodes are transferred to fresh medium. For the first 2 weeks of treatment, the medium contained 120 µM FUDR to inhibit progeny production. Strains that are used are wildtype (N2) (**a**) and CF1038 (*daf-16(mu86); loss-of-function*) (**b**). Kaplan–Meier survival analysis with log-rank test (Mantel–Cox); corresponding data are summarised in Table 2

Since increased ROS may have adverse effects on the lifespan of *C. elegans*, we have analysed the effect of IX on the lifespan of the DAF-16 *loss-of-function* strain CF1038 (Fig. 4b). The mean lifespan of DMSO-treated nematodes in the mutant strain is significantly shorter (19.1 ± 0.3 days) compared with wildtype nematodes (28.8 ± 0.7 days). Even though IX caused no prolongation of the lifespan in wildtype nematodes, it has a detrimental effect in the *daf-16(mu86)* mutant strain at a concentration of 200 µM: the mean lifespan was decreased significantly (DMSO: 19.1 ± 0.3 days, IX 200 µM: 18.2 ± 0.3 days).

Table 2 Summary of the lifespan data of wildtype (N2) and *daf-16(mu86)* (CF1038) nematodes after IX treatment (depicted in Fig. 4)

Treatment	Adult survival (days)			*p* value versus DMSO (log-rank)
	Mean (±SEM)	Median (±SEM)	*n* (censored)	
N2 (wildtype)				
DMSO	28.8 ± 0.68	28.0 ± 0.89	154 (13)	
IX 100 µM	29.5 ± 0.60	28.0 ± 0.79	156 (26)	0.965
IX 200 µM	28.2 ± 0.60	28.0 ± 0.80	162 (11)	0.251
CF1038 (*daf-16(mu86)*)				
DMSO	19.1 ± 0.34	18.0 ± 0.50	161 (31)	
IX 100 µM	19.0 ± 0.34	18.0 ± 0.46	158 (35)	0.704
IX 200 µM	18.2 ± 0.30	18.0 ± 0.42	155 (13)	0.021

Discussion

Dietary supplements from the hop plant are frequently used due to their postulated antioxidative and chemopreventive properties, as alternatives for the management of menopausal symptoms and to combat various effects of the ageing process. However, there are conflicting data from various in vitro and some in vivo studies on the physiological relevance of these phytochemicals [20, 21]. In the last years, the model organism *C. elegans* has gained increasing importance in pharmacological and toxicological sciences due to the availability of various genetically modified nematode strains, the simplicity of modulating gene expression by RNAi and the relatively short lifespan. Several studies have been performed demonstrating that secondary plant compounds influence ageing, stress resistance as well as distinct signalling pathways in this nematode (reviewed by Koch et al. [22]). To our best knowledge, no reports about effects of IX in this important model organism exist in the literature; therefore, we have analysed (1) the antioxidative capacity of IX in vivo, (2) the effect on the stress resistance of the nematode and (3) on its lifespan.

In *C. elegans*, a prolongation of lifespan is often associated with an improvement in stress resistance against various kinds of stresses, e.g. thermal stress. By using a semi-automated assay system that employs the fluorescent probe SYTOX Green as a marker of irreversibly damaged cells, we were the first to show that IX treatment strongly increased the survival of *C. elegans* against lethal thermal stress. Furthermore, we could show an antioxidative effect caused by IX in this model organism (in vivo DCF assay). These findings are in accordance with results obtained with other secondary plant compounds, e.g. myricetin, quercetin, kaempferol, fisetin and theanine [18, 23–27].

Since only marginal antioxidative effects of IX were detectable in the TEAC assay, we conclude that this compound exerts its antioxidant effect in *C. elegans* indirectly, i.e. by an improvement of the antioxidative network in *C. elegans* by induction of antioxidative enzymes. For example, catalase is regulated via the transcription factor DAF-16 [28]. To find first hints concerning the molecular mechanism of IX in *C. elegans*, we have used a strain expressing GFP-tagged DAF-16 and analysed the amount of nuclear localised DAF-16, a necessary prerequisite for the transcriptional activity of DAF-16. Under physiological conditions, DAF-16 is mainly located in the cytoplasm, while only a small amount is located in the nuclei. However, after incubation with IX, more than 50 % of the animals show a nuclear localisation phenotype, suggesting an activation of this important signalling pathway. A modulation of this signalling pathway has been reported for other flavonoids, e.g. quercetin [26], epigallocatechin gallate [29] and apigenin [30].

The improved stress resistance was completely abolished in a DAF-16 *loss-of-function* mutant strain. This indicates a necessary involvement of DAF-16, the homologue of mammalian FOXO transcription factors in *C. elegans*, in the process of IX-mediated enhancement of stress resistance. Furthermore, the antioxidative effect of IX on heat-induced ROS generation was completely abolished in the DAF-16 mutant nematodes. On the contrary, IX now exhibits a prooxidative effect in the DAF-16 *loss-of-function* mutant.

Activation of the transcription factor DAF-16 in *C. elegans* is associated with an increase in lifespan [31]. Examples for secondary plant compounds which increase the lifespan via activation of DAF-16 are, for example, icariin [32], myricetin [18] and caffeic acid phenethyl ester [33]. Since IX caused a prominent nuclear translocation of DAF-16, we have analysed the effect of this flavanone on the lifespan of the nematodes. However, no prolongation of lifespan was detectable, even at the highest concentration of 200 µM. This was not expected, especially with regard to the prominent increase in stress resistance caused by this compound.

On the contrary, in the DAF-16 *loss-of-function* strain, a significant toxic effect of IX was detectable. These results suggest that IX mediates two different effects in *C. elegans*:

(1) a pro-oxidative effect, which reduces stress resistance and lifespan as well as (2) an antioxidative effect which enhances stress resistance and lifespan that is mediated by the transcription factor DAF-16. Since the DAF-16-mediated effect prevails, we see the detrimental effects only in a DAF-16 *loss-of-function* mutant strain.

Furthermore, it has to be concluded that different molecular mechanisms exist for prolongation of lifespan as well as for the enhancement of stress resistance [34, 35]: the death of the nematodes detected in the SYTOX assay is induced by an increase in temperature to 37 °C, while the lifespan analysis was performed at 20 °C. The transcription factor DAF-16 plays an important role in both experimental settings, since (1) the IX-mediated increase in stress resistance is completely blocked in a DAF-16-mutated strain and (2) IX causes a decrease in lifespan in DAF-16-mutated nematodes. However, it may be possible that IX also influences different other transcription factors related to the ageing process or stress resistance, e.g. the Nrf-2 homologue SKN-1 [36]. Furthermore, isoxanthohumol may be metabolised in humans by the intestinal microbiota, for example, to compounds like 8-prenylnaringenin [37] which has to be taken into account estimating its biological effects. Since we used living bacteria in our experiments, a formation of bacterial metabolites is also possible. We analysed the effect of living versus inactivated bacteria on the physiological effects of IX in the SYTOX assay: Using inactive bacteria for cultivation, IX caused no enhancement of stress resistance in N2 nematodes and furthermore a reduction in stress resistance in the *daf-16* mutant nematodes (supplementary figure 2). This result suggests a formation of active bacterial metabolites in our experiments. Based on the presented evidence, the potential influence of IX on the ageing process as well as the stress resistance warrants further investigation.

Conclusion

Isoxanthohumol or bacterial metabolites of IX had no effect on the lifespan of *C. elegans*, but they cause a strong increase in stress resistance as well as antioxidative effects. These beneficial effects are mediated via activation of the DAF-16 signalling pathway and are completely blocked in a DAF-16 mutant strain. This pathway may have implications on the molecular mechanism of IX in mammals. Our findings concerning this mechanism may be important due to the frequent use of IX as part of dietary hop supplements.

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Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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Materials and Methods Supplement

Measurement of ROS *in vivo* using the fluorescence probe CellROX Deep Red

Gravid adults were dissolved using the hypochlorite method to obtain age synchronized eggs that were maintained at 20°C for three days. Thereafter, young adult animals were treated with IX in liquid S-medium for two days (daily medium exchange) as described above. After 48 hours, all animals were transferred into 1.5 ml PBST (PBS containing 0.1% Tween 20) for one hour at 20°C to wash off bacteria and residual compounds. Then, single nematodes were transferred in 1 µl of PBST into each well of a 384-well plate (384-well µClear® plate, Greiner Bio-One; Frickenhausen, Germany) containing 7 µl PBS. Subsequently, 2 µl CellROX Deep Red (Molecular Probes, Leiden, The Netherlands) were added to each well to obtain a final concentration of 5 µM CellROX Deep Red. A black backing tape (Perkin Elmer; Wellesley, MA, USA) was applied to the top of the plate to avoid evaporation. ROS accumulation was induced by thermal stress at 37°C and recorded using a monochromator based fluorescence spectrophotometer (Synergy MX, BioTek; Bad Friedrichshall, Germany) using the following excitation and emission wavelengths: ex. 625/9 nm and em. 665/9 nm. The experiment was repeated three times.

Stress resistance using heat inactivated *E. coli* as the food source

Survival of individual nematodes at the temperature of 37°C was monitored with a semi-automated assay according to Gill et al. [17] with slight modifications as described in Büchter et al. [18] and the change of using heat inactivated *E. coli* OP50-1 (30 min at 65°C) The fluorescence intensity was determined using a fluorescence spectrophotometer (Synergy MX, BioTek; Bad Friedrichshall, Germany). Experiments were repeated three times.

Life span analyses without use of 5´fluorodeoxyuridine (FUDR)

Life span analyses of the strain CF1038 (*daf-16(mu86)*) were performed as described above with the modification of not using 5´fluorodeoxyuridine to prevent viable

offspring. For preventing overcrowding and to discriminate test animals from their progeny, test nematodes were transferred to fresh medium for the first five days of treatment. Thereafter, animals were transferred three times a week into fresh medium. The experiment was repeated two times.

Supplementary Figure Legends

Figure S1: Measurement of *in vivo* ROS accumulation using CellROX Deep Red

(A) Treatment of wild type nematodes (N2) with 100 µM IX results in a statistically non-significant ($p > 0.05$; paired t-test; three experiments with 16 nematodes per group and experiment) tendency towards reduced heat induced ROS formation. (B) Treatment of *daf-16(mu86)* mutant nematodes results in a statistically non-significant ($p > 0.05$; paired t-test; three experiments with 16 nematodes per group and experiment) heat induced ROS formation.

Figure S2: Measurement of heat-stress resistance using heat inactivated *E. coli* as the food source

(A) Treatment of wild type nematodes (N2) with 100 µM IX and heat inactivated *E. coli* abolishes the increased stress resistance compared to the use of live bacteria (Kaplan Meier survival analysis; three experiments with 16 nematodes per group and experiment). (B) Treatment of CF1038 nematodes (*daf-16(mu86)*) with 100 µM IX and heat inactivated *E. coli* decreases stress resistance compared to DMSO treated animals (*p* value vs. DMSO = 0.040; Kaplan Meier survival analysis; three experiments with 16 nematodes per group and experiment). Data are summarized in Table S1.

Figure S3: Life span analysis of IX treated *daf-16(mu86)* mutant animals without the use of FUDR

Treatment of *daf-16(mu86)* mutant animals with IX (without FUDR) during the complete adult life span does not change their survival compared to DMSO treated animals (without FUDR). ($p>0.05$; Kaplan Meier survival analysis; two experiments with at least 50 nematodes per group and experiment). Data are summarized in Table S2.

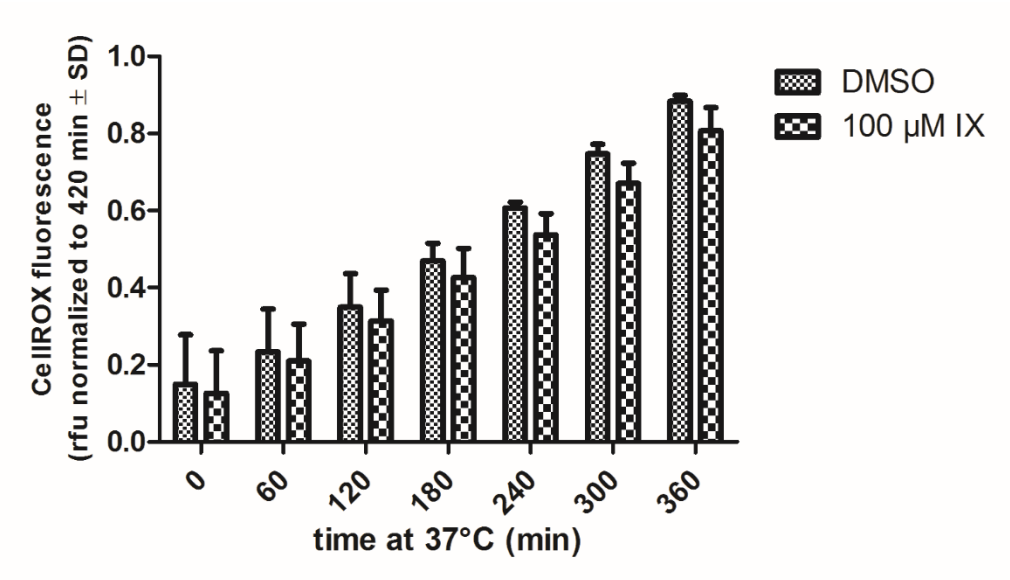
Table S1: Summary of the heat-stress survival depicted in Figure S2.

Table S2: Summary of the life span data depicted in Figure S3.

Supplementary Figures

Figure S1

A



B

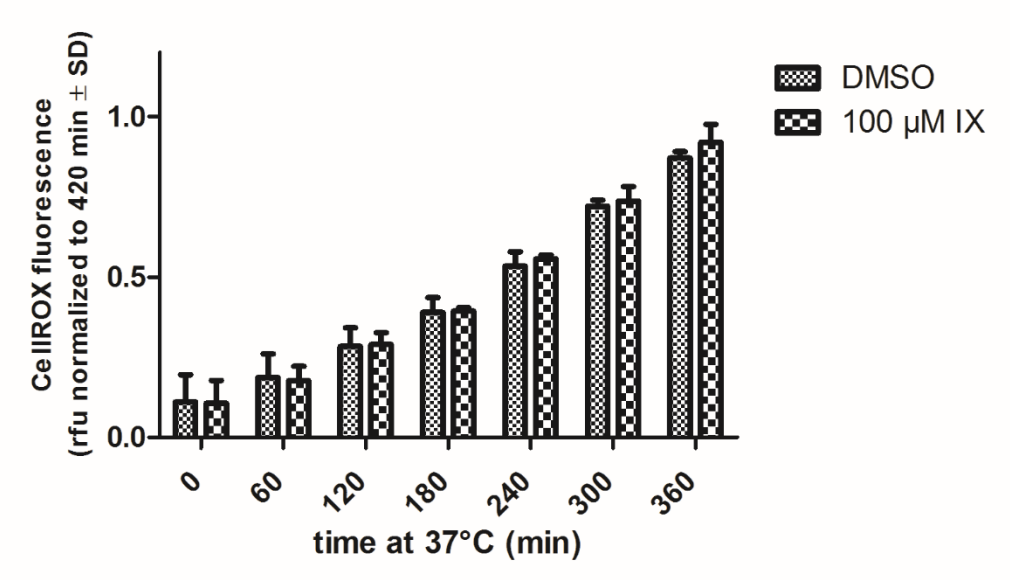
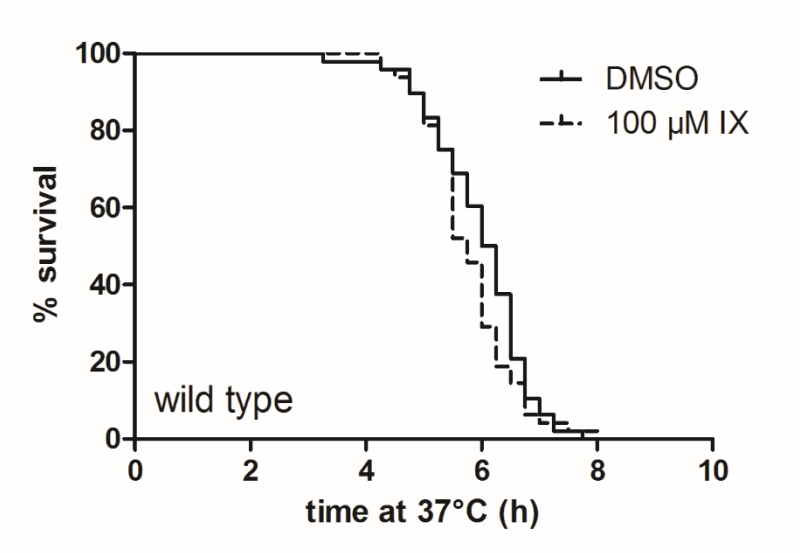


Figure S2

A


100
80
60
40
20
0
% survival
wild type
DMSO
100 µM IX
0
2
4
6
8
10
time at 37°C (h)

B

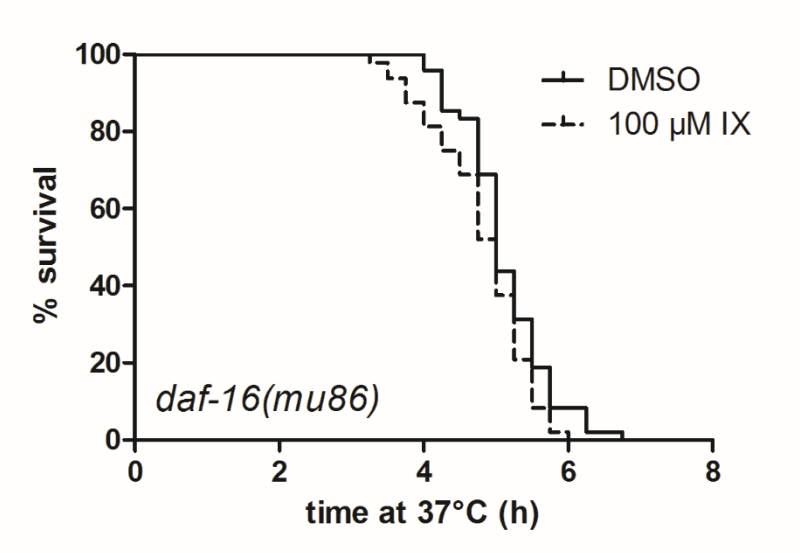
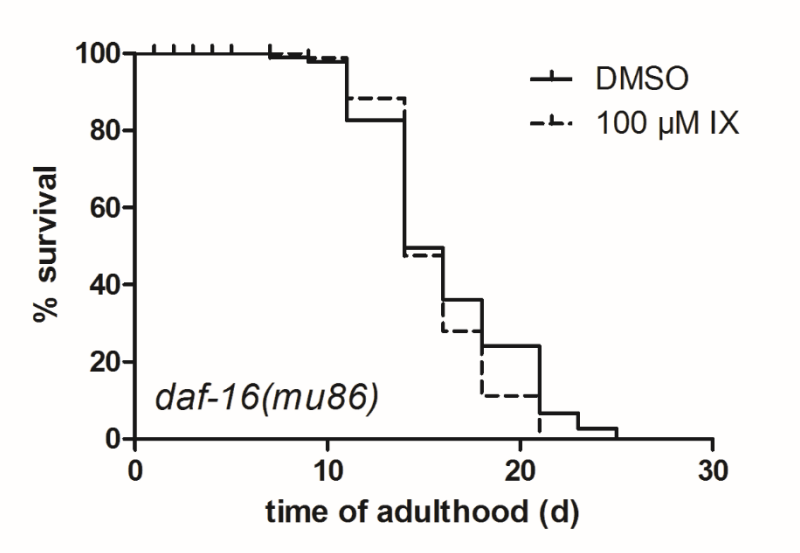

100
80
60
40
20
0
% survival
daf-16(mu86)
DMSO
100 µM IX
0
2
4
6
8
time at 37°C (h)

Figure S3


100
80
60
40
20
0
% survival
daf-16(mu86)
0
10
20
30
time of adulthood (d)
DMSO
100 µM IX

Supplementary Tables

Table S1

N2 (wildtype)	**adult survival at 37 °C (h)**			
treatment	**mean (± SEM)**	**median (± SEM)**	**n**	***p* value vs. DMSO (log-rank)**
DMSO	5.97 ± 0.12	6.00 ± 0.16	48	
IX 100 µM	5.79 ± 0.11	5.75 ± 0.12	48	0.219
CF1038 (*daf-16(mu86)*)	**adult survival at 37 °C (h)**			
treatment	**mean (± SEM)**	**median (± SEM)**	**n**	***p* value vs. DMSO (log-rank)**
DMSO	5.12 ± 0.09	5.00 ± 0.07	48	
IX 100 µM	4.81 ± 0.10	5.00 ± 0.11	48	0.040

Table S2

CF1038 (*daf-16(mu86)*)	**adult survival (d)**			
treatment	**mean (± SEM)**	**median (± SEM)**	**n (censored)**	***p* value vs. DMSO (log-rank)**
DMSO	16.0 ± 0.45	14.0 ± 0.61	114 (37)	
IX 100 µM	15.5 ± 0.33	14.0 ± 0.48	118 (44)	0.141

3.4 Studie 4: TSG (2,3,5,4´-Tetrahydroxystilbene-2-O-β-D-glucoside) from the Chinese Herb *Polygonum multiflorum* Increases Life Span and Stress Resistance of *Caenorhabditis elegans*

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Research Article

TSG (2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside) from the Chinese Herb *Polygonum multiflorum* Increases Life Span and Stress Resistance of *Caenorhabditis elegans*

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2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (TSG) was isolated from *Polygonum multiflorum*, a plant which is traditionally used as an anti-ageing drug. We have analysed ageing-related effects of TSG in the model organism *C. elegans* in comparison to resveratrol. TSG exerted a high antioxidative capacity both in a cell-free assay and in the nematode. The antioxidative capacity was even higher compared to resveratrol. Presumably due to its antioxidative effects, treatment with TSG decreased the juglone-mediated induction of the antioxidative enzyme SOD-3; the induction of the GST-4 by juglone was diminished slightly. TSG increased the resistance of *C. elegans* against lethal thermal stress more prominently than resveratrol (50 μM TSG increased mean survival by 22.2%). The level of the ageing pigment lipofuscin was decreased after incubation with the compound. TSG prolongs the mean, median, and maximum adult life span of *C. elegans* by 23.5%, 29.4%, and 7.2%, respectively, comparable to the effects of resveratrol. TSG-mediated extension of life span was not abolished in a DAF-16 loss-of-function mutant strain showing that this ageing-related transcription factor is not involved in the effects of TSG. Our data show that TSG possesses a potent antioxidative capacity, enhances the stress resistance, and increases the life span of the nematode *C. elegans*.

1. Introduction

Ageing is defined as an accumulation of diverse deleterious changes occurring in cells and tissues with advancing age that are responsible for the increased risk of several diseases and finally death [1]. Oxidative stress is believed to play a role in both physiological and pathological ageing processes, for example, age-related neurodegenerative diseases. However, besides the free radical theory of ageing [2], several other theories exist, for example, the hyperfunction theory of ageing [3], the inflammation theory of ageing [4], and the mitochondrial theory of ageing [5], which shows the complexity of the ageing process. A number of herbal medicines have been used traditionally to increase longevity and health. For example, extracts of *Polygonum multiflorum* are used as an anti-ageing treatment in East Asian countries. Besides the traditional use in folk medicine, extracts of *Polygonum multiflorum* have been shown to possess anti-ageing effects in different species: Chan et al. [6] reported that mice fed with *Polygonum multiflorum* extract had less lipofuscin in the hippocampus and lower MDA concentrations in the brain. An extract consisting of *Polygonum multiflorum* reduced the lipofuscin content of liver and brain tissues in both young (1 month old) and adult (11 months old) mice [7]. Li et al. [8] showed neuroprotective effects of an extract on nigrostriatal degeneration in mice. *Polygonum multiflorum* (root) was able to lower Aβ generation by modulating APP processing *in vitro* [9] and to prevent Aβ-induced increase of thiobarbituric acid

reactive substances and cognitive deficits in mice [10]. Steele et al. [11] reported cytoprotective effects of an extract of *Polygonum multiflorum* in astroglia cells. Furthermore, an improvement of cognitive performance in senescence accelerated mice [12] and an attenuation of glutamate-induced neurotoxicity [13] were demonstrated. Since many of the constituents of herbal extracts possess an antioxidative capacity, it is believed that this property may be involved, at least in parts, in the anti-ageing mechanism of the herbal extract. The stilbene glucoside 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside (TSG) is the main bioactive component of *Polygonum multiflorum* [14]; other compounds isolated were physcion, apigenin, hyperoside, rutin, vitexin, beta-amyrin, beta-sitosterol, and daucosterol. TSG is a potent antioxidant: Chen et al. [15] and Ryu et al. [16] investigated the antioxidant activity of compounds isolated from *Polygonum multiflorum* (DPPH-assay) showing TSG as an active constituent. TSG exerts protective effects on experimental colitis through diminishing the level of oxygen and nitrogen free radicals [17]. Wang et al. [18] showed that TSG has protective effects against cerebral ischemia by modulation of JNK, SIRT1, and NF-κB pathways. TSG mediates antagonistic effects on oxidation of lipoprotein, proliferation, and decrease of NO content of coronary arterial smooth muscle cells, which partially explains the antiatherosclerosis mechanism of *Polygonum multiflorum* [19]. Lv et al. [20] reported that TSG efficiently inhibits the formation of advanced glycation end products (AGEs). TSG has been shown to exert positive effects on learning and memory in animal models. This compound was shown to be protective against apoptosis in rat adrenal pheochromocytoma cells by modulating the ROS-NO pathway [21]. Sun et al. [22] showed that TSG protects human neuroblastoma SH-SY5Y cells against MPP^+-induced cell death through improving mitochondrial function, decreasing oxidative stress and inhibiting apoptosis which may be relevant for treatment of Parkinson's disease. Protective effects of TSG against MPP^+-toxicity in PC12 cells were reported to be mediated via modulation of the phosphoinositide-3-kinase/Akt signaling pathway [23] and the JNK pathway [24]. Various studies were performed concerning anti-ageing effects of TSG; however up to now, no direct correlation between ageing and TSG has been published. To investigate the effects of TSG on ageing processes, we used the model organism *Caenorhabditis elegans*. It is known that several polyphenols, for example, quercetin [25], myricetin [26], catechin [27], epigallocatechin gallate [28], or baicalein [29], increase the life span of *C. elegans*. As molecular mechanism for the life span extension, modulation of the insulin/IGF-like signalling pathway or an activation of Sir2 (sir-2.1 in *C. elegans*), a member of the sirtuin family of NAD^+-dependent deacetylases is discussed. Resveratrol acts on a number of target proteins [30]; for example, it was recently shown to activate AMP-kinase and to exert neuroprotective properties independent of Sir2 [31].

Aim of the Study. *Polygonum multiflorum* is widely used as a traditional anti-ageing drug in East Asian countries. The main bioactive component of the extract is 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside (TSG). The aim of this study was to evaluate the effects of TSG on oxidative stress, stress resistance, and life span in the model organism *C. elegans* in comparison to the structural analogue resveratrol and the flavonoid quercetin. For this reason, antioxidative effects were investigated *in vitro* and *in vivo*; effects on stress resistance, lipofuscin accumulation and expression of protective enzymes, and the life span were investigated.

2. Materials and Methods

2.1. Materials. Resveratrol and trolox were purchased from Calbiochem (Merck, Darmstadt, Germany), quercetin was from Extrasynthese (Genay, France), and DMSO was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and were purchased from Sigma (Deisenhofen, Germany).

2.2. Isolation of 2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside. The Chinese medicine plant *Polygonum multiflorum* was bought from Hangzhou Zhongmei Huadong Pharmaceutical Co., Ltd., Hangzhou, China. Isolation and purification of 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside were performed at the Heinrich-Heine-University, Institute of Pharmaceutical Biology and Biotechnology.

2.3. C. elegans Strains and Maintenance. The strains used in this study were N2 var. Bristol, CF1038 *[daf-16(mu86) I.]*, CF1553 *[muIs84(sod-3p::gfp)]*, and CL2166 *[pAF15(gst-4p::GFP::NLS)]*. All strains were provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Strains were maintained on nematode growth medium (NGM) agar plates at 20°C containing a lawn of *E. coli* var. OP50 (provided by the CGC) as the food source, as described previously [32]. Treatment of *C. elegans* with the substances was performed in 2 mL of liquid NGM containing 1% (w/v) bovine serum albumin, 50 μg/mL streptomycin, and 10^9 OP50-1/mL (provided by the CGC) in 35 mm petri dishes (Greiner Bio-One, Frickenhausen, Germany). Stock solutions (100 mM) were prepared with DMSO. In all assays, the substances were used in a final concentration of 50 or 100 μM; 0.1% (v/v) DMSO was used as the solvent control. Age synchronous animals were obtained by sodium hypochlorite treatment of gravid adults. Briefly, gravid adults were rinsed off from NGM agar plates with liquid NGM, collected in 0.5 mL liquid NGM and mixed with 0.5 mL bleaching solution (50% 5 M NaOH/50% NaClO). Nematodes were then incubated at room temperature for three minutes, occasionally vortexed, pelleted by centrifugation (5000 rpm/4°C/1 min) and the supernatant was discarded. The animal pellet was washed three times with liquid NGM and transferred onto fresh NGM agar plates (containing OP50 lawn) and maintained for three days at 20°C to obtain an age synchronous population of mainly L4 larvae.

2.4. Determination of Antioxidative Capacity In Vitro. To determine the antioxidative capacity of quercetin, resveratrol, and TSG, the TEAC assay was used. This assay is a cell-free method for the measurement of radical scavenging

properties of compounds [33]. The principle of this reaction is a reductive conversion of a stable, blue-green radical by an antioxidant. The solution decolorizes when an antioxidant is added and can be quantified spectrophotometrically. The decolorisation of the radical solution indicates the antioxidative capacity of a compound which is compared to the potency of the reference substance trolox, which is a synthetic vitamin E derivative. The reference- and test-substances were measured in a concentration range from 0 to 25 μM. The radical scavenging activity was measured at 734 nm spectrophotometrically (Lambda 25 UV/VIS Spectrometer, Perkin Elmer, Wellesley, MA, USA) two minutes after starting the reaction.

2.5. Measurement of Intracellular ROS Accumulation In Vivo. The fluorescent probe H_2DCF-DA (2′,7′-dichlorodihydrofluorescein-diacetate) was used to detect the intracellular ROS level in living individual nematodes. H_2DCF-DA is able to freely cross cell membranes; however, after entering the cell, nonfluorescent H_2DCF-DA becomes deacetylated to form the nonfluorescent derivative H_2DCF that is trapped within the cell. Then H_2DCF can quickly be oxidised by intracellular ROS to form fluorescent DCF that can be measured in a fluorescence spectrophotometer (excitation wavelength 485 nm; emission wavelength 535 nm). The fluorescence intensity correlates with the intracellular amount of ROS; no saturation of the DCF fluorescence was observed up to 8 h of persistent thermal stress. The experiment was performed as described elsewhere [34]. Briefly, L4 larvae were incubated in liquid NGM ± the compounds (50/100 μM) or 0.1% DMSO for 48 hours at 20°C. During the incubation period, nematodes were transferred to fresh culture media daily. After 48 hours, all animals were transferred into M9T (M9 buffer containing 0.1% Tween 20) for one hour. Then single nematodes were transferred individually in 1 μL M9T into each well of a 384-well plate (384-well μClear plate, Greiner Bio-One, Frickenhausen, Germany) containing 7 μL M9 buffer. Subsequently, when all animals were transferred, 2 μL H_2DCF-DA (250 μM in PBS) was added into each well to obtain a final concentration of 50 μM H_2DCF-DA. A black backing tape (Perkin Elmer) was applied to the top of the plate to avoid evaporation. ROS accumulation was induced by thermal stress at 37°C and recorded every 15 min for a period of 12 hours in a fluorescence spectrophotometer (Wallac Victor^2 1420 Multilabel-Counter, Perkin Elmer, Wellesley, MA, USA).

2.6. Determination of Lipofuscin Accumulation. Over the lifetime of *C. elegans*, the autofluorescent pigment lipofuscin accumulates in gut granules and serves as an established marker of ageing. Randomly picked L4 larvae were placed in liquid NGM ± the compounds (50/100 μM) as described above and incubated for 72 hours at 20°C, followed by 24 hours of incubation in compound free medium. During the incubation period, nematodes were transferred to fresh culture media daily. Lipofuscin fluorescence of seven-day-old nematodes was detected by fluorescence microscopy (excitation wavelength 360–370 nm; emission wavelength 420–460 nm; Olympus BX43; Olympus, Hamburg, Germany) and analysed densitometrically (ImageJ, National Institutes of Health, Bethesda, MD, USA). The experiment was repeated three times and 20 animals per group and experiment were analysed.

2.7. Induction of Antioxidative Enzymes. Immediately after the bleaching procedure, synchronised eggs of the transgenic strains CF1553 (expressing a *sod-3p::gfp* reporter) or CL2166 (expressing a *gst-4p::gfp::nls* reporter) were incubated in liquid NGM containing 100 μM of quercetin, resveratrol, TSG, or 0.1% DMSO, respectively, as described above and incubated for five days at 20°C. After five days of drug treatment, nematodes were placed in PBST (phosphate buffered saline containing 0.1% Tween 20) for 30 minutes to wash off residual bacteria. Each group was then separated into two groups, exposing one of each group to 150 μM of the juglone for 3 hours. The naphthoquinone juglone is a redox cycler, which results in the generation of reactive oxygen species. A cyclic process consisting of (a) reduction of a compound, followed by (b) autoxidaton of the reaction product leads to a prolonged production of ROS. 10 to 15 animals from each group were placed onto microscope slides, anesthetized in 10 μL of 10 mM levamisole, and covered with cover slips. Epifluorescence images were collected from an Axiolab fluorescence microscope (Zeiss, Göttingen, Germany) using a suitable filter set with a CoolSnap CF Digital Monochrome Camera (Intas, Göttingen, Germany) equipped with the Image Pro-Plus software (version 4.5, MediaCybernetics, Silver Spring, MD, USA). Densitometric analysis of GFP expression of the head and anterior intestinal area of at least 10 animals per group and experiment was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Thermotolerance Assay. Survival of individual nematodes at the lethal temperature of 37°C was monitored with an assay according to Gill et al. [35] and Kampkötter et al. [34]. After treating wild type L4 larvae for 48 hours with the compounds (50/100 μM) or 0.1% DMSO (daily transfer of the animals into fresh culture medium), nematodes were washed in PBST for one hour and then individually transferred in 1 μL PBST into the wells of a 384-well plate (384-well μClear plate, Greiner Bio-One, Frickenhausen, Germany) containing 9 μL PBS. Following the complete transfer of the nematodes, 10 μL of 2 μM SYTOX Green Nucleic Acid Stain (Molecular Probes Inc.; Leiden, The Netherlands) in PBS were added to each well and the plate was sealed using black backing tape (Perkin Elmer) to avoid evaporation. SYTOX Green Nucleic Acid Stain is unable to pass the membranes of intact cells. However, thermal stress causes an impairment of the cellular membrane, thereby enabling the dye to enter the cells. There the dye binds to DNA and exerts a bright fluorescence that can be used as a marker for cellular damage and thus for the viability of individual nematodes [35]. The fluorescence intensity was determined with a fluorescence spectrophotometer (Wallac Victor^2 1420 Multilabel-Counter, Perkin Elmer, Wellesley, MA, USA) and was recorded every 15 min for 12 hours (excitation wavelength 485 nm; emission

wavelength 535 nm). The fluorescence curve of each nematode was calculated and the individual cut-off value was determined by multiplying the average fluorescence of the first four measurements by the factor 3. The time point when the fluorescence exceeded the cut-off value for each well was defined as the point of death of the respective nematode. The factor 3 in the calculation of the cut-off value was previously shown to be adequate [35]. Survival curves and mean survival times were determined from these individual times of death (Kaplan-Meier survival analysis, IBM SPSS 19). Experiments were repeated at least three times.

2.9. Life Span Assays. Life span analyses were performed with N2 (three independent experiments) and CF1038 *[daf-16(mu86) I.]* (two independent experiments). About 30–50 L4 larvae per group and experiment were placed in liquid NGM ⊥ compounds (50/100 μM) as described above and incubated at 25°C. The starting day in liquid culture was considered as day 0 of the life span. Nematodes were transferred daily to new culture dishes during their fertile period to prevent overcrowding and to discriminate the test nematodes from their progeny. After the fertile period, nematodes were transferred to fresh medium every other day. Nematodes were scored as dead when they did not respond to gentle prodding and when they showed no pharyngeal pumping movement. Lost nematodes and animals containing hatched larvae were censored. Kaplan-Meier survival analysis was used to detect statistical differences.

2.10. Statistics. Data are given as mean ± S.E.M of at least 3 independent experiments. Statistical analysis was performed with SPSS 19 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., LaJolla, USA) software. Statistical significance was determined by one-way ANOVA with Bonferroni posttest. Life span analyses were performed using Kaplan-Meier survival analysis; animals that were lost, killed, or showed internal hatching were censored. Differences were considered to be significant at $P < 0.05$.

3. Results and Discussion

3.1. Antioxidative Capacity of TSG. To investigate if TSG may exert antioxidative effects in *C. elegans*, we have analysed the radical scavenging capacity of this compound in the trolox equivalent antioxidative capacity (TEAC) assay. In this cell-free assay, the potency of a substance to reduce and thereby decolourise a green radical is detected. We have compared the antioxidative capacity of TSG with the structural analogue resveratrol and quercetin, a major flavonoid with a well-known antioxidant capacity. TSG possesses a strong antioxidative capacity in this system (Figure 1(b)). Even at the lowest concentration analysed (5 μM), TSG showed a strong antioxidative effect; about 30% of the ABTS radical was reduced. The compound reduced the ABTS radical even more efficiently than resveratrol over the complete concentration range. Up to a concentration of 15 μM, TSG shows a higher antioxidative capacity than trolox, the synthetic vitamin E derivative that was used as a reference substance. Out of the compounds analysed, only the flavonoid quercetin showed stronger antioxidative effects over the complete concentration range. The superior antioxidative capacity of quercetin in the TEAC assay can be explained by differences in the molecular structure of this compound compared to resveratrol and TSG. In contrast to the both stilbene derivatives, the flavonoid possesses redox-active moieties which facilitate the reduction of the ABTS radical. These groups are, for example, the catechol group in ring B in combination with the 3-OH-group in ring C. These groups are able to donate electrons to the ABTS radical forming stable semiquinone radicals and quinoid structures. In the case of the stilbene derivatives, the stabilisation of the oxidized form is not favoured as in case of quercetin.

Next, we investigated the antioxidative effects of TSG in the nematode *C. elegans*. To analyse the antioxidative potential *in vivo*, the DCF assay was used. DCF is a probe that becomes highly fluorescent after oxidation; therefore the DCF fluorescence was taken as a marker for the formation of reactive oxygen species within the organism. We induced the formation of reactive oxygen species in *C. elegans* by application of thermal stress (37°C). As shown in Figure 2, the amount of fluorescent DCF increases over time after the onset of stress conditions. After 180 minutes, the DCF fluorescence of DMSO-treated nematodes was approximately 12.4-fold higher compared to the DMSO value at $t = 0$ (3276 ± 22 rfu → 40600 ± 2055 rfu). Treatment of the nematodes for 48 h with 100 μM TSG reduced the stress-induced increase in ROS: after 180 min, the rfu-value was 36378 ± 1926. The experiments performed with resveratrol showed no significant modulation of the DCF fluorescence. However, an incubation with the well-known antioxidant flavonoid quercetin caused a significant reduction of DCF fluorescence at 100 μM.

Antioxidative effects of resveratrol are extensively described in the literature: Jang and Surh [36] described protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells; also in H4IIE rat hepatoma cells antioxidative effects were reported in the DCF assay [37]. In a more recent paper, Vanaja et al. [38] described an improvement of the antioxidative properties of resveratrol after loading into liposomes. The antioxidative properties of resveratrol are further reviewed by, for example, Farghali et al. [39]. Concerning the antioxidative effects of TSG, less information is available: Kim et al. [40] reported protective effects of an extract of *Polygonum multiflorum* against oxidative toxicity in HT22 hippocampal cells without showing active components of the extract. Chen et al. [15] identified antioxidative components of *Polygonum multiflorum* using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (similar to TEAC assay). They reported a strong antioxidative capacity by only three compounds: gallic acid, catechin, and TSG. Further antioxidative effects are reported by Zhang et al. [41] demonstrating protective effects of TSG against hydrogen peroxide-induced dysfunction and oxidative stress in osteoblastic MC3T3-E1 cells; Tao et al. [21] report a protective effect of TSG on 6-OHDA-induced apoptosis in PC12 cells and Li et al. [24] demonstrate that TSG attenuates

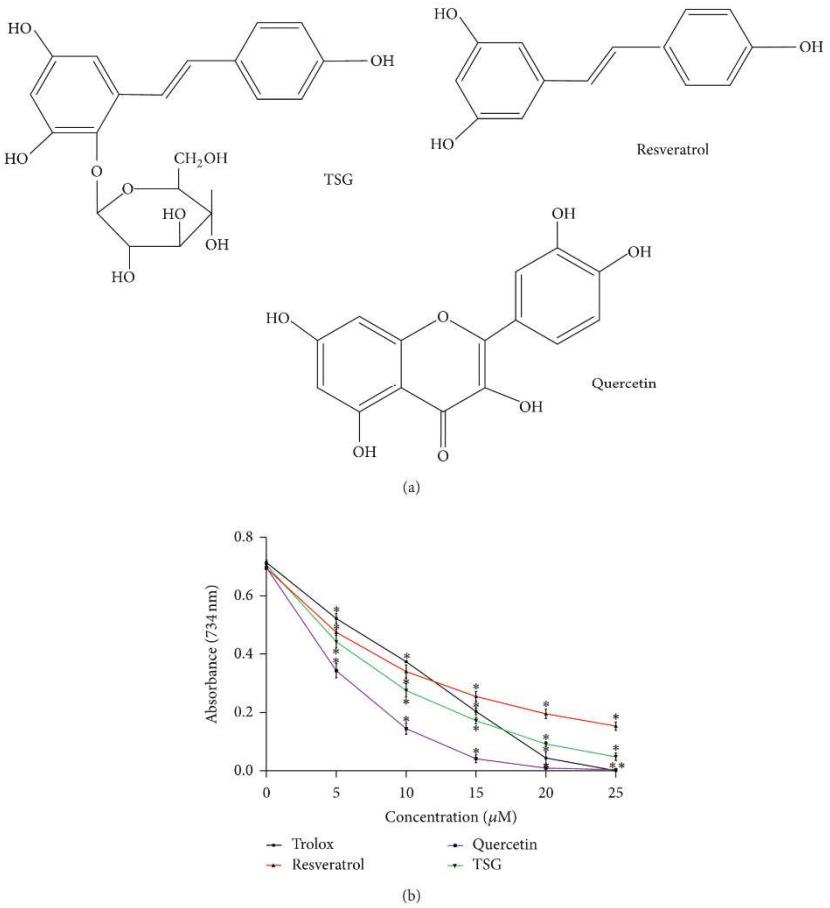

FIGURE 1: Radical scavenging properties (TEAC assay) of TSG. Chemical structures of resveratrol, quercetin, and TSG are shown in (a); the antioxidative capacity of the substances measured by the TEAC assay is shown in (b). The decolourisation of the radical-solution was detected spectrophotometrically at 734 nm (mean values ± SD, $n = 3$, $^{*}P < 0.05$ versus control value, Student's t-test).

MPP^{+}-induced apoptosis in PC12 cells by inhibiting ROS generation.

3.2. Modulation of Antioxidative Enzyme Expression by TSG. Polyphenols like TSG may protect against oxidative stress either by directly scavenging radicals or by indirectly increasing the stress resistance of the organism, for example, by induction of antioxidative enzymes. We have investigated the effects of TSG on the induction of glutathione-S-transferase-4 (GST-4) and superoxide dismutase-3 (SOD-3), two enzymes that are known to be inducible by oxidative stress.

To analyse the induction of SOD-3::GFP expression in *C. elegans*, we used the transgenic strain CF1553 (*muIs84 [pAD76(sod-3::gfp)]*). Treatment with TSG, resveratrol, or quercetin showed no significant influence on the expression of SOD-3 under basal conditions (Figure 3). Under stress conditions (150 μM of the redox-cycler juglone), SOD-3 expression is induced in *C. elegans*. Compared to the GFP fluorescence of nematodes under basal conditions (657.3 rfu), the fluorescence increased to 970.8 rfu under conditions of oxidative stress. This result shows that the redox-state in the nematode after application of juglone was shifted to the prooxidative state; a relatively high induction of

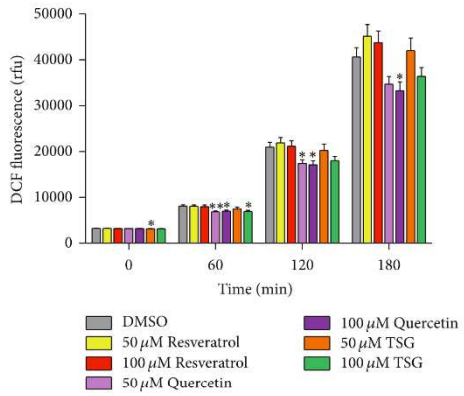

FIGURE 2: Modulation of ROS accumulation in *C. elegans* by TSG. Modulation of ROS accumulation in wild type nematodes at 37°C after incubation with TSG, quercetin, and resveratrol (50, 100 μM). The fluorescence intensity of DCF (rfu) was used as a marker for intracellular ROS; mean ± SEM, 5–7 independent experiments; at least 80 animals per concentration were analysed; $^*P < 0.05$ versus *DMSO value*, one-way ANOVA with Dunnett's post hoc test.

the antioxidative enzyme SOD-3 was needed to compensate this prooxidative condition. Treatment of the nematodes with 100 μM TSG starting 48 h prior to the application of juglone results in a significantly reduced induction of SOD-3 expression (704.1 rfu → 738.7 rfu). This may be interpreted as a reduction of the juglone-induced prooxidative status of the nematode that in consequence results in a lower expression of SOD-3. The effect of resveratrol was comparable to the effect of TSG; the highest modulation was caused by quercetin.

Next, we analysed the effects of TSG on the induction of GST-4, an enzyme that is thought to be involved in the defence against conditions of stress due to the induction via SKN-1. Similar to the SOD-3 reporter gene experiment, we used a transgenic strain (CL2166 *dvIs19 [pAF15(gst-4::gfp::NLS)]*) that expresses GFP under control of the *gst-4* promoter (Figure 4). Comparable to the expression of SOD-3, the expression of GST-4 is strongly increased under stress conditions. A 6.9-fold increase in the GFP fluorescence was detected after application of juglone (150.62 ± 5 rfu → 1043.16 ± 42 rfu). In contrast to the effect observed with the transgenic strain CF1553 (= SOD-3 reporter), TSG and resveratrol only slightly modulated the induction of GST-4; only a tendency can be suggested. Quercetin was the only compound that significantly diminished the induction of GST-4 after application of oxidative stress. The modulation of SOD-3 and GST-4 by the compounds can be explained in two ways. On the one hand, the direct antioxidative potential of the compounds reduces the oxidative stress which is generated by the redox-cycler juglone. The reduction in oxidative stress consequently reduces the amount of antioxidative enzymes which are induced. On the other hand, the reduction of SOD-3 induction may also be due to an indirect antioxidative effect of the compounds by specific activation of the antioxidative response system of the nematode.

Robb and Stuart [42] reported a significantly increased MnSOD expression by resveratrol in mouse C2C12 and primary myoblasts. Khan et al. [43] reported that treatment with 25 μM resveratrol significantly increased SOD activity in PC-3, HepG2, and MCF-7 cells, but not in HEK293T cells. Kavas et al. [44] reported an increase of SOD activity in male Wistar rats by resveratrol (20 mg/kg in drinking water for six weeks). Li et al. [45] reported that resveratrol treatment significantly increased the mRNA expression of GSTA1 in a time-dependent manner. On the other hand, Jiang et al. [46] showed that resveratrol attenuates early diabetic nephropathy by downregulating glutathione S-transferase Mu in diabetic rats. In contrast to the vast information about resveratrol, to our best knowledge, no information about TSG and modulation of antioxidative enzymes is available.

3.3. Modulation of Stress Resistance by TSG. Since TSG reduces the abundance and induction of reactive oxygen species in *C. elegans*, we investigated if the stilbene derivative also mediates a resistance against thermal stress (37°C) which has been shown to be lethal in *C. elegans*. We have analysed the effects of TSG, resveratrol, and quercetin on the tolerance of *C. elegans* against thermal stress using the semiautomated SYTOX Green assay (Figure 5). The mean and median survival time of DMSO-treated nematodes were determined as 4.82 ± 0.14 h and 4.75 ± 0.17 h, respectively. TSG strongly increases the resistance against thermal stress: 50 μM TSG induces a 22% increase in the mean survival time. The mean and median survival time of TSG-treated nematodes were determined as 5.89 ± 0.11 h and 6.00 ± 0.1 h, respectively. In case of stress resistance, TSG was shown to be the most active compound analysed (Table 1). Combined with the experiments shown before, this result clearly indicates that TSG has a high potential to protect *C. elegans* against stress conditions. Chen et al. [47] reported that resveratrol alleviated juglone-induced lethal oxidative stress and significantly prolonged the survival time of *C. elegans* under conditions of acute oxidative damage. However, no information is available about effects of TSG on stress resistance.

3.4. Prolongation of Life Span by TSG. With increasing age, highly oxidised and cross-linked proteins accumulate in the intestine of the nematode. These modified molecules form insoluble, autofluorescent ageing pigments, for example, lipofuscin. We investigated the effect of TSG, resveratrol, and quercetin on this phenomenon (i) to verify the antioxidative effects of the compounds in the nematode and (ii) to estimate effects of these compounds on the ageing process in *C. elegans*.

Treatment with TSG reduces the intestinal lipofuscin accumulation in *C. elegans* by 20 ± 1% compared to the solvent control (DMSO: 952 ± 19 rfu; TSG 817 ± 16 rfu). Resveratrol showed a slightly weaker decrease in lipofuscin fluorescence (846.8 rfu), while quercetin again showed the strongest

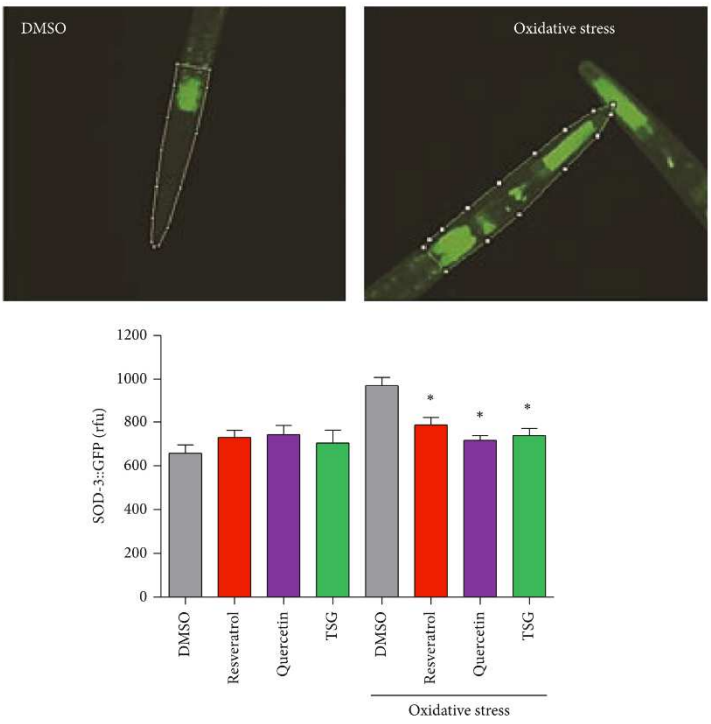

FIGURE 3: Modulation of SOD-3 expression by TSG. The images show GFP fluorescence of the head and anterior part of the intestine of CF1553 transgenic nematodes pretreated with 100 μM of the compounds without (DMSO) or with oxidative stress (150 μM juglone; 3 h). Data represent mean values ± SEM, $n = 3$, $^*P < 0.05$ versus corresponding DMSO value, one-way ANOVA with Dunnett's post hoc test. At least 10 animals per group and experiment were analysed.

reduction in this parameter. Incubation with 100 μM quercetin reduces the lipofuscin fluorescence by 27.85% (686.9 ± 14 rfu). These results show that TSG is capable of decreasing lipofuscin accumulation in *C. elegans* (Figure 6(a)).

Since it is known that a reduction of lipofuscin mostly correlates with an increase in life span of *C. elegans*, we investigated the effect of TSG, resveratrol, and quercetin on the life span of *C. elegans*. All three compounds exerted similar effects (Figure 6(b)). The mean life span of DMSO-treated nematodes was 17.4 ± 0.56 days; in case of TSG, resveratrol, and quercetin, the mean life span was 21.1 ± 0.58 d, 20.7 ± 0.67 d, and 21.3 ± 0.5 d, respectively (Table 2). The median life span of DMSO-treated nematodes was 18.0 ± 0.61 days; in case of TSG, resveratrol, and quercetin, the median life span was 22.0 ± 0.81 d, 22.0 ± 0.71 d, and 23.0 ± 1.01 d, respectively.

From the results of the DCF assay we can conclude that the radical scavenging effects of the compounds cannot be the only explanation for their positive effects on the life span of *C. elegans*. The antioxidative capacity of resveratrol was negligible in comparison to TSG and quercetin, but the effect on the life span of the nematode is comparable to the other two compounds.

To clarify this point, we analysed if the compounds may modulate intracellular pathways to prolong the life span of *C. elegans*. Therefore we used a mutant strain that contains a deletion in the *daf-16* gene leading to a loss-of-function of the corresponding protein. This transcription factor (FoxO homologue in *C. elegans*) has a crucial function in the regulation of ageing. By using a mutant strain defective in this pathway, we analysed if this factor is involved in the life span-prolonging effect of TSG. As we see in Figure 7, the life span extending effect of TSG, quercetin, and resveratrol was not abolished. We conclude that this pathway is not necessary to mediate the effects of TSG, quercetin, and resveratrol.

Resveratrol has been reported to be beneficial in cases of ageing-related cardiovascular and neurodegenerative diseases. However, previous studies on the longevity promoting effect of resveratrol have been partly inconclusive. Upadhyay et al. [48] reported an increase of life span after treatment

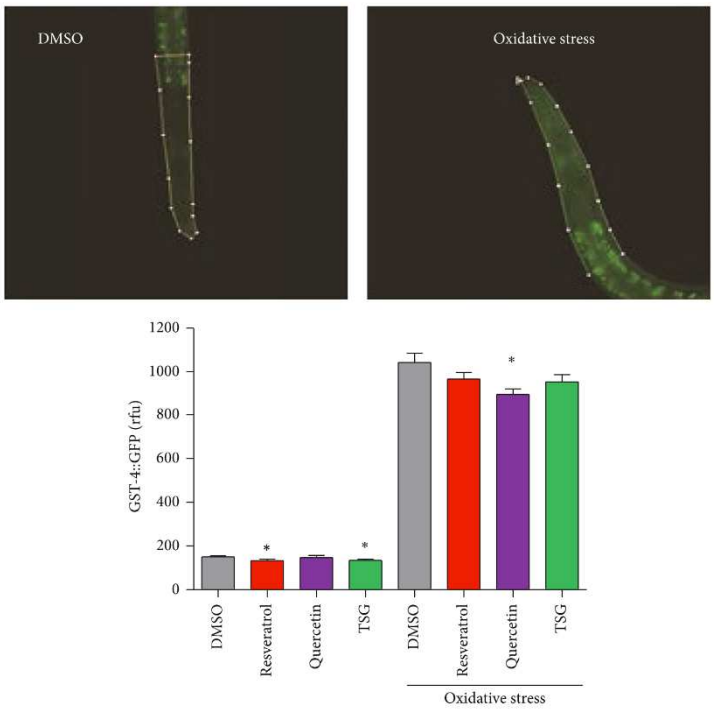

FIGURE 4: Modulation of GST-4 expression by TSG. The images show GFP fluorescence of the head and the anterior part of the intestine of CL2166 transgenic nematodes pretreated with 100 μM of the compounds followed by an incubation under physiological conditions or under conditions of oxidative stress (150 μM Juglone; 3 h). Data represent mean values ± SEM, $n = 3$, $^{*}P < 0.05$ versus corresponding DMSO value, one-way ANOVA with Dunnett's post hoc test. At least 10 animals per group and experiment were analysed.

with resveratrol (100 μM). Zarse et al. [49] reported that resveratrol significantly extends *C. elegans* life span already at a concentration of 5 μM by 3.6% (mean life span) and 3.4% (maximum life span). On the other hand, Chen et al. [47] observed no extension of the normal life span of *C. elegans* in either liquid or solid growth media containing different concentrations of resveratrol. Bass et al. [50] also analysed effects of resveratrol in *C. elegans* (wild type and sir-2.1 mutant nematodes) but results were variable. Resveratrol treatment results in slight increases in life span in some trials but not others (wild type and sir-2.1 mutant animals). As an explanation for the different effects there may be variations from one study to another concerning the delivery of the compounds to the nematodes. The use of liquid or solid growth media containing different concentrations of resveratrol makes it also difficult to compare results between studies. In our study, we confirm results of Upadhyay et al. [48] showing an extension of life span after incubation with resveratrol. Furthermore, we were the first to show that also application of TSG results in a prolongation of life span comparable to resveratrol. This finding may be a hint for the active component in the *Polygonum multiflorum* extract, which is traditionally used as an anti-ageing drug. It has to be mentioned that no adverse effects of the compounds (TSG, resveratrol, and quercetin) were detectable in the experimental assays up to a concentration of 100 μM. This is important since it was reported that other natural compounds cause toxic effects to the nematode: Mukai et al. [51] reported that a gallate of tannin isolated from the tea plant *Camellia sinensis* L. is toxic to *C. elegans* (LC_{50}: 49 μM).

4. Conclusion

2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (TSG) is the main bioactive component of *Polygonum multiflorum*, a plant which is traditionally used as an anti-ageing agent in many East Asian countries. This compound causes antioxidative effects in *C. elegans*, alleviates the accumulation of lipofuscin, and prolongs the mean life span by 23.5% independently of DAF-16. Furthermore, the stress resistance of the nematode is strongly enhanced by this compound. In addition to direct antioxidative effects of TSG, this compound

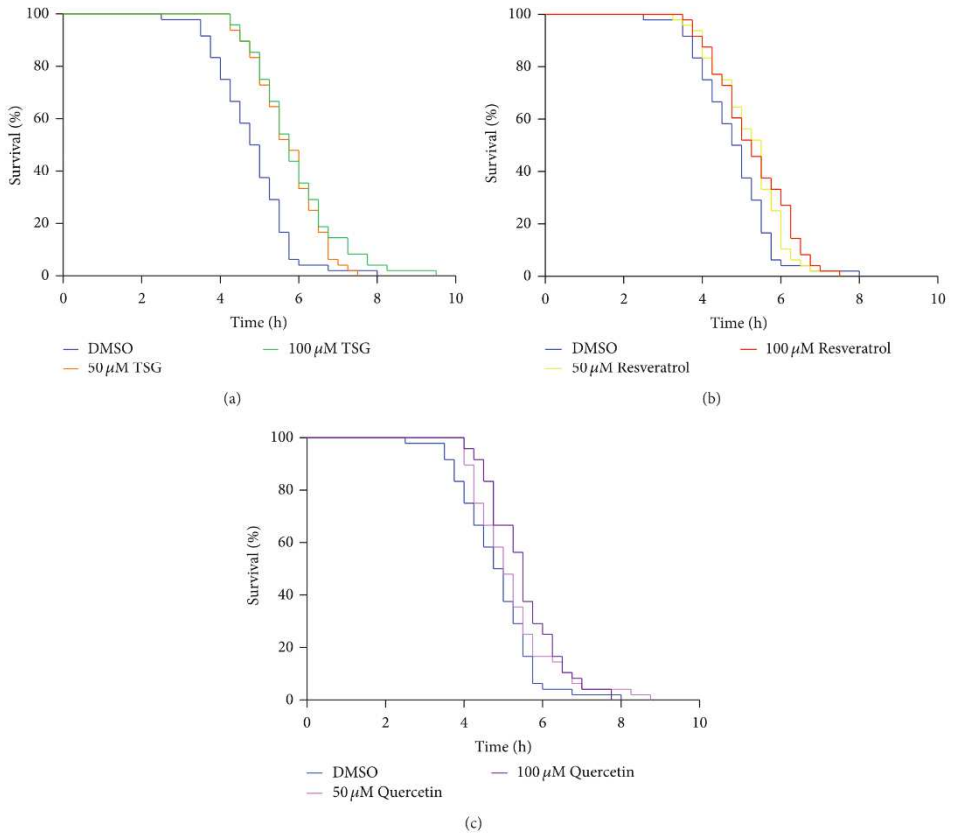

FIGURE 5: Increased resistance against lethal heat-stress by treatment with TSG. TSG treatment increases the resistance against thermal stress at 50 μM and 100 μM (a). Resveratrol treatment (b) and quercetin treatment effectively increased the survival only at 100 μM. Survival curves represent the data of 4 independent experiments with a total of 48 animals per group (Kaplan-Meier survival analysis); corresponding data are summarised in Table 1.

TABLE 1: Summary of the heat-stress survival data depicted in Figure 5.

	Adult survival [$h \pm$ SEM] at 37°C			
Treatment	Mean	Median	n	P versus DMSO (log-rank)
DMSO	4.82 ± 0.14	4.75 ± 0.17	48	
Quercetin (50 μM)	5.22 ± 0.15	5.00 ± 0.16	48	0.10
Quercetin (100 μM)	5.50 ± 0.13	5.50 ± 0.09	48	<0.001
Resveratrol (50 μM)	5.20 ± 0.13	5.25 ± 0.16	48	0.05
Resveratrol (100 μM)	5.29 ± 0.14	5.25 ± 0.24	48	<0.05
TSG (50 μM)	5.89 ± 0.11	6.00 ± 0.10	48	<0.001
TSG (100 μM)	5.94 ± 0.17	5.75 ± 0.12	48	<0.001

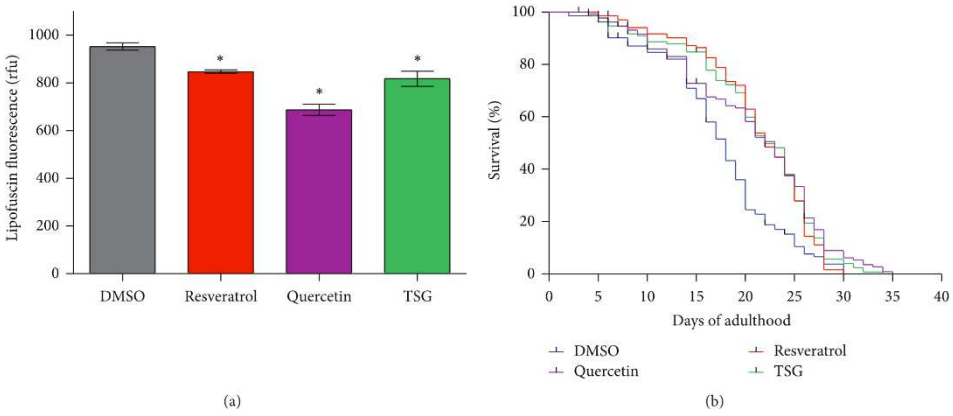

FIGURE 6: Prolongation of life span by treatment with TSG. (a) Pretreatment with 100 μM of TSG, resveratrol, and quercetin reduced the accumulation of the ageing marker lipofuscin (mean values ± SEM, $n = 3$, $^*P < 0.05$ versus control value, Student's t-test). 20 animals per group and experiment were analysed. (b) All compounds (100 μM) induced a prolongevity effect during the complete adult life (Kaplan-Meier survival analysis of three independent experiments with at least 30 animals per group/experiment; survival data are summarised in Table 2).

TABLE 2: Summary of the life span analyses of wild type nematodes (Figure 6(b)) and *daf-16* loss-of-function mutants (strain CF1038) (Figure 7).

Wild type	Adult life span [d ± SEM]			
Treatment	Mean	Median	n (censored)	P versus DMSO (log-rank)
DMSO	17.39 ± 0.56	18.00 ± 0.61	121 (14)	
Quercetin (100 μM)	20.68 ± 0.67	23.00 ± 1.00	118 (17)	<0.001
Resveratrol (100 μM)	21.31 ± 0.50	22.00 ± 0.71	129 (6)	<0.001
TSG (100 μM)	21.14 ± 0.58	22.00 ± 0.81	128 (7)	<0.001
daf-16(mu86)	Adult life span [d ± SEM]			
Treatment	Mean	Median	n (censored)	P versus DMSO (log-rank)
DMSO	11.05 ± 0.35	11.00 ± 0.37	96 (4)	
Quercetin (100 μM)	12.64 ± 0.43	14.00 ± 0.51	94 (6)	<0.001
Resveratrol (100 μM)	12.01 ± 0.40	11.00 ± 0.38	97 (3)	0.018
TSG (100 μM)	12.21 ± 0.41	12.00 ± 0.63	94 (6)	0.02

also causes indirect antioxidative effects in *C. elegans* via modulation of SOD-3 and GST-4. Our results strongly confirm the potential of TSG to be used as a pharmaceutical anti-ageing drug.

Abbreviations

DCF: 2′,7′-Dichlorofluorescein
DMSO: Dimethylsulfoxid
GST: Glutathione-S-transferase
rfu: Relative fluorescence unit
ROS: Reactive oxygen species
SOD: Superoxide dismutase
TCM: Traditional Chinese medicine
TEAC: Trolox equivalent antioxidative capacity assay
TSG: 2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Christian Büchter and Liang Zhao contributed equally.

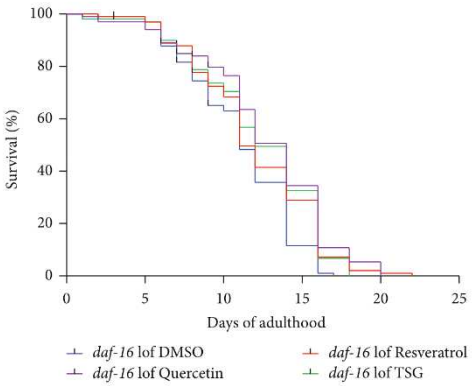

FIGURE 7: The TSG-mediated prolongation of life span is independent of DAF-16. Treatment with the compounds (100 μM) during the complete adult life induced a prolongevity effect in the *daf-16* loss-of-function mutant strain CF1038 (Kaplan-Meier survival analysis of two independent experiments with at least 30 animals per group/experiment; survival data are summarised in Table 2).

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3.5 Studie 5: The resveratrol derivatives trans-3,5-dimethoxy-4-fluoro-4´-hydroxystilbene and trans-2,4´,5-trihydroxystilbene decrease oxidative stress and prolong lifespan in *Caenorhabditis elegans*

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The resveratrol derivatives trans-3,5-dimethoxy-4-fluoro-4′-hydroxystilbene and trans-2,4′,5-trihydroxystilbene decrease oxidative stress and prolong lifespan in *Caenorhabditis elegans*

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Keywords
ageing; insulin-signalling; Nrf2; oxidative stress; secondary plant compounds

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Abstract

Objectives Resveratrol (*trans*-3,4′,5-trihydroxystilbene (**1**)) was previously shown to extend the lifespan of different model organisms. However, its pharmacological efficiency is controversially discussed. Therefore, the bioactivity of four newly synthesized stilbenes (*trans*-3,5-dimethoxy-4-fluoro-4′-hydroxystilbene (**3**), *trans*-4′-hydroxy-3,4,5-trifluorostilbene (**4**), *trans*-2,5-dimethoxy-4′-hydroxystilbene (**5**), *trans*-2,4′,5-trihydroxystilbene (**6**)) was compared to (**1**) and pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene (**2**)) in the established model organism *Caenorhabditis elegans*.

Methods Trolox equivalent antioxidant capacity (TEAC), 2′,7′-dichlorofluorescein (DCF), thermotolerance assays, *C. elegans* lifespan analyses.

Key findings All compounds exert a strong in-vitro radical scavenging activity (**6** > **1** > **5** > **2** = **3** = **4**), but *in vivo*, only (**3**) and (**6**) reduce reactive oxygen species (ROS) accumulation. Furthermore, (**3**) and (**6**) increased the mobility of aged nematodes and prolonged their mean lifespans, while these compounds decreased the thermal stress resistance. Using *daf-16* (FoxO), *skn-1* (Nrf2) and *sir-2.1* (sirtuin) loss-of-function mutant strains, the *in vivo* antioxidant effects of compounds (**3**) and (**6**) were abolished, showing the necessity of these evolutionary highly conserved factors. However, short-time treatment with stilbenes (**3**) and (**6**) did not modulate the cellular localization of the transcription factors DAF-16 and SKN-1.

Conclusion In contrast to resveratrol, the synthetic stilbene derivatives (**3**) and (**6**) increase the lifespan of *C. elegans*, rendering them promising candidates for pharmacological anti-ageing purposes.

Introduction

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a naturally occurring phytoalexin which can be found in various plants, for example grapes. This compound is discussed to mediate numerous beneficial effects to human health, such as protection against morbus Alzheimer,[1] macular degeneration[2] as well as cancer.[3,4] As this compound can be found in relatively high concentrations in red wine, it is claimed to be responsible for beneficial effects associated with a modest consumption of red wine (the so-called French paradox[5]). Diverse molecular mechanisms are discussed for the beneficial effects induced by this natural compound, such as anti-inflammatory and antioxidant effects as well as modulation of different intracellular signalling pathways.[6] Antioxidant and radical scavenging activity of resveratrol may be important for the possible life-prolonging effects of resveratrol as both physiological and pathological ageing processes are characterized by changes in the amount of reactive oxygen species (ROS).[7]

Resveratrol is able to enhance the lifespans of different organisms, for example *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Nothobranchius furzeri* and mice.[8–12] Up to now, several studies have been performed to demonstrate the anti-ageing effects of resveratrol in the nematode *Caenorhabditis elegans*; however, the results obtained so far are manifold. Compared with resveratrol, structurally related stilbene derivatives like 2,3,4′,5,-tetrahydroxystilbene-2-*O*-β-D-glucoside (TSG) were shown to induce even stronger effects in *C. elegans*: while resveratrol was unable to reduce the ROS level at 50 μM, administration of 50 μM TSG strongly decreased (−26%) the ROS accumulation.[13] This leads to the hypothesis that derivatives of resveratrol may be more potent than resveratrol itself and it also raises the question, which structural element(s) of resveratrol's basic stilbene structure need to be changed to improve its beneficial health effects. In this study, we evaluated the bioactivity of four newly synthesized compounds (*trans*-3,5-dimethoxy-4-fluoro-4′-hydroxystilbene (**3**), *trans*-4′-hydroxy-3,4,5-trifluorostilbene (**4**), *trans*-2,5-dimethoxy-4′-hydroxystilbene (**5**) and trans-2,4′,5-trihydroxystilbene (**6**)), and compared it to the well-known natural compounds resveratrol (**1**) and pterostilbene (**2**) (Figure 1). *In vitro*, compounds (**5**) and (**6**) have previously shown to exert stronger antioxidant activity (DPPH assay) compared with resveratrol[14] and all new derivatives (**2–6**) induced much stronger toxic effects in NIH 3T3 mouse embryonic fibroblast cells: The IC_{50} values for compounds (**2**)–(**6**) were calculated as 12.2, 11.5, 6.9, 7.4 and 9.6 μM, respectively, and they were much lower than the IC_{50} value of resveratrol (24.2 μM).[15] Furthermore, compound (**6**) was shown to be a more potent inhibitor of acetylcholinesterase and butyrylcholinesterase compared with resveratrol, which may be relevant for application as an anti-Alzheimer therapeutic.[16] We investigated the effects of newly synthesized resveratrol analogues on stress resistance, antioxidant effects and the ageing process in *C. elegans* as well as signalling pathways involved in these effects. The comparison of these newly synthesized derivatives with the established compound resveratrol is very promising in terms of structure–activity relationship, as no data are available concerning molecular effects of these resveratrol derivatives *in vivo*.

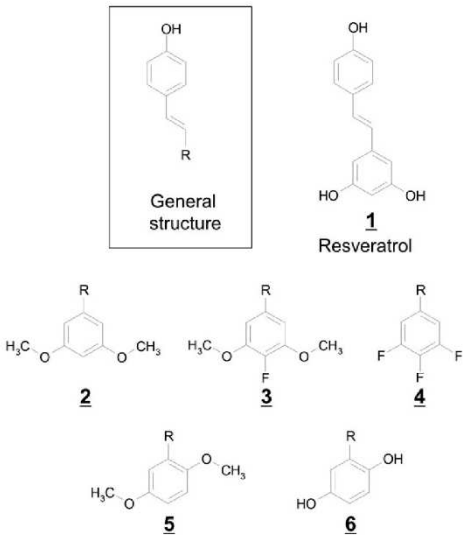

Figure 1 Structure of resveratrol and derivatives used. Compound (**1**): 3,4′,5-trihydroxystilbene (resveratrol), compound (**2**): *trans*-3,5-dimethoxy-4′-hydroxystilbene (pterostilbene), compound (**3**): *trans*-3,5-dimethoxy-4-fluoro-4′-hydroxystilbene, compound (**4**): *trans*-4′-hydroxy-3,4,5-trifluorostilbene, compound (**5**): *trans*-2,5-dimethoxy-4′-hydroxystilbene, compound (**6**): *trans*-2,4′,5-trihydroxystilbene.

Materials and Methods

Materials

SYTOX® Green nucleic acid stain was obtained from Molecular Probes Inc. (Leiden, the Netherlands). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich (Deisenhofen, Germany).

Caenorhabditis elegans strains and maintenance

The strains used in this study were the wild-type N2 var. Bristol, CF1038 *[daf-16(mu86) I.]*, EU1 *[skn-1(zu67) IV/nT1 [unc-?(n754) let-?] (IV;V).]*, VC199 *[sir-2.1(ok434) IV.]*, TJ356 *[zIs356 IV (pdaf-16-daf-16::gfp; rol-6)]* and LD001 *[ldIs007 (pskn-1::skn-1b/c::gfp; rol-6)]*. Nematodal and bacterial strains were provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Strain maintenance was performed at 20 °C on nematode growth medium (NGM) agar plates containing a lawn of *Escherichia coli* var. OP50 as the food source as described elsewhere[17] with slight modifications (heat-inactivated, non-proliferating *E. coli* were used as the food source).

Stress resistance

Due to the poikilothermic nature of *C. elegans*, the nematodes react very sensitive on environmental temperature changes.[18] To test the influence of compound treatment on survival of individual nematodes at 37 °C, we

conducted an assay adapted from Gill *et al.* and Kampkötter *et al.*[18,19] which employs the nucleic acid stain SYTOX® Green with slight modifications.[17]

Measurement of intracellular reactive oxygen species accumulation in vivo

The intracellular production of reactive oxygen species in *C. elegans* can be induced by the application of thermal stress and this feature has been described in various studies.[17,20] We used the fluorescent probe DCF to measure the amount of ROS in living nematodes according to Büchter *et al.*[17]

Trolox equivalent antioxidant capacity assay

The trolox equivalent antioxidant capacity (TEAC) assay is a cell-free method for the detection of radical scavenging properties of compounds in comparison to the synthetic vitamin E derivative trolox. Experimental procedures were performed according to Büchter *et al.*[17]

Intracellular localization of DAF-16::GFP and SKN-1::GFP

The transgenic strains TJ356 *[zIs356 IV (pdaf-16-daf-16::gfp; rol-6)]* and LD001 *[ldIs007 (pskn-1::skn-1b/c::gfp; rol-6)]* were used to detect the intracellular localization of the green fluorescent protein (GFP)-tagged transcription factors. Experimental procedures were performed according to Büchter *et al.*[17]

Lifespan and locomotion assays

For the analysis of the lifespan and the mobility of aged animals, the wild-type strain N2 was used. Forty L4 larvae per test compound were transferred into liquid media (day 0 of lifespan). During the first 13 days of the lifespan (the fertile period), 120 μM 5′fluorodeoxyuridine (FUDR) was added to prevent the hatching of viable progeny. The survival of the animals was measured five times per week by touch-provoked movement.[17] The locomotion/mobility of the nematodes was tested at days 11 and 18 during the lifespan experiments. Therefore, the animals were classified into three categories: A – nematodes moved freely after briefly shaking the Petri dishes; B – nematodes moved after a gentle touch stimulus; and C – nematodes moved with head or tail only after rigorous touch stimulus.

Statistics

Statistical analysis was conducted of at least three independent experiments with data given as mean ± SD. PASW Statistics for Windows, version 18.0 (SPSS Inc.; Chicago, Illinois, USA) and GraphPad Prism 6 (La Jolla, California, USA) software were used to compute the statistical analyses. Statistical significance was determined by one-way ANOVA with Dunnett's post-test while lifespan analyses and thermal stress resistance were calculated using Kaplan–Meier survival analyses with log-rank test. Statistical differences were considered to be significant at a level of $P < 0.05$.

Results

Synthesis of resveratrol analogues

The stilbene derivatives used in this study were synthesized by a Wittig reaction followed by a Mizoroki–Heck reaction. Identification and structural elucidation of synthesized compounds were performed based on UV, 1D and 2D NMR spectroscopy as well as mass spectrometry. Spectral data of the compounds were in accordance with published data.[14–16]

Antioxidant effects of resveratrol analogues

All compounds exert strong radical scavenging activity *in vitro* (TEAC assay). The antioxidant capacity of compounds (**2**), (**3**) and (**4**) was almost identical, while compound (**5**) was slightly more potent. Compounds (**1**) and (**6**) were the most powerful radical scavenging compounds: analysing the antioxidative effects at a concentration of 10 μM, the antioxidant effect of compound (**1**) was stronger than trolox, the synthetic vitamin E derivative used as reference compound. The antioxidative effect of compound (**6**) at 10 μM was not significantly different from trolox (Figure 2a).

Contrary to the findings in the *in vitro* situation, the results in the model organism *C. elegans* are different: administration of even higher concentrations (100 μM vs 50 μM) only compounds (**3**) and (**6**) reduced the *in vivo* ROS accumulation significantly starting at time points 240 min (**3**) and 360 min (**6**) (reduction in the ROS level to 60% and 61% of the corresponding control values, respectively). Application of the stilbene derivatives (**2**) and (**5**) resulted in a tendency to reduce the ROS level to 74% and 72% (6 h) of the control value, while treatment with compounds (**1**) and (**4**) showed only marginal effects.

Modulation of lifespan by resveratrol analogues

We further investigated whether the stilbenes (**3**) and (**6**), both of which are displaying prominent antioxidant activity in *C. elegans*, were able to modulate the lifespans of wild-type nematodes when continuously treated during the

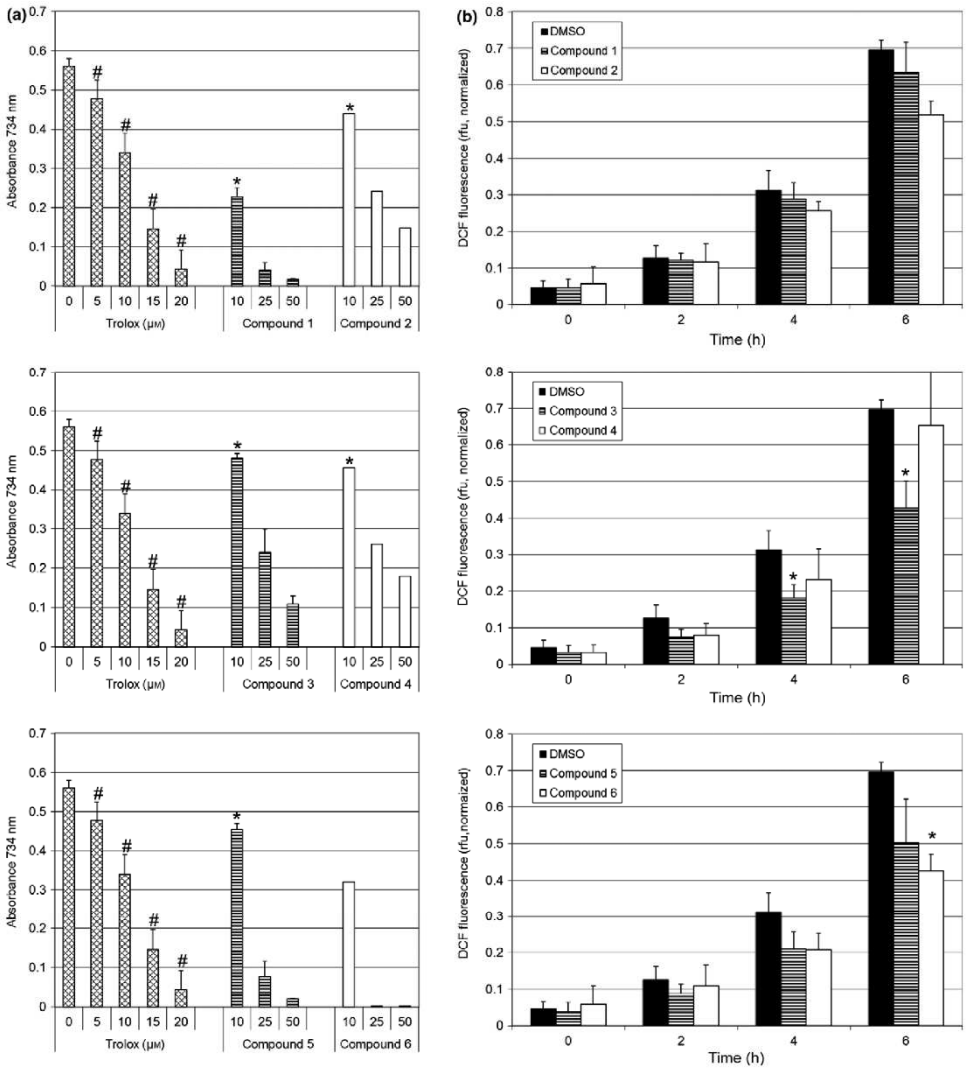

Figure 2 Antioxidative capacity of resveratrol derivatives. (a) The radical scavenging properties (=antioxidative capacity) of compounds (**1**–**6**) were measured using the trolox equivalent antioxidant capacity assay *in vitro*. The decolorization of the radical solution was detected spectrophotometrically at 734 nm (mean values ± SD, $n = 3$–4, $^{\#}P < 0.05$ vs corresponding control value (only trolox), $^{*}P < 0.05$ vs corresponding trolox value (only 10 µM); one-way ANOVA with Dunnett's post-hoc test. (b) Three days after hatching, age-synchronized, wild-type nematodes were treated with compounds (100 µM) or 0.1% dimethylsulfoxid; solvent control DMSO in nematode growth medium for 48 h. After a washing step, individual nematodes were placed in each well of a 384-well plate containing 50 µM $H_2$2′,7′-dichlorofluorescein -DA, kept at 37 °C and the reactive oxygen species accumulation was measured by an increase in 2′,7′-dichlorofluorescein fluorescence. Mean ± SD; three experiments with eight individuals per group and experiment; $^{*}P < 0.05$ vs corresponding DMSO-treated control value.

complete adult life (mean lifespan of control animals 19.58 ± 0.3 days). In contrast to the parent compound resveratrol (**1**), which did not prolong the lifespan in our experimental setting, both (**3**) and (**6**) were able to significantly extend the mean lifespans of the nematodes to 20.29 ± 0.38 and 20.84 ± 0.37 days, respectively. The maximum lifespan was increased by 4.76% and 9.52% (Figure 3a). In addition to the data obtained from the lifespan experiments, we were further interested in gathering information on the impact of the animals' locomotion system during ageing as a marker for health span, an ever increasing topic in ageing research. Here we were able to demonstrate that (**3**) and (**6**) increased the locomotion activity of the nematodes at day 11 (Figure 3b), although this difference diminished at a later time point. However, resveratrol (**1**) treatment had no effect on locomotion activity.

Modulation of stress resistance by resveratrol analogues

To investigate whether the administration of stilbene derivatives could induce further beneficial health effects in the nematodes, we analysed the thermal stress resistance of *C. elegans*. In analogy to the results obtained in the lifespan and DCF assays, application of 100 μM resveratrol caused no protection against thermal stress (Figure 3c). In

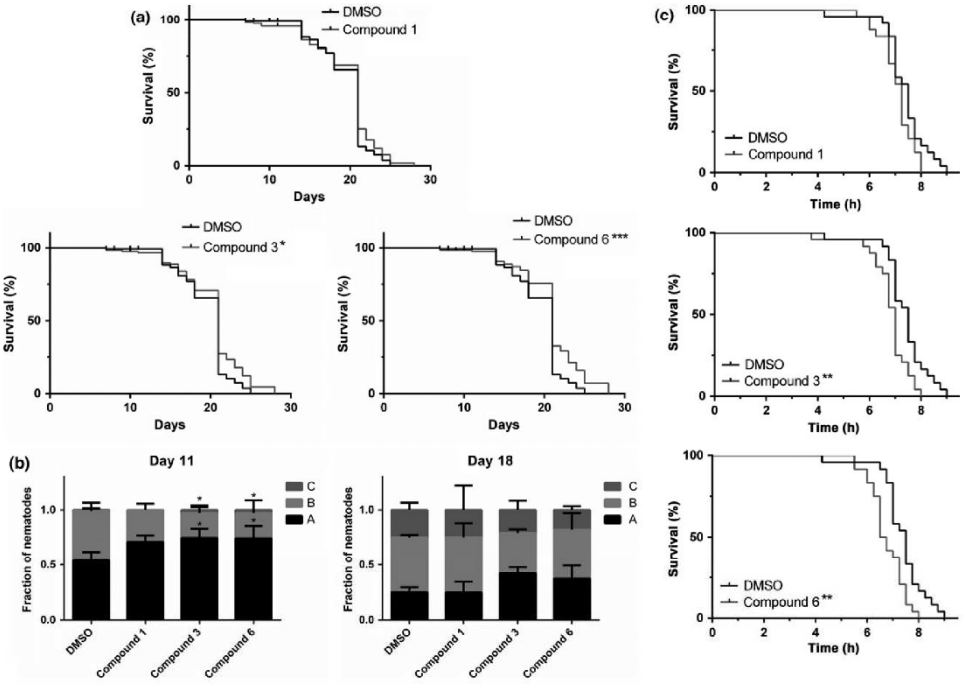

Figure 3 Modulation of lifespan and stress resistance by resveratrol derivatives. (a) Three days after hatching, age-synchronized animals were transferred into nematode growth medium containing 100 μM of compounds (**1**), (**3**) and (**6**) or 0.1% DMSO. The survival at 25 °C was monitored five times per week and living nematodes were transferred to fresh medium thrice per week. For the first thirteen days of treatment, the medium contained 120 μM 5-fluoro-2′-deoxyuridine to inhibit progeny production. Kaplan–Meier survival analysis with log-rank test (Mantel–Cox); data are pooled from three independent experiments with 40 animals per group and experiment. (b) The mobility of animals in Figure 4a was checked at day 11 and day 18 by touch-provoked movement. Data are mean values ±SD; $n = 3$; one-way ANOVA with Dunnett's post-hoc test; $^{*}P < 0.05$. (c) Modulation of thermal stress resistance by compounds (**1**), (**3**) and (**6**): 3 days after hatching, age-synchronized, wild-type nematodes were treated with the compounds or 0.1% DMSO in liquid nematode growth medium for 48 h. After a washing step, single nematodes were placed in the wells of a 384-well plate containing 2 μM SYTOX Green. The microplate was kept at 37 °C and the deaths of the nematodes were measured via the increase in fluorescence. The graph shows the percentage of viable nematodes. Kaplan–Meier survival analysis with log-rank test (Mantel–Cox); $n = 3$ with eight individuals per group and experiment; $^{**}P < 0.01$ vs DMSO.

addition, and to a certain extent contrary to our expectations, the compounds (**3**) and (**6**) decreased the thermal stress resistance of the nematodes: a reduction in mean survival time was detected by 2.58% and 8.15%, respectively.

Modulation of redox-active signalling pathways by resveratrol analogues

We used the *C. elegans* strains CF1038, EU1 and VC199, which are loss-of-function mutants of *daf-16*, *skn-1* and *sir-2.1*, respectively, to analyse whether these important cellular molecules are, at least in part, necessary factors for the aforementioned effects of the stilbene derivatives (**3**) and (**6**). Using the *skn-1*- and *sir-2.1*-deficient strains, compounds (**3**) and (**6**) no longer caused a reduction in the DCF fluorescence (Figure 4c and 4d). In case of the *daf-16*-deficient strain, significant antioxidant effects were detected only at a relatively early time point (Figure 4b). However, in spite of the statistically non-significant effects at 6 h, compounds (**3**) and (**6**) both induced a reduction in DCF fluorescence by 28.64% and 24.78%, which is comparable to the observed effects in wild-type nematodes. We used the transgenic strains TJ356 (DAF-16::GFP) and LD001 (SKN-1::GFP) to analyse whether the compounds induce a prominent shift towards a nuclear localization phenotype of the transcription factors DAF-16 (Figure 5a) or SKN-1 (Figure 5b), a necessary prerequisite for their transcriptional activity. However, after short-term treatment (1 h), no significant change in the intracellular localization of these transcription factors could be detected. Neither compounds (**3**) and (**6**) nor compound (**1**) significantly increased the amount of animals displaying a nuclear localization phenotype of DAF-16 or SKN-1, respectively. However, the positive controls used (10 min 37 °C in case of DAF-16 or 10 mM sodium arsenite in case of SKN-1) were able to cause a significant increase in the nuclear localization of the transcription factors.

Discussion

The life-prolonging effects of the stilbene resveratrol have been shown in different species, but at least in case of the model organism *C. elegans*, the results are contradictory and therefore controversially discussed. We used four newly synthesized resveratrol derivatives (*trans*-3,5-dimethoxy-4-fluoro-4′-hydroxystilbene (**3**), *trans*-4′-hydroxy-3,4,5-trifluorostilbene (**4**), *trans*-2,5-dimethoxy-4′-hydroxystilbene (**5**) and *trans*-2,4′,5-trihydroxystilbene (**6**)) to analyse their effects on stress resistance, ROS generation and lifespan in the model organism *C. elegans*.

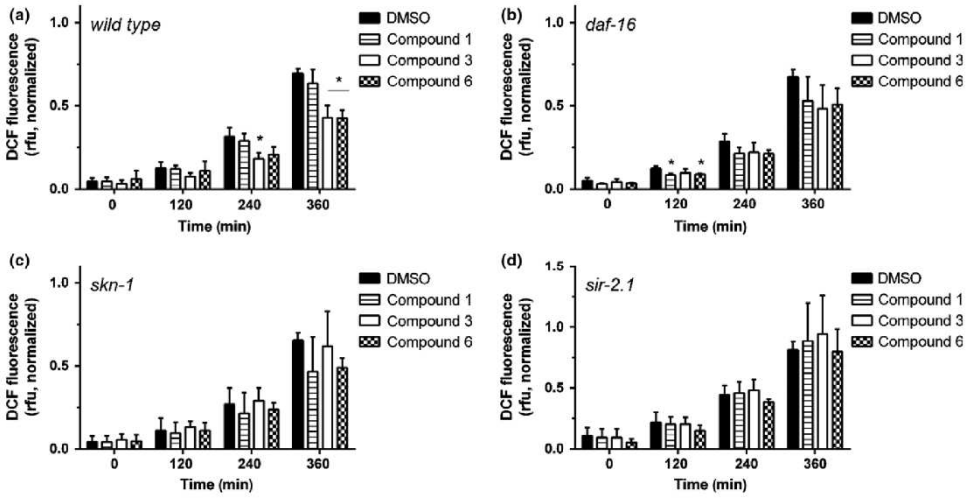

Figure 4 Antioxidative capacity of resveratrol derivatives in mutant strains. The antioxidative potential of compounds (**1**), (**3**) and (**6**) was analysed using *Caenorhabditis elegans* strains deficient in DAF-16, SKN-1 or SIR-2.1. Three days after hatching, age-synchronized, nematodes were incubated with compounds (100 μM) or 0.1% DMSO in liquid nematode growth medium for 48 h. After a washing step, single nematodes were placed in each well of a 384-well plate containing 50 μM $H_2$2′,7′-dichlorofluorescein-DA, kept at 37 °C and the reactive oxygen species accumulation was measured by an increase in 2′,7′-dichlorofluorescein fluorescence. Mean ± SD; $n = 3$ with eight individuals per group and experiment; *$P < 0.05$ vs DMSO; one-way ANOVA with Dunnett's post-hoc test.

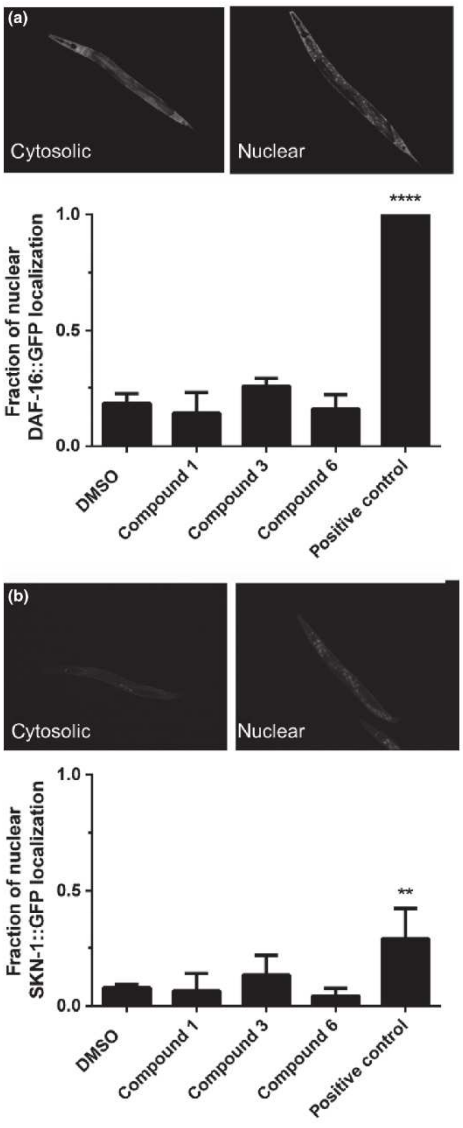

Figure 5 Modulation of DAF-16/SKN-1 intracellular localization by resveratrol derivatives. (a) The nuclear localization of DAF-16::GFP is visible by distinct fluorescent dots in the nematode (right). Young adult nematodes were treated with (**1**), (**3**), (**6**) or 0.1% DMSO for 1 h followed by microscopic determination of the GFP localization phenotype; positive control: 10 min at 37 °C. Mean ± SD of four independent experiments with 30 animals per group; ****$P < 0.0001$ vs DMSO; one-way ANOVA with Dunnett's post-hoc test. (b) The nuclear localization of SKN-1::GFP is visible by distinct fluorescent dots in the nematodes intestine (right). Three-day-old synchronized transgenic nematodes (SKN-1::GFP, strain LD001) were used to analyse the localization of SKN-1::GFP. Young adult nematodes were treated with (**1**), (**3**), (**6**) or 0.1% DMSO for 1 h followed by microscopic determination of the GFP localization phenotype; positive control: 10 mM sodium arsenite. Mean ± SD of four independent experiments with 30 animals per group; **$P < 0.01$ vs DMSO; one-way ANOVA with Dunnett's post-hoc test.

All resveratrol derivatives showed a prominent *in vitro* radical scavenging activity in the TEAC assay; in case of resveratrol (**1**) and the newly synthesized compound (**6**), the antioxidant effects were in a comparable range of trolox. These antioxidant effects of resveratrol *in vitro* were shown by diverse working groups.[13,21] However, these potent antioxidant effects were not directly transferable to the *in vivo* system *C. elegans*. On the one hand, out of all resveratrol derivatives tested, only the compounds *trans*-3,5-dimethoxy-4-fluoro-4′-hydroxystilbene (**3**) and *trans*-2,4′,5-trihydroxystilbene (**6**) significantly reduced the accumulation of ROS *in vivo* after administration of relatively high concentrations in the treatment media (100 μM); on the other hand, resveratrol (**1**) itself was not able to induce antioxidant effects in this experimental setting. The lack of protection against thermally induced ROS accumulation by resveratrol has previously been shown.[13] It was reported that resveratrol protects *C. elegans* from oxidative stress induced by paraquat or juglone.[21,22]

As antioxidant effects are claimed to be linked to lifespan extension, the effects on lifespan modulation by the potent *in vivo* antioxidant compounds (**3**) and (**6**) as well as resveratrol were analysed. The two compounds which were effective antioxidants also increased the lifespans of the nematodes, while resveratrol had no such effect on *C. elegans* lifespan. Analogous to our results, Chen *et al.*[21] reported that resveratrol had no life-prolonging effect in wild-type *C. elegans*: 100 μM resveratrol treatment caused either a non-significant reduction in lifespan (−4.1%) or a strong reduction (−18.6%) depending on the concentration of DMSO used and the type of incubation media that was chosen (NGM agar plates vs liquid S-medium). They further analysed the effects of resveratrol in a *mev-1*-mutant *C. elegans* strain, which is characterized by an enhanced level of oxidative stress. However, 100 μM resveratrol had no effect on longevity, while higher concentrations (200 μM) reduced the lifespan of the oxidatively stressed nematodes by 9.6%.[21] In contrast to the results presented in this study, we previously could show that 100 μM resveratrol

significantly increased mean and median lifespan of wild-type nematodes, when living *E. coli* in liquid medium were used as the food source.[13] Lee *et al.*[23] showed a prolongation of lifespan after incubation with 100, 500 and 1000 μM resveratrol (mean lifespan of DMSO-treated control: 13.5 ± 0.21 days, mean lifespan of nematodes treated with 1000 μM resveratrol: 17.6 ± 0.23 days). Similarly, Gruber *et al.*[22] reported that resveratrol increased the lifespan of wild-type *C. elegans* by 50.8%. In contrast to our recent experimental design, Gruber *et al.*[22] used NGM agar plates, not liquid medium but they also used heat-inactivated *E. coli* to administer resveratrol. The advantage for usage of liquid medium was the possibility for a proper adjustment of concentrations. These contradictory results dramatically illustrate why there is such a controversial debate about resveratrol's *in vivo* bioactivity. As the lifespan extending effect of compounds may simply be due to starvation of nematodes, Gruber *et al.*[22] showed that resveratrol had no effect on the pharyngeal pumping rate, indicating that the animals most likely did not suffer from caloric restriction. In our study, we excluded the possibility of caloric restriction phenomena by controlling the body size of the nematodes. In contrast to resveratrol, *trans*-3,5-dimethoxy-4-fluoro-4′-hydroxystilbene (**3**) and *trans*-2,4′,5-trihydroxystilbene (**6**) were able to enhance the lifespan of *C. elegans* significantly in our experimental setting. In case of compound (**6**), this may be due to the strong antioxidant potential (*para*-quinoid system) of this compound.

Both compounds (**3**) and (**6**) decreased the thermal stress resistance of the nematodes, while the other compounds showed no effect. In accordance with this result, Gruber *et al.*[22] reported that resveratrol was not capable to protect the nematodes against thermal stress. However, we have to conclude that the beneficial effect in lifespan prolongation not necessarily implies an enhancement of other parameters, like thermal stress tolerance. However, the possible metabolism of the compounds by bacteria, for example by the human intestinal microbiome, seems to be highly relevant: analysing the effect of the compounds in *C. elegans* with living *E. coli* as experimental setting, all antioxidant effects *in vivo* as well as the modulation of thermal stress tolerance completely abolished compared with the experiments conducted with heat-inactivated *E. coli* (data not shown).

Different *C. elegans* loss-of-function strains (*daf-16*, *skn-1*, *sir-2.1*) were used to evaluate whether these factors are at least in part necessary to exert the *in vivo* antioxidant effects induced by the stilbene derivatives in wild-type *C. elegans*. None of the compounds caused significant antioxidant effects in these mutant strains that were detectable for more than just one time point, showing the importance of these factors. However, despite their necessity for *in vitro* antioxidant effects, a short-time treatment with compounds (**3**) and (**6**) did not lead to an increased nuclear localization of DAF-16 and SKN-1, respectively. These observations not only raise the interesting question of how these compounds regulate the activity of the transcription factors under certain environmental conditions (heat stress vs ambient temperature), it would also be of interest if these distinct factors are also necessary for the lifespan extension induced by compounds (**3**) and (**6**).

Conclusions

Using the model organism *C. elegans*, we showed for the first time that the newly synthesized stilbenoid compounds *trans*-3,5-dimethoxy-4-fluoro-4′-hydroxystilbene (**3**) and *trans*-2,4′,5-trihydroxystilbene (**6**) possess a higher bioactivity compared with the established phytoalexin resveratrol (*trans*-3,4′,5-trihydroxystilbene (**1**)) and its naturally occurring *O*-methylated derivative pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene (**2**)): only (**3**) and (**6**) were able to increase the lifespan and to reduce the ROS accumulation of the nematodes. Using *daf-16*, *skn-1* and *sir-2.1*-loss-of-function strains, we demonstrated that the antioxidant effects were dependent on these factors. As the compounds (**3**) and (**6**) showed a highly desired increased bioactivity compared to the established and worldwide merchandized anti-ageing compound resveratrol, these structures may be interesting candidates for pharmacological anti-ageing purposes.

Declarations

Conflict of interest

The Authors declare that there is no conflict of interests regarding the publication of this paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

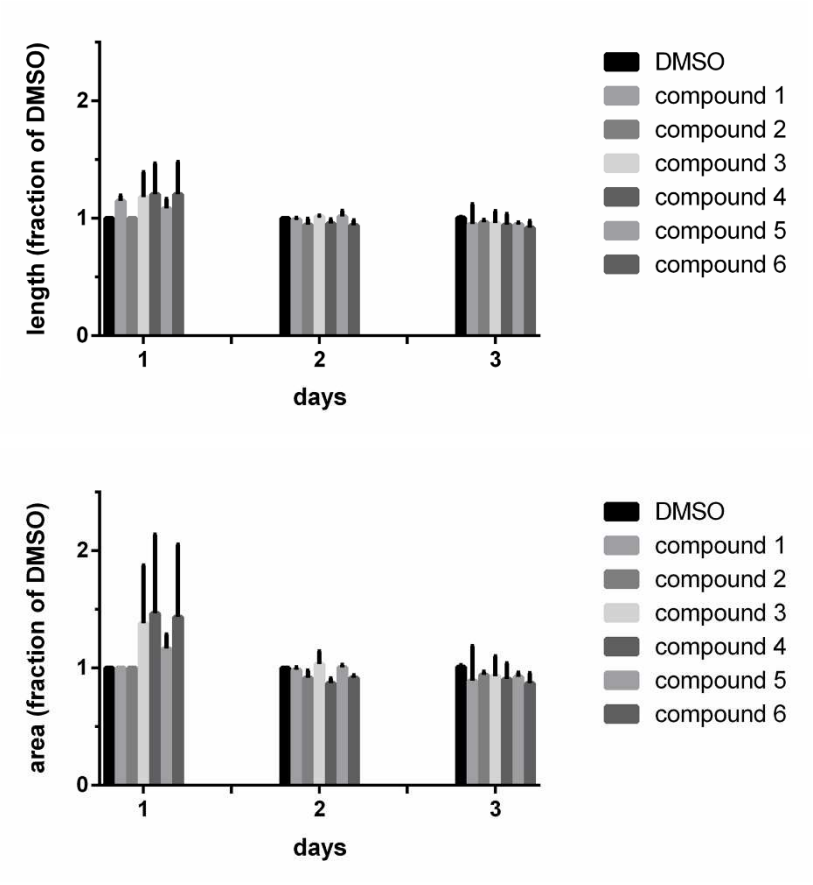
Figure S1. Effects of the resveratrol derivatives are not mediated by caloric restriction.

Figure S2. Antioxidative capacity and modulation of stress resistance (no inactivation of bacteria).

Figure S3. The newly synthesized stilbene derivatives (**3**) and (**6**) but not resveratrol show antioxidant effects *in vivo* and increase the life span of *Caenorhabditis elegans*.

Table S1. antioxidant capacity and modulation of life span by resveratrol derivatives.

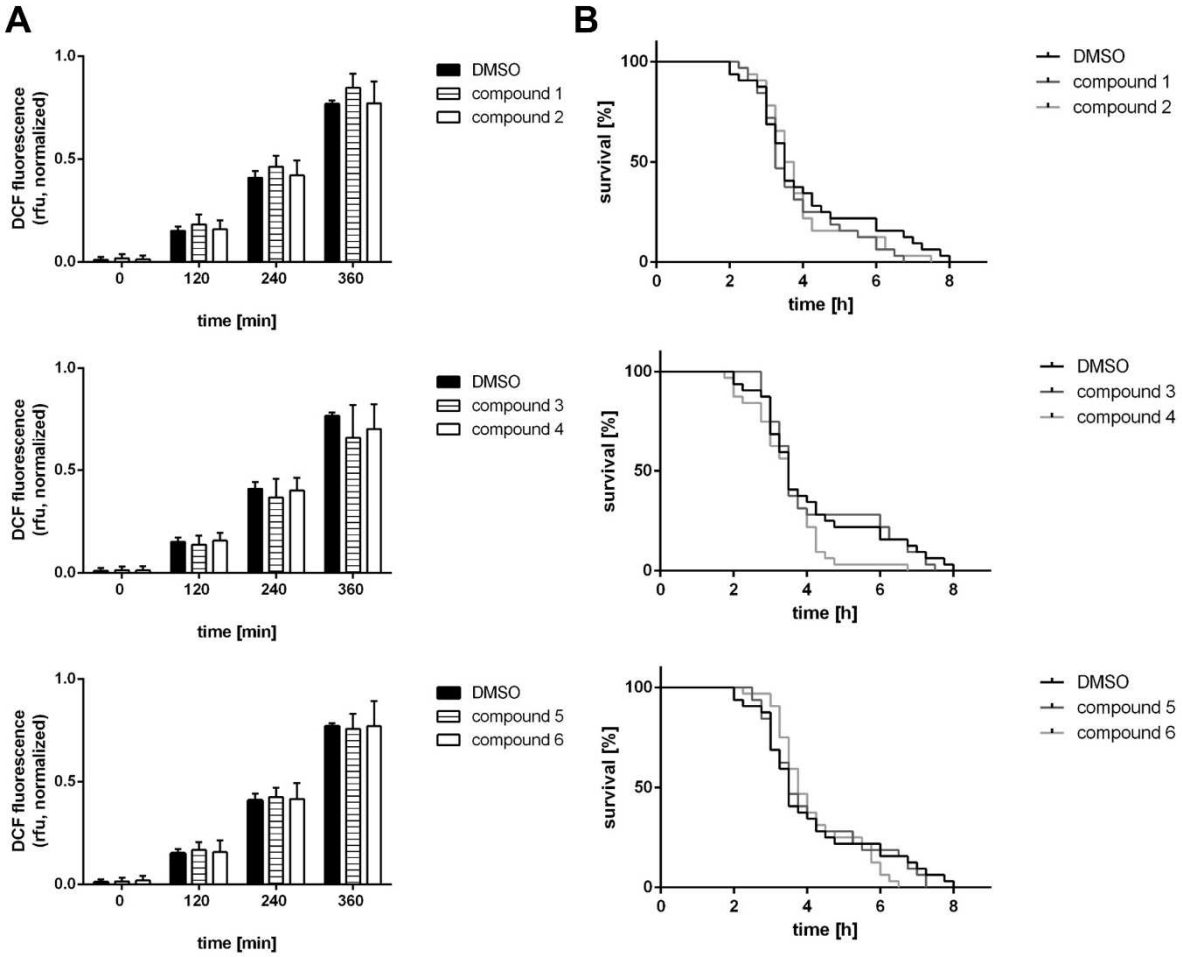
SUPPLEMENTAL FIGURE 1



Effects of the resveratrol derivatives are not mediated by caloric restriction:

Treatment of L1 larvae with the compounds (100 µM) for up to 3 days did not reduce animal length or the area of the nematodes. Data are mean values ± SD (normalized to DMSO value = 1), two independent experiments, for each data point, 40 nematodes were analyzed.

SUPPLEMENTAL FIGURE 2



Antioxidative capacity and modulation of stress resistance (no inactivation of bacteria)

Compound treatment of *C. elegans* was conducted in 1.5 ml of liquid NGM containing 1% (w/v) bovine serum albumin, 50 µg/ml streptomycin and E. coli OP50^{-1} (10^9 cfu/ml) in 35 mm petri dishes (no inactivation of bacteria). A: Three days after hatching age-synchronized, wildtype nematodes were treated with compounds (100 µM) or 0.1% DMSO in liquid NGM for 48 h. After a washing step, individual nematodes were placed in each well of a 384 well plate containing 50 µM H_2DCF-DA, kept at 37 °C and the ROS accumulation was measured by an increase in DCF fluorescence. Mean ± SD; 3 experiments with 8 individuals per group and experiment; *, $p<0.05$ vs. corresponding DMSO-treated control value. B: Modulation of thermal stress-resistance by compounds: Three days after hatching age-synchronized, wildtype nematodes were treated with the compounds or 0.1% DMSO in liquid NGM for 48 h. After a washing step, single nematodes were placed in the wells of a 384 well plate containing 2 µM SYTOX Green. The microplate was kept at 37 °C and the deaths of the nematodes were measured via the increase in fluorescence. The graph shows the percentage of viable nematodes. Kaplan Meier survival analysis with log rank test (Mantel-Cox); n=3 with 8 individuals per group and experiment; **, $p<0.01$ vs. DMSO.

SUPPLEMENTAL TABLES

A **TEAC-Assay**

	10 µM	25 µM	50 µM
trolox	0.313 ± 0.028	n.d.	n.d.
compound **1**	0.229 ± 0.023*	0.041 ± 0.019	0.017 ± 0.003
compound **2**	0.440 ± 0.012*	0.243 ± 0.062	0.149 ± 0.024
compound **3**	0.481 ± 0.011*	0.241 ± 0.060	0.104 ± 0.024
compound **4**	0.457 ± 0.009*	0.261 ± 0.012	0.177 ± 0.003
compound **5**	0.454 ± 0.015*	0.076 ± 0.040	0.019 ± 0.003
compound **6**	0.320 ± 0.008	0.003 ± 0.001	0.002 ± 0.001

B **DCF-Assay**

	0 h	2 h	4 h	6 h
DMSO	0.05 ± 0.02	0.13 ± 0.04	0.31 ± 0.05	0.70 ± 0.03
compound **1**	0.05 ± 0.02	0.12 ± 0.02	0.29 ± 0.04	0.63 ± 0.08
compound **2**	0.06 ± 0.05	0.12 ± 0.05	0.26 ± 0.02	0.52 ± 0.04
compound **3**	0.03 ± 0.02	0.07 ± 0.02	0.18 ± 0.04*	0.43 ± 0.07*
compound **4**	0.03 ± 0.02	0.08 ± 0.03	0.23 ± 0.08	0.65 ± 0.21
compound **5**	0.04 ± 0.03	0.09 ± 0.03	0.21 ± 0.05	0.50 ± 0.12
compound **6**	0.06 ± 0.05	0.11 ± 0.06	0.21 ± 0.04	0.43 ± 0.05*

C **life span**

	median ± S.E.	mean ± S.E.	n (censored)	change in mean survival (%)	*p*-value vs. DMSO
DMSO	21.00 ± 0.19	19.58 ± 0.30	120 (13)		
compound **1**	21.00 ± 0.29	19.75 ± 0.37	120 (11)	+0.87	0.1745
compound **3**	21.00 ± 0.30	20.29 ± 0.38	120 (15)	+3.63	0.0176
compound **6**	21.00 ± 0.31	20.84 ± 0.37	120 (11)	+6.44	0.0004

Antioxidative capacity and modulation of life span by resveratrol derivatives
A: Data of TEAC assay (corresponding figure: 2A); mean values ± SD, n = 3-4, one way ANOVA with Dunnett´s post hoc test; *, $p<0.05$ vs. 10 µM trolox (significance only analyzed for 10 µM concentration).
B: Data of DCF-assay (corresponding figure: 2B); mean values ± SD; 3 experiments with 8 individuals per group and experiment; *, $p<0.05$ vs. corresponding DMSO-treated control value.
C: Data of life span experiment (corresponding figure: 3A); Kaplan Meier survival analysis with log-rank test (Mantel-Cox); data are pooled from three independent experiments with 40 animals per group and experiment.

4. Diskussion

4.1 Untersuchungen zu molekularen Mechanismen der protektiven Wirkungen von Flavonoiden in *C. elegans* (Studien 1 bis 3)

Flavonoiden werden zahlreiche gesundheitsförderliche Wirkungen zugesprochen, wobei in epidemiologischen Studien nachgewiesen werden konnte, dass die Inzidenz von altersassoziierten Erkrankungen wie kardiovaskulären Krankheiten (Wang et al., 2013), koronaren Herzerkrankungen (Hertog et al., 1993; Hertog et al., 1995) und Typ-2-Diabetes (Zamora-Ros et al., 2014) invers mit der Aufnahme von Flavonoiden korreliert. Inwiefern Flavonoide die Lebensspanne im Menschen verlängern, konnte bislang allerdings nicht gezeigt werden. Um experimentelle Nachweise zu lebensverlängernden Wirkungen von Flavonoiden zu erhalten werden daher Modellorganismen zur Untersuchung herangezogen, die eine deutlich kürzere Lebensspanne besitzen als der Mensch. Als Säugetiermodell werden häufig Mäuse eingesetzt, da die evolutionären Nähe zum Menschen eine relativ gute Übertragbarkeit von pharmakologischen Wirkungen ermöglicht. So wurde z.B.im Mausmodell für einige über die Nahrung aufgenommene Flavonoide eine lebensverlängernde Wirkung festgestellt: Epicatechin Supplementierung (Si et al., 2018) oder Icariin Supplementierung (Zhang et al., 2015) erhöhten die Überlebensrate bzw. die mittlere Lebensspanne von männlichen Mäusen, während für Fisetin eine verlängerte mittlere und maximale Lebensspanne von gemischtgeschlechtlichen Mauspopulationen gezeigt werden konnte (Yousefzadeh et al., 2018). Auch in Studien mit *C. elegans* konnte gezeigt werden, dass viele Flavonoide lebensverlängernde Effekte induzieren, darunter z.B. Icariin, Quercetin, Isorhamnetin oder Baicalein, (Cai et al., 2011; Kampkötter et al., 2008; Surco-Laos et al., 2011; Havermann et al., 2013). Häufig jedoch bleiben die der lebensverlängernden Wirkung der Flavonoide zugrunde liegenden molekularen Effekte unklar.

Physiologische Wirkungen von Myricetin in C. elegans und zugrunde liegende molekulare Mechanismen

Es konnte in dieser Arbeit gezeigt werden, dass eine Behandlung mit Myricetin die durchschnittliche Lebensspanne von wildtypischen Nematoden erhöht. Dies bestätigt die Ergebnisse weiterer Arbeitsgruppen, welche in wildtypischen Tieren ebenfalls eine

Lebensspannenverlängerung durch Myricetin beobachten konnten (Grünz et al.; 2012; Jung et al., 2017). Obwohl die experimentellen Bedingungen stark unterschiedlich waren (Agar vs. Flüssigmedium, 20 °C vs. 25 °C, Ethanol/ Tween 80 vs. DMSO), ist die Myricetin-induzierte Verlängerung der Lebensspanne ein robuster Effekt in *C. elegans*, der für andere Flavonoide nicht gezeigt werden konnte. Für Catechin beispielsweise sind bei gleicher Konzentration (200 µM) sowohl das Auftreten wie auch das Ausbleiben von lebensverlängernden Effekten beschrieben (Saul et al., 2009; Surco-Laos et al., 2012). Einen wichtigen Schritt zum Verständnis der Effekte, die zu einer gesteigerten Lebensspanne in *C. elegans* und zu möglichen gesundheitsförderlichen Wirkungen im Menschen beitragen, ist die Untersuchung der Bioverfügbarkeit. Dass Myricetin grundsätzlich vom Nematoden resorbiert wird, konnte in der hier vorliegenden Arbeit durch eine zeitabhängige Aufnahme von Myricetin, mit Hilfe des Fluoreszenzverstärkers 2-Aminoethyldiphenylborinat (Naturstoffreagenz-A; NSRA) nachgewiesen werden, wobei nach 30 min bereits eine Fluoreszenz in intestinalen Zellen beobachtet wurde, die im zeitlichen Verlauf noch deutlich anstieg. Dieses Ergebnis bestätigt die Befunde von Grünz et al., die einen konzentrationsabhängigen Anstieg der Fluoreszenz mittels NSRA zeigen konnten (Grünz et al., 2012). Somit kann davon ausgegangen werden, dass die beobachteten Wirkungen tatsächlich durch resorbiertes Myricetin bzw. durch Myricetinmetabolite verursacht wurden und nicht auf unspezifischen Effekten beruhen, wie z.B. einer kalorischen Restriktion durch eine verringerte Nahrungsaufnahme. Dies wäre z.B. der Fall, wenn Myricetin einen für Nematoden unangenehmen Geschmack besitzt. Der lebensverlängernde Effekt einer kalorischen Restriktion (verringerte Energiezufuhr ohne Mangel an wichtigen Nährstoffen) ist für diverse Spezies und auch in *C. elegans* nachgewiesen (Fontana et al., 2010). Dies kann im Fall von Myricetin jedoch nicht bestätigt werden, da weder die Nahrungsaufnahme (bestimmt über die Pumprate des Pharynx), noch die Körpergröße der Nematoden reduziert war, welche als Indikatoren für eine kalorische Restriktion angesehen werden.

Nach der Frage der Bioverfügbarkeit von Myricetin, stellt sich weiterhin die Frage, welche molekularen Mechanismen in der Myricetin-induzierten Verlängerung der Lebensspanne involviert sind. Ein möglicher Ansatz zur Klärung der Frage liegt in der „*free radical theory of ageing*“ nach Denham Harman begründet. Danach wird postuliert, dass über die Zeit eine Akkumulation von oxidativ geschädigten Molekülen stattfindet, welche zu einem Funktionsverlust von Zellen und Geweben und letztlich zum Tod eines Organismus führt (Harman, 2003). Für Myricetin konnte in der vorliegenden Arbeit gezeigt werden, dass es sowohl *in vitro* (im zellfreien System und in HepG2-Zellen) als auch *in vivo* in *C. elegans* antioxidativ wirkt. Unter physiologischen Bedingungen führte eine Behandlung mit Myricetin zu einer signifikant reduzierten Akkumulation von Lipofuscin. Lipofuscin wird als speziesübergreifender biologischer Alterungsmarker angesehen, welcher sich aus oxidativ

geschädigten Makromolekülen wie Proteinen und Lipiden zusammensetzt und weder lysosomal noch proteasomal abgebaut werden kann, so dass er sich innerhalb der Zellen ansammelt (Hosokawa et al., 1994; Gerstbrein et al., 2005; Jung et al., 2007). Somit führt die Behandlung mit Myricetin nicht nur zu einer verlängerten Lebensspanne, die erhaltenen Ergebnisse sprechen auch dafür, dass auf molekularer Ebene eine Verlangsamung der Zellalterung stattfindet. Ebenso wurde die thermal induzierte Produktion von ROS durch die Behandlung mit Myricetin reduziert (im DCF-Assay) wodurch auf zwei Arten eine deutliche antioxidative Wirkung *in vivo* nachgewiesen werden konnte.

Die erhaltenen Ergebnisse erklären jedoch nicht hinreichend, wie Myricetin seine antioxidative Wirkung *in vivo* ausübt. Zum einen ist die direkte Interaktion mit freien Radikalen möglich, zum anderen kann aber auch eine Interaktion mit zellulären Signalwegen eine entscheidende Rolle spielen, welche einen indirekten Schutzmechanismus auslösen. Durch die veränderte Expression von antioxidativ wirkenden Enzymen, die direkt mit ROS interagieren (z.B SOD, CTL) oder die am Phase-II-Fremdstoffmetabolismus beteiligt und z.B. das antioxidativ wirksame Tripeptid Glutathion synthetisieren oder übertragen (Glutamylcysteinyl-Synthetase GCS, Glutathion-Synthetase GSS, Glutathion-S-Transferase GST), könnte Myricetin indirekte antioxidative Wirkungen ausüben. In einem ersten Schritt wurden dazu transgene Tiere mit Myricetin behandelt, welche GFP-gekoppelte Reportergene in Form von DAF-16-GFP und SKN-1-GFP exprimieren. Der redox-aktive Transkriptionsfaktor Nrf2 bindet an das so genannte *antioxidant responsive element* (ARE), ein DNS-Motiv im Promotorbereich von Genen, welche für Proteine kodieren, die antioxidative Wirkung besitzen oder am Phase-II-Fremdstoffmetabolismus beteiligt sind (Zhang et al., 2015). SKN-1 ist das *C. elegans* Homolog des Nrf2 der Säugetiere und übt einerseits Funktionen während der Embryonalentwicklung der Nematoden aus, andererseits ist SKN-1 in späteren Stadien an der zellulären Antwort auf oxidativen Stress beteiligt (An & Blackwell, 2003). Die Behandlung mit Myricetin führte unter den experimentellen Bedingungen jedoch nicht zu einer nukleären Translokation von SKN-1, welche eine Grundvoraussetzung für dessen transkriptionelle Aktivität darstellt. Für andere Flavonoide, wie Baicalein oder Epigallocatechingallat (EGCG), konnte hingegen in Studien eine Aktivierung des Transkriptionsfaktors in *C. elegans* gezeigt werden (Zhang et al., 2009; Havermann et al., 2013). Hieraus lässt sich die Hypothese ableiten, dass SKN-1 vermutlich nicht an der Verlängerung der Lebensspanne in *C. elegans* beteiligt ist. Weitergehende Untersuchungen zur Lebensspanne sollten jedoch mit *skn-1 loss-of-function* (*lof*) Mutanten durchgeführt werden, um die Hypothese zu überprüfen, da in *in vitro* Studien (Cho et al., 2016; Liao et al., 2019; Pan et al., 2019) und im Rattenmodell (Wu et al., 2016) eine verstärkte Expression oder Kernlokalisation von Nrf2 durch Myricetin gezeigt werden konnte.

Eine Behandlung mit Myricetin führte jedoch zu einer signifikant verstärkten Kernlokalisation des Transkriptionsfaktors DAF-16, dem Homolog der FoxO Transkriptionsfaktoren der Säuger. Eine nukleäre Translokation von DAF-16, einem zentralen Faktor des Insulin/IGF-1 ähnlichen Signalwegs, wurde auch in der Studie von Grünz et al. beschrieben in der zudem eine verstärkte Expression des DAF-16 Zielgens *sod-3* gezeigt werden konnte (Grünz et al., 2012). Darüber hinaus konnten Pillai und Mini in β-Zellen des Pankreas (RIN-m5F Zellen der Ratte) zeigen, dass *in vitro* die Inhibition der FoxO1 mRNA-Expression durch hohe Glukoselevel, nach Myricetin Behandlung wieder aufgehoben wurde (Pillai & Mini, 2018). Die oben dargestellten Daten sprechen dafür, dass Myricetin neben seiner direkten antioxidativen Wirkung als Radikalfänger auch durch die Modulation des Insulin/IGF-1 ähnlichen Signalwegs antioxidative und lebensverlängernde Wirkungen ausübt. Durch den Einsatz einer *daf-16* Funktionsverlustmutante konnte dies bestätigt werden: In der vorliegenden Arbeit wurde gezeigt, dass die antioxidative Wirkung *in vivo* wahrscheinlich auf einer Kombination aus Radikalfängeraktivität und Modulation der Signaltransduktion besteht, da in der *daf-16 lof* Mutante ein biphasischer Effekt zu beobachten war. Die durch thermalen Stress induzierte Produktion von ROS war zu frühen Messpunkten (nach zwei bzw. drei Stunden) in Myricetin behandelten Tieren geringer als in den Kontrollen, während zu den späteren Messpunkten keine signifikanten Unterschiede zu beobachten waren. Aus diesen Daten lässt sich schlussfolgern, dass nach dem „Verbrauch“ als Radikalfänger zu frühen Zeitpunkten, durch das Fehlen von DAF-16 kein zusätzlicher protektiver Effekt vermittelt werden konnte.

Auch die Lebensverlängerung durch Myricetin ist zumindest in Teilen von funktionsfähigem DAF-16 abhängig, da in der *daf-16 lof* Mutante die im Wildtypen induzierte Lebensverlängerung vollständig aufgehoben wurde. Dieses Ergebnis steht im Kontrast zu Daten aus anderen Studien, die eine von DAF-16 unabhängige Verlängerung der Lebensspanne beobachtet haben (Grünz et al., 2012; Jung et al., 2017). Eine mögliche Erklärung für die beobachteten Unterschiede in der *daf-16 lof* Mutante in Bezug auf die lebensverlängernden Effekte von Myricetin könnte das unterschiedliche experimentelle Vorgehen sein. Da die Aktivität von DAF-16 die Expression einer großen Bandbreite von Zielgenen reguliert, welche unter anderem an der Entwicklung, dem Metabolismus und der Stressantwort beteiligt sind (Murphy et al., 2003; Tullet, 2014), könnte die Behandlung mit Myricetin in der ohnehin kurzlebigeren und stressanfälligeren *daf-16 lof* Mutante zu einem deutlich weniger robusten Effekt geführt haben. Eine verlängerte Lebensspanne korreliert häufig mit einer erhöhten Resistenz gegen oxidativen und thermalen Stress und dies konnte sowohl für langlebige Mutanten (Lithgow et al., 1995; Johnson et al., 2002; Baumeister et al., 2006) als auch für eine Behandlung mit Flavonoiden (Kampkötter et al., 2008; Surco-Laos et al., 2012) in diversen Studien gezeigt werden. Eine Behandlung mit Myricetin führte jedoch nicht zu einer erhöhten Resistenz gegen thermalen Stress und es gab zwischen behandelten

und Kontrolltieren keine Unterschiede in der Überlebensspanne, weder im Wildtypen, noch in der *daf-16 lof* Mutante.

Einfluss funktioneller Gruppen von Flavonoiden auf ihre protektive Wirkung in C. elegans I: Hydroxylierung und Methylierung im B-Ring am Beispiel des Myricetin

Zwei Parameter sind maßgeblich an der Ausübung von physiologischen Effekten von über die Nahrung aufgenommenen Substanzen wie Polyphenolen in Organismen beteiligt, zum einen die Bioverfügbarkeit und zum anderen die Bioaktivität. In beiden Fällen sind die strukturellen Eigenschaften der Substanzen ausschlaggebend. Die Bioverfügbarkeit ergibt sich aus der Polarität der Substanz (d.h. ist passive Diffusion durch die Zellmembranen möglich), aus der Affinität zu Transportproteinen (d.h. sind strukturelle Merkmale vorhanden, die eine Bindung an Transporter ermöglichen) und aus der Metabolisierung der Verbindung (d.h. trifft die Ausgangssubstanz am Wirkort ein). Für die Bioaktivität gelten ähnliche Voraussetzungen, da es auch hier auf die Strukturmerkmale ankommt. So können Substanzen mit Proteinen in Wechselwirkung treten, die das Eintreffen am Wirkort verhindern oder eine Metabolisierung verändert die Wirkung der Ausgangssubstanz (siehe Kapitel 1.2).

Um die zuvor gezeigten physiologischen Wirkungen von Myricetin in *C. elegans* in einen Struktur-Wirkungs-Zusammenhang zu bringen, wurden seine methylierten Derivate Laricitrin, Syringetin und Myricetintrimethylether untersucht. Die so erhaltenen Daten lassen Rückschlüsse über die Bedeutung der 3´,4´,5´-Trihydroxylierung am B-Ring des Myricetin zu. In der vorliegenden Arbeit konnte gezeigt werden, dass alle untersuchten methylierten Myricetinderivate die Lebensspanne von *C. elegans* verlängerten. Hieraus lässt sich ableiten, dass die Anwesenheit von freien Hydroxylgruppen im B-Ring von Myricetin nicht ausschlaggebend für dessen lebensverlängernde Wirkung sind. In Übereinstimmung mit diesen Daten konnten Havermann et al. zeigen, dass das Flavonoid Baicalein, welches keine funktionelle Gruppe im B-Ring besitzt, ebenfalls die Lebensspanne von *C. elegans* verlängern konnte, wobei der dort beobachtete Effekt in einen Zusammenhang mit dem Transkriptionsfaktor SKN-1 gebracht wurde, da eine nukleäre Lokalisation nach Behandlung mit diesem Flavonoid aufgetreten ist (Havermann et al., 2013).

Die antioxidative Wirkung von Myricetin *in vivo* ist ebenfalls unabhängig von der B-Ring Hydroxylierung und steht in keinem direkten Zusammenhang mit der antioxidativen Wirkung im zellfreien System. Im zellfreien TEAC-Assay zeigte sich eine direkte Abhängigkeit in der antioxidativen Wirkung durch die Anzahl freier Hydroxylgruppen im B-Ring (Myricetin>Laricitrin>Syringetin>Myricetintrimethylether). Dieser Zusammenhang bestätigte sich in *C. elegans* jedoch nicht, da die antioxidative Wirkung unter physiologischen

Bedingungen (durch die Bestimmung von Lipofuscin) und unter thermalem Stress (durch die Messung der intrazellulären ROS-Konzentration im DCF-Assay) nicht mit den Daten aus dem zellfreien System korrelierten. Die methylierten Myricetinderivate zeigten in etwa das gleiche Niveau in der ROS-Akkumulation wie das Myricetin, mit einem etwas stärkeren Effekt bei Laricitrin und einem etwas schwächeren Effekt beim Myricetintrimethylether. Unter nicht-Stress Bedingungen ließ sich sogar ein dem zellfreien System entgegengesetzter Trend beobachten, so dass mit der Abnahme der Hydroxylgruppen im B-Ring eine Verringerung der Lipofuscinakkumulation einherging (Myricetin≈Laricitrin>Syringetin>Myricetintrimethylether).

Der Hydroxylierungsgrad von Myricetin bestimmt auch dessen Effektivität zur Modulation der zellulären Signaltransduktion, da die nukleäre Lokalisation des Transkriptionsfaktors DAF-16 durch die methylierten Derivate deutlich stärker ausgeprägt war als bei Myricetin selbst. Hierbei scheint die Anzahl der Substituierungen für die Modulation der DAF-16 Translokation allerdings keine Rolle zu spielen, denn die Kernlokalisation ist bei allen Derivaten in etwa gleich stark ausgeprägt. Eine entscheidende Frage die sich an dieser Stelle anschließt ist, ob die gesteigerte biologische Wirkung durch eine erhöhte Bioverfügbarkeit oder eine erhöhte Bioaktivität (oder eine Kombination aus beiden) zu Stande kommt. Ein Erklärungsansatz besteht darin, dass die Bioverfügbarkeit und/oder die Bioaktivität von Myricetin, bzw. der methylierten Derivate, vom Hydroxylierungs- bzw. Methylierungsgrad abhängig sind. So konnte z.B. gezeigt werden, dass die Bioaktivität der Flavone Chrysin und Apigenin durch Methylierung erhöht war und die methylierten Derivate (5,7-Dimethoxyflavon und 5,7,4´-Trimethoxyflavon) *in vitro* stärkere cytotoxische Effekte in Krebs vs nicht-Krebs-Zellen aufzeigten; zusätzlich war eine gesteigerte orale Bioverfügbarkeit in Ratten zu beobachten (Walle et al., 2007). Die Substitution der Hydroxylgruppen durch Methylgruppen erhöht zudem die Membranpermeabilität und steigert die metabolische Stabilität, indem Glucuronidierungs- oder Sulfatierungsreaktionen verhindert werden (Walle et al., 2009). Demgegenüber konnte in *C. elegans* eine höhere Bioverfügbarkeit durch Methylierung nicht bestätigt werden. Surco-Laos et al. konnten zeigen, dass Quercetin, im Vergleich mit Isorhamnetin (Quercetin-3´-O-methylether) und Tamarixetin (Quercetin-4´-O-methylether) eine höhere Bioverfügbarkeit besaß (Surco-Laos et al., 2011). In der gleichen Studie konnte auch gezeigt werden, dass eine Metabolisierung der Substanzen stattgefunden hat. So wurden für Quercetin Methylierung, Glykosylierung und Sulfatierung festgestellt, Isorhamnetin wurde glykosyliert und demethyliert, während für Tamarixetin nur eine Demethylierung festgestellt wurde. In der vorliegenden Arbeit konnte zwar der qualitative Nachweis der Resorption von Myricetin bzw. der methylierten Derivate mittels NSRA gezeigt werden, jedoch war der quantitative Nachweis einer Aufnahme und Metabolisierung von Myricetin und den methylierten Derivaten mittels HPLC-Analyse nicht möglich, da es während der Aufarbeitung bereits zur Degradation des Myricetin-Standards kam. Daher lässt sich aufgrund der in *C.*

elegans ermittelten Ergebnisse von Surco-Laos et al. schlussfolgern, dass die stärkeren biologischen Effekte der methylierten Myricetinderivate in *C. elegans* wahrscheinlich durch eine gesteigerte Bioaktivität verursacht wurden.

Durch die geänderte Bioaktivität spielen neben der Modulation von DAF-16 vermutlich weitere Signalwege, die an der Stressantwort und der Verlängerung der Lebensspanne beteiligt sind, eine Rolle bei der Wirkung der methylierten Derivate. So konnte in der vorliegenden Arbeit gezeigt werden, dass Syringetin die Lebensspanne in der *daf-16 lof* Mutante verlängert hat. Trotz der starken Aktivierung von DAF-16 durch Syringetin zeigt dieses Ergebnis, dass die Lebensspannenverlängerung nicht hauptsächlich auf einer Aktivierung von DAF16 beruht. Darüber hinaus ist ein weiterer Hinweis auf die Beteiligung anderer Signalwege, eine durch Myricetin nicht induzierbare Erhöhung der thermalen Stressresistenz. Alle untersuchten methylierten Derivate führten zu einer gesteigerten Resistenz, wobei DAF-16 eine entscheidende Rolle bei Laricitrin und Syringetin ausübt, da in der *daf-16* lof Mutante die Resistenzsteigerung ausblieb. Im Fall des Myricetintrimethylethers hingegen ist keine oder nur eine untergeordnete Beteiligung von DAF-16 vorhanden, da die gesteigerte Hitzeresistenz trotz des Fehlens von funktionsfähigem DAF-16 weiterhin aufgetreten ist. Ein wichtiger Faktor, der an der Stressresistenz und Langlebigkeit beteiligt ist und den es in weitergehenden Untersuchungen zu überprüfen gilt, ist der Transkriptionsfaktor SKN-1. Obwohl Myricetin SKN-1 nicht aktiviert hat, ist es nicht auszuschließen, dass die Modifikation durch Methylierung auch eine Aktivierung von SKN-1 bedingt. So konnte z.B. eine Aktivierung von SKN-1 durch strukturell diverse Flavonoide beschrieben werden (Havermann et al., 2013; Link & Wink, 2019; Chen et al., 2019). Ein weiterer Faktor, der sowohl die Lebensspanne als auch die Stressresistenz beeinflusst ist das *C. elegans* Homolog der Kinase mTOR (*mechanistic target of rapamycin*), LET-363 (Blackwell et al., 2019). So führt eine Inhibition von LET-363 durch genetischen *knock-out* (Vellai et al., 2003) oder pharmakologisch durch Rapamycin (Calvert et al., 2015) zu einer gesteigerten Lebensspanne.

Eine weitere wichtige Frage, die es zu beantworten gilt, ist wie die Aktivität von DAF-16 auf molekularer Ebene beeinflusst wird. Hier müsste der Einfluss von Myricetin und seinen Derivaten auf die DAF-16 vorgeschalteten Kinasen untersucht werden, da für Myricetin *in vitro* und *in vivo* inhibitorische Wirkungen auf PI3K und Akt-Kinase beschrieben sind (Walker et al., 2000; Li et al., 2016; Kim, 2017). Daher wäre es interessant in weitergehenden Untersuchungen herauszufinden, ob die *C. elegans* PI3K AGE-1 ein Ziel von Myricetin und den methylierten Derivaten darstellt. Darüber hinaus sollte untersucht werden, ob weitere Kinasen in *C. elegans* ein Ziel darstellen, da z.B. *in vitro* eine Inhibition von mTOR durch Myricetin beschrieben wurde (Cui et al., 2015). Darüber hinaus wäre es weiterführend, das Expressionsprofil zwischen Myricetin behandelten Tieren und den mit methylierten Derivaten

behandelten Nematoden zu vergleichen, um die Verlängerung der Lebensspanne noch genauer zu verstehen. So wäre es wichtig herauszufinden, welche DAF-16 abhängigen Gene differentiell reguliert werden, um einerseits zu verstehen, wie dieser Mechanismus in *C. elegans* wirkt und andererseits könnten die so gewonnenen Erkenntnisse Rückschlüsse auf den Menschen zulassen, da einerseits eine Genvariante des humanen Homologs FoxO3a ebenfalls mit Langlebigkeit assoziiert ist (Wilcox et al., 2008), andererseits zeigen Daten einer prospektiven Kohortenstudie (Tianjin Chronic Low-grade Systemic Inflammation and Health (TCLSIHealth)), dass die Aufnahme von Myricetin über die Nahrung invers mit der Häufigkeit von Typ-II-Diabetes assoziiert ist (Yao et al., 2019).

Einfluss funktioneller Gruppen von Flavonoiden auf ihre protektive Wirkung in C. elegans II: Prenylierung und Methylierung des A-Rings am Beispiel des Isoxanthohumol

Hopfeninhaltsstoffe sind seit langer Zeit Teil der menschlichen Ernährung, als geschmacksgebende Komponente in der Herstellung von Bier. Darüber hinaus werden Hopfenpräparate bei der Behandlung von Schlafstörungen und menopausalen Beschwerden eingesetzt (Piersen, 2003). Hopfeninhaltsstoffe werden in Verbindung gebracht mit antioxidativen, chemopräventiven, antimikrobiellen, cytotoxischen und östrogenen Wirkungen (Übersicht in Chadwick et al., 2006). Zu den polyphenolischen Hauptbestandteilen der weiblichen Hopfenblüten zählt vor allem das prenylierte Chalkon Xanthohumol (0,01%-0,5%) und in geringeren Konzentrationen die prenylierten Flavanone Isoxanthohumol und 8-Prenylnaringenin (Chadwick et al., 2006). In Bier ist Isoxanthohumol die hauptsächlich auftretende prenylierte Verbindung, da es während des Brauprozesses durch Isomerisation aus Xanthohumol hervorgeht (Gerhäuser, 2005). Der überwiegende Anteil an Studien zu pharmakologischen Wirkungen von prenylierten Polyphenolen bezieht sich jedoch auf *in vitro* Daten und es fehlt an Nachweisen zu möglichen Gesundheitsförderlichen Wirkungen *in vivo*. In der vorliegenden Arbeit wurde daher *C. elegans* als *in vivo* Modell genutzt, um physiologische Wirkungen von Isoxanthohumol und die zugrunde liegenden molekularen Mechanismen zu untersuchen.

Es konnte gezeigt werden, dass Isoxanthohumol sowohl die Resistenz gegen thermalen Stress erhöht als auch die durch Hitze induzierte Akkumulation von ROS reduziert und somit auch *in vivo* gesundheitsförderliche Wirkungen ausüben kann. Diese Effekte sind ein Alleinstellungsmerkmal von Isoxanthohumol, da für andere prenylierte Flavonoide keine oder

adverse Wirkungen in *C. elegans* beobachtet wurden (Koch et al., 2019). So zeigten die prenylierten Flavonoide Abyssinon-4´-O-methylether, Sigmoidin-B-4´-O-methylether, Glabranin, Exiguaflavanon und 8-Prenylnaringenin keine antioxidative Wirkung *in vivo*, während Abyssinon V einen prooxidativen Effekt induzierte. Für das nicht prenylierte und nicht methylierte Naringenin, welches die gemeinsame Grundstruktur für Isoxanthohumol und 8-Prenylnaringenin darstellt, konnte in *C. elegans* ebenfalls keine antioxidative Wirkung nachgewiesen werden (Grünz et al., 2012). Ebenso führte keines der prenylierten Flavonoide zu einer gesteigerten Stressresistenz, während Abyssinon V, Glabranin und 8-Prenylnaringenin die Resistenz gegen thermalen Stress sogar reduzierten. Besonders die stark unterschiedliche Wirkung im Vergleich von Isoxanthohumol und 8-Prenylnaringenin ist überraschend, da sich beide Verbindungen nur an Position 5 des A-Rings unterscheiden (5-OH: 8-Prenylnaringenin; 5-O-methyl: Isoxanthohumol). Wie bereits für das Flavonoid Myricetin gezeigt (Studie 2), führt auch bei Isoxanthohumol eine Methylierung zu einer deutlichen Änderung der biologischen Aktivität in *C. elegans*. Diese Daten aus *C. elegans* könnten somit dazu beitragen, ein besseres Verständnis zur Wirkung von 8-Prenylnaringenin-haltigen pflanzlichen Präparaten im Menschen zu erhalten, da eine große Anzahl an humanen Proteinen mit Methyltransferaseaktivität beschrieben ist und eine Metabolisierung wahrscheinlich ist (Petrossian & Clarke, 2010).

Um die molekularen Mechanismen der antioxidativen und Stressresistenz erhöhenden Wirkungen von Isoxanthohumol zu erklären, wurde zuerst die antioxidative Kapazität im zellfreien System getestet, wobei Isoxanthohumol nur eine sehr schwache antioxidative Kapazität im TEAC-Assay zeigte. Hieraus lässt sich schlussfolgern, dass die protektiven Eigenschaften nicht auf einer Wirkung als Radikalfänger beruhen kann, sondern es muss ein indirekter Effekt vorliegen z.B. durch die Modulation von zellulären Schutzmechanismen.

Wie bereits für Myricetin und seine methylierten Derivate gezeigt, ist DAF-16 eine wichtige Komponente in der Vermittlung der Stressresistenz und Langlebigkeit. In der vorliegenden Arbeit konnte gezeigt werden, dass DAF-16 ebenfalls eine wichtige Rolle in der Isoxanthohumol induzierten protektiven Wirkung einnimmt, da es erstens zu einer gesteigerten Kernlokalisation des Transkriptionsfaktors kam und zweitens, sowohl die antioxidative Wirkung als auch die gesteigerte Thermotoleranz in der *daf-16 lof* Mutante vollständig aufgehoben wurden. Die Induktion der DAF-16 Kernlokalisation ist dabei allerdings unabhängig von der Prenylierung bzw. Methylierung des A-Rings, da auch für Naringenin eine nukleäre Translokation beschrieben wurde (Grünz et al., 2012). Die Modulation von DAF-16 durch Isoxanthohumol ist auf unterschiedliche Arten denkbar: Einerseits ist die Inhibition der DAF-16 vorgeschalteten AKT-Kinasen möglich, da Negrão et al. *in vitro* eine Reduktion von phosphoryliertem AKT nach Isoxanthohumol Behandlung zeigen konnten (Negrão et al.,

2013). Allerdings konnte in einer weiteren *in vitro* Studie die AKT-Inhibition durch Isoxanthohumol nicht bestätigt werden (Krajnović et al., 2016), weshalb weitere Untersuchungen einer möglichen AKT-Inhibition in *C. elegans* zielführend wären. Andererseits wäre auch eine hormetische Wirkung durch Isoxanthohumol möglich und anhand der erhaltenen experimentellen Daten wahrscheinlicher, da in der *daf-16 lof* Mutante eine tendenziell prooxidative Aktivität von Isoxanthohumol aufgetreten ist, die zum letzten Messpunkt auch statistische Signifikanz erreichte. Diese Annahme beruht auf dem Konzept der Hormesis. Hormesis wird definiert wird als ein Prozess, bei dem die Exposition gegenüber einer geringen Dosis einer Chemikalie oder eines Umweltfaktors einen adaptiven, vorteilhaften Effekt in Zellen oder Organismen auslöst, während hohe Dosen schädlich wirken (Mattson, 2008). So konnte z.B. gezeigt werden, dass eine verstärkte Produktion von Superoxidradikalanionen, durch eine niedrige Konzentration des *redox-cyclers* Juglon (5-Hydroxy-1,4-naphthoquinone), zu einer DAF-16 und SIR-2.1 abhängigen Verlängerung der Lebensspanne in *C. elegans* führte und die Expression des Hitzeschockproteins HSP-16.2 induziert wurde (Heidler et al., 2009; Hartwig et al., 2009). Zusammengenommen lässt dies die Schlussfolgerung zu, dass Isoxanthohumol wahrscheinlich eine hormetische Wirkung in *C. elegans* ausübt, woraus eine DAF-16 abhängige, adaptive Stressantwort resultiert, die sich in einer verringerten Akkumulation von ROS und einer erhöhten Thermotoleranz manifestiert.

Die in *C. elegans* mit einer gesteigerten Stressresistenz oftmals einhergehende Verlängerung der Lebensspanne trat durch eine Behandlung mit Isoxanthohumol nicht auf. In der höchsten getesteten Konzentration von 200 µM Isoxanthohumol trat hingegen eine Reduktion der Lebensspanne in der *daf-16 lof* Mutante auf. Dieses Ergebnis, zusammen mit den zuvor beschriebenen prooxidativen Effekten, bekräftigt noch einmal die Annahme, dass Isoxanthohumol einen Stressreiz verursacht, der eine adaptive, DAF-16 abhängige protektive Stressantwort zur Folge hat. Daher sollte in weiterführenden Untersuchungen die Expression der beteiligten DAF-16 Zielgene bestimmt werden, die an der ROS Detoxifizierung (z.B. SOD, CTL, GST) oder an der Thermoresistenz (z.B. HSPs) beteiligt sind (Murphy et al., 2003; Tullet, 2014).

Ein weiteres interessantes, jedoch nicht eindeutig zu erklärendes Ergebnis in Bezug auf die Stressresistenz erbrachte die Ernährung der Tiere mit Hitze-inaktivierten Bakterien (zur Elimination des bakteriellen Stoffwechsels). Unter diesen experimentellen Bedingungen induzierte Isoxanthohumol in Wildtypen keine erhöhte Thermotoleranz und reduzierte die Stressresistenz in *daf-16 lof* Mutanten. Eine mögliche Schlussfolgerung ist, dass die Tiere durch die nicht optimale Diät mit inaktivierten Bakterien in einem Stresszustand sind, der eine DAF-16 abhängige Protektion durch Isoxanthohumol verhindert oder überlagert, da für *C. elegans* beschrieben ist, dass wichtige Nährstoffe nur von metabolisch aktiven Bakterien

gebildet werden oder durch die Erhitzung verloren gehen (Lenaerts et al., 2008). Darüber hinaus lassen die erhaltenen Ergebnisse aber auch den Schluss zu, dass die zuvor beschriebenen Effekte nicht durch Isoxanthohumol, sondern durch dessen bakterielle Metabolite verursacht worden sein können. Daher wäre es zielführend in weitergehenden Untersuchungen herauszufinden, welche Metabolite des Isoxanthohumols durch die eingesetzten *E. coli* Bakterien produziert werden und ob die Metabolite für die beobachteten Effekte in *C. elegans* verantwortlich sind. Gerade auch mit Hinblick auf die Metabolisierung von Isoxanthohumol durch das menschliche Mikrobiom lassen sich so neue Erkenntnisse gewinnen, die zu einem besseren Verständnis der Bioaktivität von Isoxanthohumol im Menschen beitragen können.

4.2 Untersuchungen zu molekularen Mechanismen der Erhöhung der Stressresistenz und Lebensspanne durch Modifikation der funktionellen Gruppen des Stilbens Resveratrol in *C. elegans* (Studien 4 und 5)

Vergleich der protektiven Wirkungen von Resveratrol mit dem natürlich vorkommenden glykosylierten Derivat TSG

Das Resveratrolderivat 2,3,5,4′-Tetrahydroxystilben-2-O-β-D-glucosid (TSG) ist einer der biologisch aktiven Hauptinhaltsstoffe, der in der traditionellen chinesischen Medizin eingesetzten Pflanze *Polygonum multiflorum*. In Studien konnten zahlreiche pharmakologische Wirkungen von TSG nachgewiesen werden, wie antioxidative, antiinflammatorische, antiatherosklerotische, chemopräventive, neuroprotektive sowie anti-ageing Effekte (Übersicht in Zhang & Chen, 2018; Qian et al., 2020). Es ist von großem Interesse, biologisch aktive Substanzen zu finden, die die gesellschaftlichen und ökonomischen Belastungen einer alternden Bevölkerung und den mit dem Altern assoziierten Erkrankungen, entgegen zu wirken. Eine Reduktion des durch ROS induzierten oxidativen Stresses, welcher mit dem Altern in Verbindung gebracht wird, ist eine Möglichkeit dem Alterungsprozess entgegen zu wirken. So konnte in der vorliegenden Arbeit gezeigt werden, dass TSG im zellfreien TEAC-Assay eine deutliche antioxidative Wirkung zeigte, die bis zu einer Konzentration von 15 µM stärker war als die Kontrollsubstanz Trolox und zudem eine stärkere Aktivität aufwies als Resveratrol. Nur das zum Vergleich der Aktivität untersuchte Quercetin zeigte eine noch stärkere antioxidative Kapazität. Diese Ergebnisse sind vergleichbar mit Studien, in der für TSG ebenfalls eine stärkere antioxidative Wirkung im Vergleich mit Resveratrol *in vitro* beschrieben wurde (Lv, 2007; Liu et al., 2018).

Vielversprechende Daten aus *in vitro* Studien erweisen sich dabei leider oftmals als nicht direkt übertragbar auf die *in vivo* Situation, da häufig die Bioverfügbarkeit *in vivo* eingeschränkt ist oder die Bioaktivität, durch Metabolisierung, modifiziert ist. Im verwendeten Modellorganismus *C. elegans* konnte hier jedoch gezeigt werden, dass TSG auch in der *in vivo* Situation eine antioxidative Wirkung ausübt. So war die durch thermalen Stress induzierte Akkumulation von intrazellulären ROS durch TSG teilweise signifikant geringer als in der Kontrolle und in der Konzentration von 100 µM etwa auf dem gleichen Niveau wie das Flavonoid Quercetin. Das zum Vergleich eingesetzte Resveratrol zeigte unter diesen Bedingungen jedoch keine antioxidative Aktivität. Dies könnte darin begründet sein, dass die *in vitro* festgestellte niedrigere antioxidative Kapazität von Resveratrol unter den verwendeten experimentellen Bedingungen nicht ausreichend war, um die stark erhöhte Produktion von ROS unter

thermalen Stressbedingungen zu reduzieren. Für diese Annahme spricht die Tatsache, dass unter physiologischen Bedingungen sowohl TSG als auch Resveratrol die Akkumulation des Alterungspigments Lipofuscin verringert haben und hierbei einen vergleichbaren Effekt erzielten, der jedoch geringer ausfiel als bei dem Flavonoid Quercetin. Ähnliche Ergebnisse konnten auch im Mausmodell, in Bezug auf die mit ROS assoziierte Bildung von *Advanced Glycation Endproducts* (AGE), gezeigt werden, wobei dort TSG, im Vergleich mit Resveratrol und dem methylierten Resveratrolderivat Pterostilben, die stärkste Inhibition der AGE-Bildung aufwies (Lv et al., 2010).

In der vorliegenden Arbeit konnte zudem gezeigt werden, dass TSG die Resistenz gegen letalen thermalen Stress in *C. elegans* erhöht. Hierbei zeigte TSG im Vergleich mit Resveratrol und Quercetin, die jeweils auch zu einer Erhöhung der Stressresistenz führten, den stärksten Effekt. Ebenso führte die Behandlung mit TSG, Resveratrol und Quercetin zu einer Verlängerung der Lebensspanne in *C. elegans*. Für das Flavonoid Quercetin sind in *C. elegans* eine Reihe von Studien durchgeführt worden, die eine Erhöhung der Stressresistenz und eine Verlängerung der Lebensspanne gezeigt haben, jedoch ist der Wirkmechanismus noch nicht hinreichend geklärt, da einerseits eine Translokation von DAF-16 gezeigt werden konnte, andererseits in einer *daf-16 lof* Mutante weiterhin eine Verlängerung der Lebensspanne, sowie eine erhöhte Resistenz gegen thermalen und oxidativen Stress beschrieben ist (Kampkötter et al.; 2007 und 2008; Pietsch et al., 2007; Saul et al., 2008). Für Resveratrol sind ebenfalls keine eindeutigen Wirkungen in der Literatur beschrieben. So zeigten Chen et al. z.B. eine erhöhte Resistenz gegen oxidativen Stress durch Resveratrol, während die Lebensspanne nicht verändert wurde, weitere Studien zeigten hingegen eine Verlängerung der Lebensspanne in *C. elegans* (Chen et al., 2013; Zarse et al., 2010; Upadhyay et al., 2013). In der vorliegenden Arbeit konnte erstmalig nachgewiesen werden, dass TSG die Stressresistenz und die Lebensspanne von *C. elegans* erhöht. Diese Ergebnisse könnten auch für eine Verwendung von TSG im Menschen eine Relevanz besitzen, da im Mausmodell durch die Behandlung mit TSG zum einen D-Galaktose-induzierte Alterungsprozesse reduziert wurden (Verbesserung des Erinnerungsvermögens und Regulation des Körpergewichts), zum anderen die Lebensspanne von SAMP8 (*Senescence Accelerated Mouse Prone 8*) Mäusen verlängert wurde (Zhou et al., 2013 und 2015). In den untersuchten Mausmodellen wurden als molekulare Mechanismen die Reduktion von ROS, NO und Insulin/IGF-1 Signalen und erhöhte Level von Ca^{2+}, Klotho und SOD diskutiert. In *C. elegans* ist Langlebigkeit ebenfalls mit einer verringerten Aktivität des Insulin/IGF-1 Signalwegs assoziiert wobei es auch zu einer gesteigerten *sod-3* mRNA Expression kommt (Honda & Honda, 1999). Das *C. elegans* Homolog von Klotho ist ebenfalls an der Regulation der Langlebigkeit und Stressresistenz beteiligt, wobei die Regulation der Langlebigkeit eine funktionierende DAF-2/DAF-16 Signalleitung erfordert, während die Regulation der Resistenz gegen oxidativen Stress nur die

Aktivität von DAF-16 erfordert (Château et al., 2010). Inwiefern Resveratrol oder TSG die Aktivität von Klotho in *C. elegans* moduliert und dies an den protektiven Wirkungen in *C. elegans* beteiligt ist, bleibt eine wichtige Frage für weitergehende mechanistische Untersuchungen. Es könnte sich hierbei jedoch um einen speziesübergreifenden anti-ageing Mechanismus von Resveratrol bzw. von Resveratrolderivaten handeln.

Um einen ersten Hinweis auf molekulare Mechanismen der protektiven Wirkungen von TSG in *C. elegans* zu erhalten, wurde die Expression der an der oxidativen Stressantwort beteiligten Enzyme SOD-3 und GST-4, mit Hilfe von transgenen GFP-gekoppelten Reporterstämmen untersucht. Hierbei zeigte sich, dass die Expression der SOD unter basalen Bedingungen unverändert blieb und dies auch bei Resveratrol und Quercetin der Fall war. Somit sind die protektiven Effekte nicht auf eine gesteigerte Expression der SOD zurückzuführen, was sich auch bei zusätzlich appliziertem oxidativen Stress (durch Juglon) zeigt, da das Expressionslevel der SOD zwar allgemein angestiegen ist, durch Quercetin, Resveratrol und TSG jedoch wieder reduziert wurde. Ähnlich verhält es sich bei der untersuchten GST-4 Expression: Unter basalen Bedingungen ist die Expression durch Quercetin unverändert, während Resveratrol und TSG eine geringe Reduktion der GST induzieren; unter oxidativen Stressbedingungen ist die Expression insgesamt gesteigert, jedoch liegen die GST-4 Level unterhalb der Kontrolle. Aus diesen Ergebnissen lässt sich schlussfolgern, dass TSG seine protektiven und lebensverlängernden Wirkungen entweder durch eine direkte Radikalfängeraktivität vermittelt oder dass eine Regulation von Genen der Stressantwort stattfindet, die hier nicht untersucht wurden. Durch die Ergebnisse in Mäusen von Zhou et al. ist eine Modulation von stress-assoziierten Signalwegen jedoch wahrscheinlich. In der vorliegenden Arbeit wurde daher die Wirkung von TSG, Resveratrol und Quercetin in *daf-16 lof* Mutante durchgeführt, jedoch zeigte sich, dass die Anwesenheit von DAF-16 nicht notwendig ist für eine Verlängerung der Lebensspanne. Dieses Ergebnis unterstützt zwar im Fall von Quercetin die Befunde von Saul et al., die ebenfalls eine von DAF-16 unabhängige Verlängerung der Lebensspanne beschrieben haben, jedoch stehen die Daten im scheinbaren Gegensatz zu den Erkenntnissen aus den Studien mit Mäusen, die eine Beteiligung des IIS für TSG zeigen konnten (Saul et al., 2008; Zhou et al., 2013 und 2015). Im Mausmodell wurde allerdings nicht mit einem DAF-16 bzw. FoxO *knockout* gearbeitet, sondern es wurde nur eine verringerte IIS Aktivität über geringere Level von IGF-1 und des IGF-1 Rezeptors nachgewiesen. Wie bereits für Quercetin oder auch Isoxanthohumol gezeigt, ist eine Translokation von DAF-16 in den Zellkern nicht zwangsläufig mit einer verlängerten Lebensspanne assoziiert (Kampkötter et al., 2007; Saul et al., 2008; Büchter et al., 2015). Besonders im Hinblick auf die Nutzung von TSG als potentielles anti-ageing Mittel im Menschen und die diversen, in *in vitro* Studien festgestellten positiven Effekte, ist es von

großem Interesse, ob und wenn ja, welche weiteren protektiven Signalwege durch TSG moduliert werden, die zu einer Verlängerung der Lebensspanne führen.

Vergleich der protektiven Wirkungen von Resveratrol mit neu synthetisierten Resveratrolderivaten

Natürlich vorkommende Stilbene, wie das Resveratrol und sein 3,5-O-dimethyliertes Derivat Pterostilben, wirken in Pflanzen als so genannte Phytoalexine und werden in Folge von abiotischen und biotischen Stressreizen gebildet, um die Pflanzen vor UV-Strahlung oder mikrobiellem Befall zu schützen (Jeandet, 2015). Darüber hinaus konnten zahlreiche *in vitro* und *in vivo* Studien für die menschliche Gesundheit potenziell förderliche Wirkungen von Resveratrol und Pterostilben zeigen. Für Resveratrol sind z.B. antioxidative, kardioprotektive, chemopräventive, sowie neuroprotektive Wirkungen beschrieben (Jang et al., 1997; Bradamante et al., 2004; Sengottuvelan et al., 2006; Gulcin, 2010, Lofrumento et al., 2014; Regitz et al., 2015). Kontrovers diskutiert wird die Rolle von Resveratrol in Bezug auf einen Einsatz als mögliches anti-ageing Mittel. So zeigte Resveratrol in unterschiedlichen Spezies wie *S. cerevisiae*, *C. elegans*, *D. melanogaster*, oder *N. furzeri* einerseits positive Effekte auf die Lebensspanne (Howitz et al., 2003; Wood et al., 2004; Valenzano et al., 2006), andererseits wurden keine oder adverse Effekte auf die Lebensspanne von *C. elegans* beschrieben (Chen et al., 2013). Pterostilben zeigte in Studien ebenfalls antioxidative, chemopräventive und neuromodulatorische Eigenschaften, die bei der Pathogenese der Alzheimer Krankheit und bei der Alterung eine Rolle spielen (Rimando et al., 2002; Tolomeo et al., 2005; Chakraborty et al., 2010; Paul et al., 2010; Chang et al., 2012). Für die Ausübung von physiologischen Wirkungen in der *in vivo* Situation ist die orale Bioverfügbarkeit von großer Bedeutung, so könnte z.B. die in dem SAMP8 Mausmodell beobachtete neuromodulatorische Wirkung von Pterostilben, die im direkten Vergleich für Resveratrol nicht gezeigt werden konnte, auf die bessere orale Bioverfügbarkeit des Pterostilbens (in der Ratte) zurückzuführen sein (Kapetanovic et al., 2011; Chang et al., 2012).

Durch die große Anzahl an biologischen Effekten der natürlich auftretenden Stilbene und einem möglichen Einsatz als anti-ageing Mittel, besteht auch ein gesteigertes Interesse daran, synthetisch hergestellte Stilbenderivate mit besserer Bioverfügbarkeit oder gesteigerter Bioaktivität zu finden. Aus diesem Grund hat die Arbeitsgruppe von Prof. Dr. Csuk, vom Lehrstuhl für Organische und Bioorganische Chemie der Martin-Luther Universität, eine Reihe

von Stilbenderivaten synthetisiert, die antioxidative, antimikrobielle, chemopräventive sowie Acetylcholinesterase- und Butyrylcholinesteraseinhibitor-Aktivität aufweisen (Albert et al, 2011; Csuk et al., 2012; Csuk et al 2013[a], 2013[b]). Im weiteren Verlauf erfolgt die Bezeichnung der Derivate analog zu Studie 5: (**3**) Trans-3,5-dimethoxy-4-fluoro-4´-hydroxystilben, (**4**) Trans-4`-hydroxy-3,4,5-trifluorostilben, (**5**) Trans-2,5-dimethoxy-4´-hydroxystilben und (**6**) Trans-2,4´,5-trihydroxystilben.

Zunächst wurde in der vorliegenden Arbeit das antioxidative Potential der Stilbenderivate untersucht und es zeigte sich, dass alle getesteten Verbindungen im zellfreien TEAC-Assay freie Radikale abfangen können. Hierbei ergaben sich unterschiedliche Effektstärken, die durch die Art und die Stellung der funktionellen Gruppen im Stilbengrundgerüst bedingt sind. Resveratrol und die Verbindung (6) wiesen die höchste und eine mit der Kontrolle Trolox vergleichbare antioxidative Kapazität auf, wobei in höheren Konzentrationen Verbindung (6) potenter war als Resveratrol. Die starke antioxidative Kapazität von Resveratrol und von Verbindung (6) liegt darin begründet, dass beide Substanzen drei freie Hydroxylgruppen aufweisen und für Reaktionen mit Radikalen zur Verfügung stehen, wobei die unterschiedliche Stellung der Hydroxylgruppen im A-Ring (Resveratrol in *meta*-Stellung und (6) in *para*-Stellung) vermutlich für die stärkere Wirkung von Verbindung (6) verantwortlich ist. Die methylierten Derivate Pterostilben und Verbindung (5), unterscheiden sich ebenfalls durch die Stellung der Methylgruppen, mit der Folge, dass Verbindung (5) (*para*-Stellung) in hohen Konzentrationen potenter war als Pterostilben (*meta*-Stellung). Die Fluorierung der Verbindungen (3) und (4) führte zu keiner großen Veränderung im antioxidativen Potential und war mit Pterostilben vergleichbar. Eine stärkere antioxidative Wirkung von Resveratrol im Vergleich zu Pterostilben *in vitro* (Citronellal Thermooxidation, DPPH Reduktion, Lipid Peroxidation in Rattenleber Mikrosomen) ist auch in der Literatur beschrieben (Stivala et al., 2001).

Die *in vitro* ermittelte antioxidative Wirkung konnte jedoch nicht direkt auf die *in vivo* Situation übertragen werden. In *C. elegans* zeigten nur die Verbindungen (3) und (6) eine signifikante Reduktion der thermal induzierten ROS Produktion, während die methylierten Derivate Pterostilben und Verbindung (5) eine tendenzielle Reduktion hervorriefen. Resveratrol und Verbindung (4) wiesen nur minimale Effekte in diesem experimentellen System auf. Für Resveratrol wurden somit die Ergebnisse aus der vorliegenden Studie 4 bestätigt (Büchter et al., 2015). Allerdings wurde in Studien ebenfalls eine protektive Wirkung von Resveratrol bei oxidativem Stress beschrieben (Gruber et al. 2007; Chen et al., 2013). Aus diesem Grund lassen sich generelle Aussagen zur antioxidativen Wirkung von Resveratrol *in vivo* bislang nicht treffen. Die bessere antioxidative Wirkung von Pterostilben, im Vergleich mit Resveratrol, in *C. elegans* ist möglicherweise durch eine gesteigerte Bioverfügbarkeit zu erklären

(Kapetanovic et al., 2011), es liegen bislang allerdings noch keine experimentellen Daten zur Bioverfügbarkeit von Pterostilben in *C. elegans* vor. Durch die *in vivo* Aktivität der Verbindungen (3) und (6) wurden diese im weiteren Verlauf der Arbeit, im Vergleich zu Resveratrol, weiter untersucht.

Durch das große Interesse Substanzen zu finden, die einen positiven Einfluss auf das Altern ausüben, wurde in der vorliegenden Arbeit der Einfluss der Verbindungen (3) und (6) im Vergleich mit Resveratrol, auf die Lebensspanne von *C. elegans* untersucht. Unter den hier verwendeten experimentellen Bedingungen induzierten die Verbindungen (3) und (6) eine signifikante Verlängerung der Lebensspanne, während Resveratrol keinen Effekt zeigte. Für die neu synthetisierten Stilbenderivate (3) und (6) konnte somit erstmalig eine positive Wirkung auf das Altern *in vivo* nachgewiesen werden. Um einen Einfluss auf das Altern durch kalorische Restriktion auszuschließen, wurde die Körpergröße der Tiere bestimmt, wobei sich keine Unterschiede zeigten. Durch die Bestimmung der pharyngealen Pumprate, als Marker für die Nahrungsaufnahme, wurde eine kalorische Restriktion als unspezifischer Effekt für Resveratrol in einer weiteren Studie ebenfalls ausgeschlossen (Gruber et al., 2007). Ein weiterer Hinweis auf eine positive Beeinflussung des Alterns durch (3) und (6) ergab die Analyse der Mobilität von gealterten Tieren, die zum ersten Messpunkt (11 Tage) deutlich über der Bewegungsfähigkeit der Kontrolltiere lag, während bei Resveratrol nur eine tendenzielle Erhöhung festzustellen war. Eine Studie mit dem kurzlebigen Fisch *N. furzeri* zeigte hingegen eine Erhöhung der Mobilität sowie eine Verlängerung der Lebensspanne durch Resveratrol (Valenzano et al., 2006).

Weiterhin wurde der Einfluss der antioxidativ und lebensverlängernd wirkenden Stilbenderivate (3) und (6) auf die Resistenz gegen thermalen Stress untersucht. Hier zeigten die Verbindungen (3) und (6) jedoch keine protektive Wirkung, die Behandlung führte im Gegenteil zu einer signifikant verringerten Thermoresistenz, während Resveratrol keinen Effekt zeigte. Die verringerte Stressresistenz ist dabei nicht auf eine erhöhte ROS Produktion zurückzuführen, da (3) und (6) unter gleichen Stressbedingungen die geringste Belastung mit ROS aufwiesen. Dass eine antioxidative und lebensverlängernde Wirkung nicht direkt mit einer erhöhten Resistenz gegen thermalen Stress korreliert, konnte auch für Resveratrol oder Myricetin gezeigt werden (Gruber et al., 2007; Benedetti et al., 2008; Büchter et al., 2013).

Resveratrol wird in der Literatur, in Bezug auf das Altern, häufig mit Sirtuinen (NAD^+-abhängigen Deacetylasen) in Verbindung gebracht. So wurde z.B. in *S. cerevisiae* eine Sirtuin-abhängige Lebensspannenverlängerung durch Resveratrol gefunden, ebenso wie in *C. elegans* und *D. melanogaster* (Howitz et al., 2003; Wood et al., 2004). Darüber hinaus konnte gezeigt werden, dass SIR-2.1 (*C. elegans* Sirtuin), zusammen mit 14-3-3 Proteinen, DAF-16 aktivieren kann und dies zu gesteigerter Stressresistenz und Lebensspanne führt

(Berdichevsky et al., 2006). Weiterhin ist für SKN-1 die Beteiligung an der oxidativen Stressantwort und Langlebigkeit beschrieben (An & Blackwell, 2003; Wang et al., 2010). Um eine Beteiligung dieser stressresponsiven Faktoren an der gefundenen antioxidativen Wirkung der Stilbenderivate (3) und (6) zu untersuchen, wurde die Hitze-induzierte ROS Akkumulation in *daf-16*, *sir-2.1* und *skn-1 lof* Mutanten getestet. Die antioxidative Wirkung der Verbindung (3) bei thermalem Stress war *in vivo* in allen untersuchten *lof* Mutanten nicht mehr vorhanden, d.h. diese Faktoren sind essenziell für die Vermittlung der antioxidativen Wirkung in *C. elegans* unter diesen Stressbedingungen. Für die Verbindung (6) zeigte sich ein ähnliches Bild in den *skn-1* und *sir-2.1* lof Mutanten, während in der *daf-16* lof Mutante zu einem frühen Zeitpunkt noch eine antioxidative Wirkung zu erkennen war, dieser Effekt blieb aber im weiteren Verlauf aus. Hiermit zeigen die erhaltenen Ergebnisse, dass die Verbindungen (3) und (6) ihre antioxidative Wirkung *in vivo* unter thermalem Stress wahrscheinlich nicht oder nur teilweise durch das direkte Abfangen von freien Radikalen ausüben. Vielmehr ist eine Modulation der oben genannten Faktoren wahrscheinlich.

Um eine modulatorische Wirkung der Verbindungen auf die zelluläre Lokalisation der Transkriptionsfaktoren DAF-16 und SKN-1 zu untersuchen, wurden Reporterstämme eingesetzt, die GFP gekoppelt an den jeweiligen Transkriptionsfaktor exprimieren. Unter Normalbedingungen zeigten die Stilbenderivate jedoch keine Translokation der Transkriptionsfaktoren in den Zellkern. Somit bleibt noch unklar, ob DAF-16, SKN-1 und SIR-2.1 auch an der Verlängerung der Lebensspanne durch die Stilbenderivate beteiligt sind oder ob noch weitere Faktoren moduliert werden. Einen wichtigen Anhaltspunkt liefert die Studie von Yoon et al., in der gezeigt werden konnte, dass der lebensverlängernde Effekt von Resveratrol in *C. elegans* auf zwei unabhängigen Wege beruht: Auf der einen Seite kann Resveratrol die Langlebigkeit in Abhängigkeit von SIR-2.1 und DAF-16 induzieren, während auf der anderen Seite SKN-1 und MPK-1 (Mitogen-aktivierte-Proteinkinase-1; homolog der humanen MAPK1) die Lebensspanne durch Resveratrol ebenfalls verlängern können (Yoon et al., 2018). Hierzu wäre es zielführend, in nachfolgenden Untersuchungen herauszufinden ob die Lebensverlängerung durch die modifizierten Resveratrolderivate in *lof* Mutanten bzw. im RNAi vermittelten *knock-down* von *sir-2.1/daf-16* und *mpk-1/skn-1* bestehen bleibt. Durch die im Menschen vorhandenen Homologe dieser Faktoren ergeben sich somit Hinweise auf mögliche gesundheitsförderliche sowie anti-ageing Wirkungen im Menschen, die eventuell stärker ausfallen könnten als es bislang für Resveratrol in Modellen gezeigt werden konnte.

Einfluss des bakteriellen Metabolismus auf die Bioaktivität von Resveratrol und modifizierter Resveratrolderivate

In der Literatur wird der Einfluss von Resveratrol auf das Altern in *in vivo* Modellen kontrovers diskutiert. In *C. elegans* sind sowohl positive als auch adverse Effekte beschrieben, die vermutlich auf unterschiedliche experimentelle Bedingungen zurückzuführen sind. So zeigten Chen et al. bei Verwendung von Agarplatten positive Effekte für Resveratrol nur unter Glukose-Stress, während unter normalen Bedingungen keine Effekte aufgetreten sind; wurde Resveratrol in flüssigem S-Medium eingesetzt, traten adverse Effekte auf die Lebensspanne von *C. elegans* auf (Chen et al., 2013). Positive Effekte traten hingegen bei einer Studie von Lee et al. auf, bei der die Tiere unter normalen Bedingungen auf Agarplatten behandelt wurden und metabolisch aktive Bakterien als Nahrung verfüttert wurden (Lee et al., 2016). Eine Verlängerung der Lebensspanne durch Resveratrol wurde auch von Gruber et al., beschrieben, die ebenfalls Agarplatten, aber Hitze-inaktivierte Bakterien als Nahrungsquelle verwendeten (Gruber et al., 2007). Hierdurch wird ersichtlich, dass die experimentellen Bedingungen maßgeblichen Einfluss auf die erhaltenen Ergebnisse haben. Auch in der vorliegenden Arbeit hat sich dieser Einfluss gezeigt, da im Vergleich von Resveratrol und TSG (Studie 4) ein lebensverlängernder Effekt durch Resveratrol induziert wurde, der sich im Vergleich von Resveratrol und den synthetisierten Derivaten (Studie 5) nicht bestätigen konnte. Im Vergleich der beiden Studien bestand der Unterschied lediglich in im Einsatz der verwendeten Bakterien. Während in Studie 4 metabolisch aktive Bakterien verwendet wurden, sind in Studie 5 metabolisch inaktivierte Bakterien als Nahrung eingesetzt worden. Zusätzlich zeigte sich in der Studie 5 auch bei der Resistenz gegen thermalen Stress und der antioxidativen Wirkung *in vivo*, dass bakterielle Metabolite die Bioaktivität der Resveratrolderivate stark verändern können. Eine Wiederholung der Experimente mit metabolisch aktiven Bakterien hob die erhöhte Stressresistenz wieder auf und verhinderte eine antioxidative Wirkung der Resveratrolderivate. Hieraus lässt sich schlussfolgern, dass der bakterielle Metabolismus einen wesentlichen Einfluss auf die Bioaktivität der Stilbene hat. Die in *C. elegans* erhaltenen Ergebnisse sind zwar nicht direkt auf den Menschen übertragbar, es ergibt sich aber ein wichtiger Anhaltspunkt für potenzielle Wirkungen oral aufgenommener Stilbene im Menschen, da die metabolische Aktivität des menschlichen Mikrobioms für die Bioaktivität beachtet werden muss.

4.3 Schlussfolgerungen

Die in den vorliegenden Studien erhaltenen Daten zeigen, dass unterschiedliche pflanzliche Polyphenole in dem Modellorganismus *C. elegans* antioxidative und lebensverlängernde Wirkungen induzieren können. Diese im Modellsystem gefundenen gesundheitsförderlichen Effekte beruhen jedoch oftmals nicht allein auf einer Wirkung als Radikalfänger und somit als direktes Antioxidans, vielmehr sind Signalwege involviert, die an Stoffwechselprozessen und der zellulären Stressantwort beteiligt sind. Hierbei stellte sich heraus, dass die protektiven und lebensverlängernden Wirkungen der untersuchten Flavonoide häufig auf der Anwesenheit des Transkriptionsfaktors DAF-16 beruhen, einem zentralen Faktor des evolutionär konservierten Insulin/IGF-1 ähnlichen Signalwegs. Dieser Befund könnte somit eine direkte Relevanz für mögliche positive Wirkungen dieser Substanzen auf die Gesundheit des Menschen besitzen, da die Gruppe der humanen FoxO Transkriptionsfaktoren einerseits die Homologe zu DAF-16 darstellen, andererseits ist eine Genvariante von FoxO3a mit Langlebigkeit im Menschen assoziiert. Die für das Flavonoid Myricetin beobachtete Modulation von DAF-16 liefert einen wichtigen Hinweis auf einen molekularen Wirkmechanismus im Menschen, um die in der Literatur beschriebene inverse Korrelation der Myricetin-Aufnahme und der Wahrscheinlichkeit an Typ-II-Diabetes zu erkranken erklären zu können.

Weiterhin zeigen die vorliegenden Studien, dass die Art und die Position von funktionellen Gruppen der Verbindungen eine wichtige Rolle für ihre biologische Wirksamkeit spielen und zum Teil deutlich stärkere Effekte induzieren als die Ausgangssubstanz. Für das Flavonoid Myricetin zeigte sich eine Substitution der Hydroxylgruppen durch O-Methylierung im B-Ring als vorteilhaft für die Wirksamkeit *in vivo*. Ebenso zeigte die methylgruppe im A-Ring des Isoxanthohumol eine gesteigerte Bioaktivität im Vergleich mit der in der Literatur beschriebenen Wirkung von 8-Prenylnaringenin. Eine Glykosylierung des Resveratrol, ebenso wie eine *para*-Stellung der Hydroxylgruppen, oder eine Modifikation durch O-Methylierung in Kombination mit einer Fluorierung können eine Verbesserung der physiologischen Wirksamkeit induzieren. Aus diesen Daten ergibt sich sie Schlussfolgerung, dass eine Modifikation der funktionellen Gruppen protektiv wirkender Leitstrukturen die physiologische Wirksamkeit der Verbindungen deutlich steigern kann. Jedoch besteht an dieser Stelle noch Bedarf an weiterführenden Untersuchungen, um zu klären, ob eine veränderte Bioverfügbarkeit, eine veränderte Bioaktivität oder ein Zusammenspiel beider Parameter vorliegt.

Gerade im Hinblick auf mögliche gesundheitsförderliche Wirkungen von polyphenolischen Pflanzenstoffen im Menschen, die überwiegend über die Nahrung aufgenommen werden, ist die orale Verfügbarkeit der Verbindungen von herausragender Bedeutung. In den durchgeführten Studien sind sowohl aktivierende als auch inhibierende Wirkungen der

Substanzen durch den bakteriellen Metabolismus gezeigt worden. Es bleibt jedoch noch zu klären, welche Metabolite durch das menschliche Mikrobiom produziert werden und welche physiologischen Effekte sie vermitteln. Allgemeingültige Aussagen zu den unterschiedlichen Leitstrukturen, d.h. ob der bakterielle Stoffwechsel aktivierend oder inhibierend wirkt, lassen sich noch nicht treffen. Die in *C. elegans* erhaltenen Ergebnisse lassen jedoch die Schlussfolgerung zu, dass der bakterielle Metabolismus sehr wahrscheinlich auch einen entscheidenden Einfluss auf die Funktionalität der Substanzen im Menschen hat.

5. Zusammenfassung

In den letzten 150 Jahren konnte die durchschnittliche menschliche Lebensspanne durch Fortschritte in der medizinischen Versorgung, vor allem durch die Bekämpfung von Infektionskrankheiten, drastisch gesteigert werden. Die Gesellschaft ist durch diese positive Entwicklung allerdings auch mit neuen Herausforderungen konfrontiert, da mit zunehmendem Alter auch das Risiko steigt, an altersassoziierten Krankheiten zu leiden und dadurch das Gesundheitssystem stark belastet wird. Daher besteht ein gesteigertes Interesse daran Interventionen zu finden, die ein möglichst gesundes Altern ermöglichen. Einen vielversprechenden Ansatzpunkt stellen sekundäre Pflanzenstoffe dar, da Befunde aus epidemiologischen Studien zeigen, dass eine Aufnahme von polyphenolischen Pflanzenstoffen, wie z.B. Flavonoiden, die Inzidenz von altersassoziierten Erkrankungen senken kann. Ein direkter Nachweis einer gesteigerten Lebensspanne im Menschen und zugrunde liegende Funktionsprinzipien liegen bislang jedoch nicht vor und die Umsetzung in Humanstudien gestaltet sich schwierig bis unmöglich. Daher wurde in dieser Arbeit die Wirkung von polyphenolischen Verbindungen, die zu den Klassen der Flavonoide und Stilbene zählen, auf die Lebensspanne und mögliche beteiligte molekulare Mechanismen, wie eine antioxidative Wirkung und die Modulation von stressresponsiven Signalwegen, im Modellorganismus *C. elegans* untersucht. Darüber hinaus wurde geprüft, ob bestimmte Strukturmerkmale der Verbindungen für die Wirkungen in *C. elegans* verantwortlich sind und ob eine Modifikation der Substanzen die Bioaktivität steigert.

In der vorliegenden ersten Studie konnte gezeigt werden, dass das Flavonoid Myricetin das Altern in *C. elegans* positiv beeinflusst, die durchschnittliche Lebensspanne der behandelten Tiere deutlich gesteigert war und auch der speziesübergreifende Alterungsmarker Lipofuscin reduziert war, während die Stressresistenz unverändert blieb. Auf molekularer Ebene wurde nachgewiesen, dass Myricetin in *C. elegans* antioxidativ wirkt, dies jedoch ebenso wie die verlängerte Lebensspanne von dem Transkriptionsfaktor DAF-16 abhängig ist. In der folgenden Studie wurde der Fokus darauf gerichtet herauszufinden, ob Hydroxylgruppen im B-Ring des Myricetin für dessen Bioaktivität notwendig sind. Durch den Einsatz von methylierten Derivaten, in denen eine, zwei oder alle drei Hydroxyl- durch Methylgruppen substituiert waren, konnte nachgewiesen werden, dass die Hydroxylgruppen nicht nur nicht notwendig sind, sondern im Gegenteil eine Methylierung die biologische Aktivität oder Verfügbarkeit erhöht. In der dritten Studie konnte gezeigt werden, dass das prenylierte Flavonoid Isoxanthohumol wahrscheinlich eine hormetische Wirkung in *C. elegans* ausübt, die eine DAF-16 abgängige, adaptive Stressantwort zur Folge hat und sich in einer reduzierten Akkumulation von ROS und einer erhöhten Thermotoleranz ausdrückt. Es bleibt jedoch noch unklar, ob Isoxanthohumol oder bakterielle Metabolite für die beobachteten Effekte

verantwortlich sind, da die antioxidative Wirkung und die erhöhte Stressresistenz bei metabolisch inaktiven Bakterien ausblieb. In der vierten Studie wurde nachgewiesen, dass das glykosylierte Resveratrolderivat TSG die Lebensspanne von *C. elegans* verlängert, sowie die Thermotoleranz erhöht und die Hitze-induzierte ROS Akkumulation und die Menge des Alterungspigments Lipofuscin reduziert. Dabei zeigte TSG im Vergleich zu Resveratrol stärkere Effekte in der Stressresistenz und der thermal induzierten ROS Akkumulation. Ein direkter Einfluss auf molekulare Mechanismen konnte nicht nachgewiesen werden, da *daf-16 lof* Mutanten weiterhin eine verlängerte Lebensspanne aufwiesen und die Expression der an der zellulären antioxidativen Stressantwort beteiligten Enzyme SOD-3 und GST-4 nicht gesteigert war. Schließlich wurde in der letzten Studie gezeigt, dass die neu synthetisierten Stilbenderivate Trans-3,5-dimethoxy-4-fluoro-4´-hydroxystilben und Trans-2,4´,5-trihydroxystilben die Lebensspanne von *C. elegans* verlängern. Zusätzlich induzierten die Derivate *in vivo* eine deutlich stärkere antioxidative Wirkung als die Referenzsubstanz Resveratrol. Durch den Einsatz von *lof* Mutanten der stressresponsiven und an der Alterung beteiligten Faktoren DAF-16, SKN-1 und SIR-2.1 konnte eine Beteiligung dieser Faktoren an der antioxidativen Wirkung in *C. elegans* nachgewiesen werden. Zusätzlich deuten die erhaltenen Ergebnisse darauf hin, dass das Vorhandensein von metabolisch aktiven Bakterien einen Einfluss auf die Bioaktivität der Stilbenderivate haben kann.

Die in der vorliegenden Arbeit erhaltenen Daten zu protektiven und lebensverlängernden Effekten im Modellorganismus *C. elegans* geben Hinweise darauf wie polyphenolische Pflanzeninhaltsstoffe mögliche positive Wirkungen auf das Altern im Menschen ausüben könnten, besonders im Hinblick auf die Beteiligung von evolutionär konservierten Signalwegen.

6. Summary

Over the last 150 years, the average human lifespan has increased dramatically due to advances in medical care, especially by combating infectious diseases. However, this positive development also confronts society with new challenges, since the risk of suffering from age-associated diseases increases with increasing age, placing a heavy burden on the healthcare system. Therefore, there is a growing interest in finding interventions that enable people to age as healthily as possible. Secondary plant compounds represent a promising starting point, as findings from epidemiological studies show that the intake of polyphenolic plant compounds, such as flavonoids, can reduce the incidence of age-associated diseases. However, direct evidence of an increased human life span and the underlying functional principles are not yet available and the implementation in human studies is difficult or even impossible. Therefore, in this work the effect of polyphenolic compounds, which belong to the classes of flavonoids and stilbenes, on life span and possible molecular mechanisms involved, such as antioxidative effects and the modulation of stress-responsive signaling pathways, were investigated in the model organism *C. elegans*. In addition, it was examined whether certain structural features of the compounds are responsible for the effects in *C. elegans* and whether a modification of the compounds increases their bioactivity.

In the first study it could be shown that the flavonoid myricetin has a positive effect on ageing in *C. elegans*. The average life span of the treated animals was significantly increased and also the species non-specific ageing marker lipofuscin was reduced, while stress resistance remained unchanged. At the molecular level, myricetin was shown to have an antioxidant effect in *C. elegans*, but this effect, as well as the prolonged life span, was dependent on the transcription factor DAF-16. The following study was focussed on finding out whether hydroxyl groups in the B-ring of myricetin are necessary for its bioactivity. By using methylated derivatives in which one, two or all three hydroxyl groups were substituted by methyl groups, it was shown that not only are the hydroxyl groups not necessary, but on the contrary, methylation increases biological activity or availability. In the third study, it was shown that the prenylated flavonoid isoxanthohumol probably exerts a hormetic effect in *C. elegans*, resulting in a DAF-16-dependent adaptive stress response leading to a reduced accumulation of ROS and an increased thermal stress resistance. However, it remains unclear whether isoxanthohumol or bacterial metabolites are responsible for the observed effects, as the antioxidative effect and increased stress resistance did not occur in metabolically inactive bacteria as the food source. In the fourth study it was shown that the glycosylated resveratrol derivative TSG prolongs the life span of C. elegans, increases thermal tolerance and reduces heat-induced ROS accumulation as well as the amount of the ageing pigment lipofuscin. Compared to resveratrol, TSG exhibited stronger effects in stress resistance and thermally

induced ROS formation. A direct influence on molecular mechanisms could not be demonstrated, since *daf-16 lof* mutants continued to show an extended life span and the expression of the enzymes SOD-3 and GST-4, which are involved in the cellular antioxidative stress response, was not increased. Finally, the last study showed that the newly synthesized stilbene derivatives trans-3,5-dimethoxy-4-fluoro-4'-hydroxystilbene and trans-2,4',5-trihydroxystilbene prolong the life span of *C. elegans*. In addition, the derivatives induced a significantly stronger antioxidative effect *in vivo* than the reference substance resveratrol. By using loss of function mutants of the stress-responsive and aging-related factors DAF-16, SKN-1 and SIR-2.1, a participation of these factors in the antioxidative effects in *C. elegans* could be demonstrated. In addition, the results obtained in this study suggest that the presence of metabolically active bacteria may have an influence on the bioactivity of the stilbene derivatives.

The data obtained in the present work on protective and life-prolonging effects in the model organism *C. elegans* provide indications of how polyphenolic plant compounds might exert possible beneficial effects on human ageing, especially regarding the involvement of evolutionary conserved signalling pathways.

7. Literaturverzeichnis

Die in den Veröffentlichungen zitierte Literatur ist dort in dem jeweils entsprechenden Format aufgeführt. Das nachfolgende Literaturverzeichnis bezieht sich auf den deutsch-sprachigen Teil dieser Dissertationsschrift.

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8. Veröffentlichungen

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Kongressbeiträge

Posterpräsentationen

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11. Eidesstattliche Erklärung/ *Declaration under Oath*

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

Datum/ Date	Unterschrift/ Signature