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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

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

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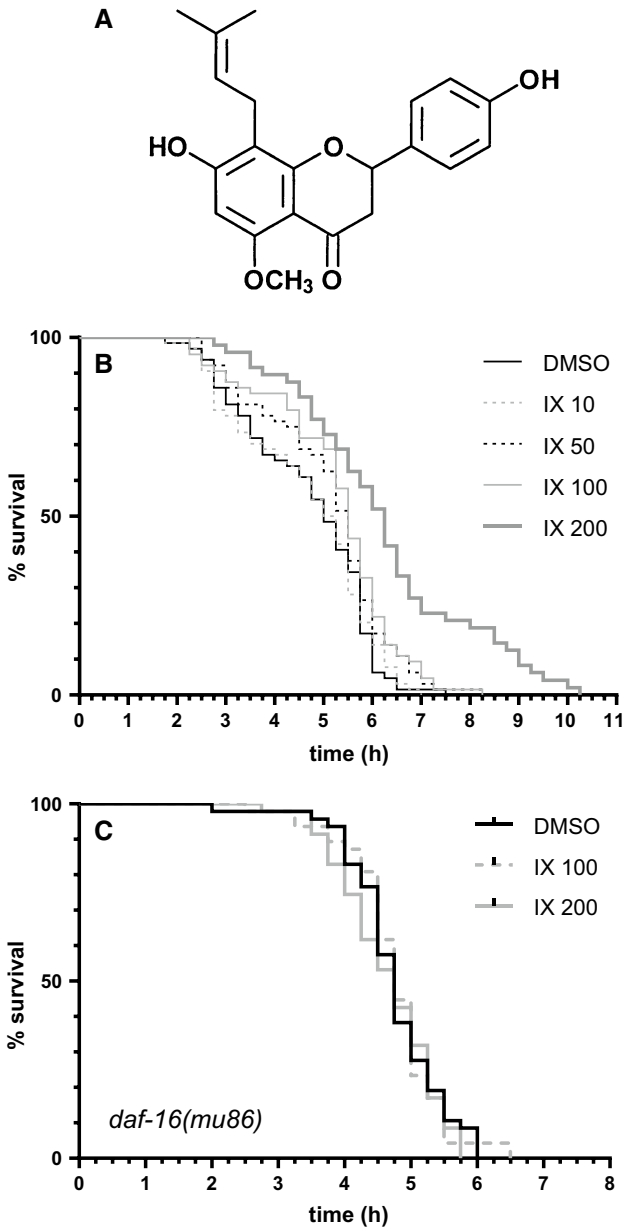

Fig. 1 Isoxanthohumol enhances the heat-stress resistance of *C. elegans* depending on DAF-16. **a** Chemical structure of isoxanthohumol; **b**, **c** modulation of stress resistance by IX: 3 days after hatching age-synchronised, wildtype nematodes (**b**) or *daf-16(mu86)* nematodes (**c**) are incubated 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).

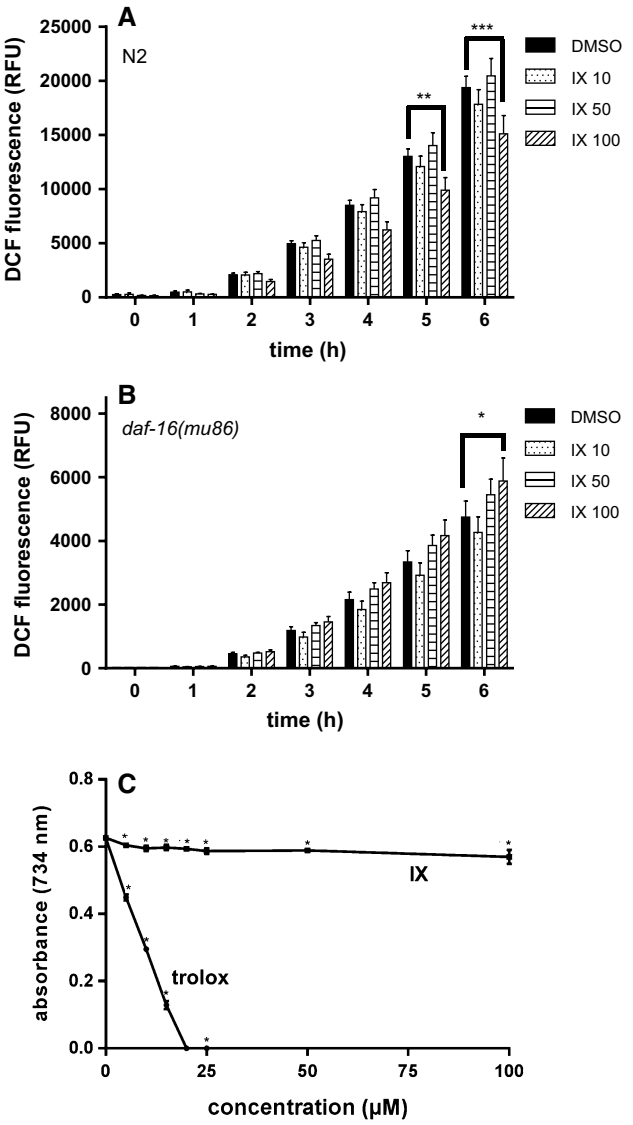

Fig. 2 Isoxanthohumol exerts antioxidative properties in *C. elegans* depending on DAF-16. Three days after hatching age-synchronised, wildtype nematodes (**a**) or *daf-16(mu86)* nematodes (**b**) are incubated with IX or 0.2 % DMSO in liquid S-medium for 48 h. After a washing step, ten nematodes are 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/

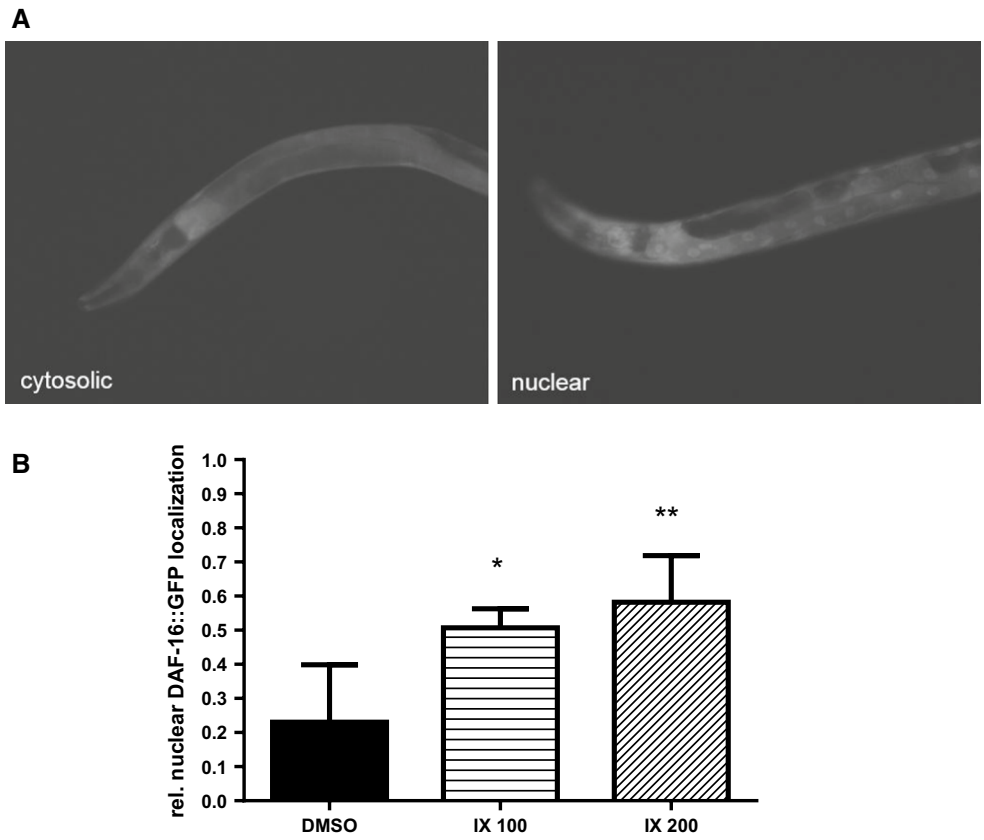

Fig. 3 Isoxanthohumol induces nuclear translocation of DAF-16. Transgenic nematodes (DAF-16-GFP; strain TJ356) are used to detect the localisation of the transcription factor DAF-16. **a** The nuclear localisation of DAF-16-GFP is visible by distinct fluorescent dots in the nematode (*right*) in contrast 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.

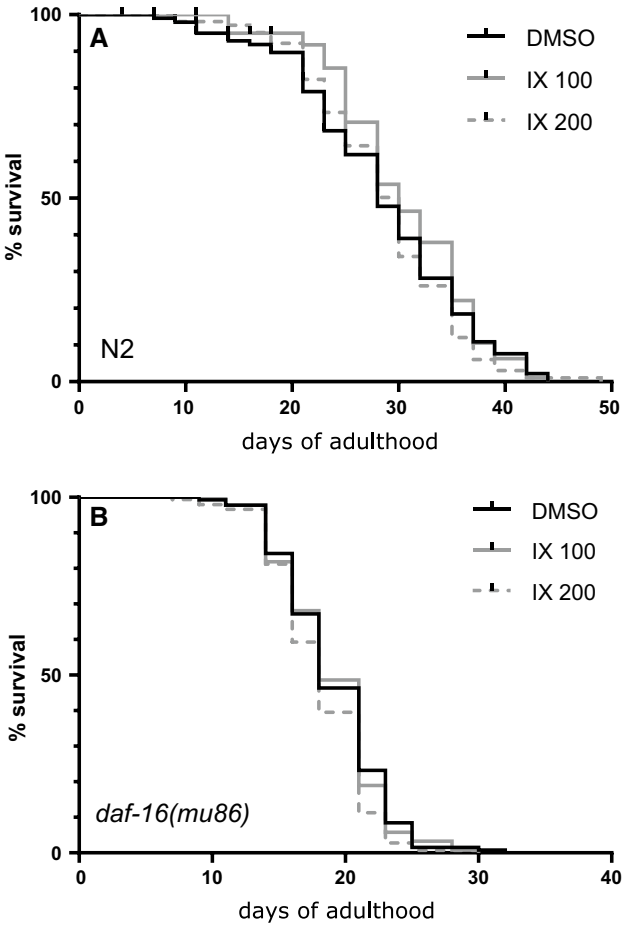

Fig. 4 Effect of isoxanthohumol on adult lifespan of *C. elegans*. Three days after hatching age-synchronised animals are transferred into liquid S-medium containing IX or 0.2 % DMSO. The survival at 20 °C is monitored three times per week and living nematodes are transferred to fresh medium. For the 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 pro-oxidative 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.

References

1. Stevens JF, Page JE (2004) Xanthohumol and related prenylflavonoids from hops and beer: to your good health! Phytochemistry 65:1317–1330
2. Gerhäuser C (2005) Beer constituents as potential cancer chemopreventive agents. Eur J Cancer 41:1941–1954
3. Possemiers S, Bolca S, Grootaert C, Heyerick A, Decroos K, Dhooge W, De Keukeleire D, Rabot S, Verstraete W, Van de Wiele T (2006) The prenylflavonoid isoxanthohumol from hops (*Humulus lupulus* L.) is activated into the potent phytoestrogen 8-prenylnaringenin in vitro and in the human intestine. J Nutr 136:1862–1867
4. Krause E, Yuan Y, Hajirahimkhan A, Dong H, Dietz BM, Nikolic D, Pauli GF, Bolton JL, van Breemen RB (2014) Biological and chemical standardization of a hop (*Humulus lupulus*) botanical dietary supplement. Biomed Chromatogr 28:729–734
5. Monteiro R, Faria A, Azevedo I, Calhau CJ (2007) Modulation of breast cancer cell survival by aromatase inhibiting hop (*Humulus lupulus* L.) flavonoids. Steroid Biochem Mol Biol 105:124–130
6. Izzo G, Söder O, Svechnikov K (2011) The prenylflavonoid phytoestrogens 8-prenylnaringenin and isoxanthohumol differentially suppress steroidogenesis in rat Leydig cells in ontogenesis. J Appl Toxicol 31:589–594
7. Bolca S, Li J, Nikolic D, Roche N, Blondeel P, Possemiers S, De Keukeleire D, Bracke M, Heyerick A, van Breemen RB, Depypere H (2010) Disposition of hop prenylflavonoids in human breast tissue. Mol Nutr Food Res 54(Suppl 2):284–294
8. Negrão R, Duarte D, Costa R, Soares R (2013) Isoxanthohumol modulates angiogenesis and inflammation via vascular endothelial growth factor receptor, tumor necrosis factor alpha and nuclear factor kappa B pathways. BioFactors 39:608–622
9. Serwe A, Rudolph K, Anke T, Erkel G (2012) Inhibition of TGF-β signaling, vasculogenic mimicry and proinflammatory gene expression by isoxanthohumol. Invest New Drugs 30:898–915
10. Tronina T, Bartmańska A, Filip-Psurska B, Wietrzyk J, Popłoński J, Huszcza E (2013) Fungal metabolites of xanthohumol with potent antiproliferative activity on human cancer cell lines in vitro. Bioorg Med Chem 21:2001–2006
11. Delmulle L, Bellahcène A, Dhooge W, Comhaire F, Roelens F, Huvaere K, Heyerick A, Castronovo V, De Keukeleire D (2006) Anti-proliferative properties of prenylated flavonoids from hops (*Humulus lupulus* L.) in human prostate cancer cell lines. Phytomedicine 13:732–734
12. Allsopp P, Possemiers S, Campbell D, Gill C, Rowland I (2013) A comparison of the anticancer properties of isoxanthohumol and 8-prenylnaringenin using in vitro models of colon cancer. BioFactors 39:441–477
13. Potaniec B, Grabarczyk M, Stompor M, Szumny A, Zieliński P, Żołnierczyk AK, Anioł M (2014) Antioxidant activity and spectroscopic data of isoxanthohomol oxime and related compounds. Spectrochim Acta A Mol Biomol Spectrosc 118:716–723
14. Yang JY, Della-Fera MA, Rayalam S, Baile CA (2007) Effect of xanthohumol and isoxanthohumol on 3T3-L1 cell apoptosis and adipogenesis. Apoptosis 12:1953–1963

15. Philips N, Bynum D, Hwang H (2014) Counteraction of skin inflammation and aging or cancer by polyphenols and flavonoids from *Polypodium leucotomos* and xanthohumol. AntiInflamm Antiallergy Agents Med Chem 9:142–149
16. Brenner S (1974) The genetics of *Caenorhabditis elegans*. Genetics 77:71–94
17. Gill MS, Olsen A, Sampayo JN, Lithgow GJ (2003) An automated high-throughput assay for survival of the nematode *Caenorhabditis elegans*. Free Radic Biol Med 35:558–565
18. Büchter C, Ackermann D, Havermann S, Honnen S, Chovolou Y, Fritz G, Kampkötter A, Wätjen W (2013) Myricetin-mediated lifespan extension in *Caenorhabditis elegans* is modulated by DAF-16. Int J Mol Sci 14:11895–11914
19. Mukhopadhyay A, Oh SW, Tissenbaum HA (2006) Worming pathways to and from DAF-16/FOXO. Exp Gerontol 41:928–934
20. Piersen CE (2003) Phytoestrogens in botanical dietary supplements: implications for cancer. Integr Cancer Ther 2:120–138
21. Chadwick LR, Pauli GF, Farnsworth NR (2006) The pharmacognosy of *Humulus lupulus* L. (hops) with an emphasis on estrogenic properties. Phytomedicine 13:119–131
22. Koch K, Havermann S, Büchter C, Wätjen W (2014) *Caenorhabditis elegans* as model system in pharmacology and toxicology: effects of flavonoids on redox-sensitive signalling pathways and ageing. Sci World J. doi:10.1155/2014/920398
23. Kampkötter A, Gombitang Nkwonkam C, Zurawski RF, Timpel C, Chovolou Y, Wätjen W, Kahl R (2007) Effects of the flavonoids kaempferol and fisetin on thermotolerance, oxidative stress and FoxO transcription factor DAF-16 in the model organism *Caenorhabditis elegans*. Arch Toxicol 81:849–858
24. Grünz G, Haas K, Soukup S, Klingenspor M, Kulling SE, Daniel H, Spanier B (2012) Structural features and bioavailability of four flavonoids and their implications for lifespan-extending and antioxidant actions in *C. elegans*. Mech Aging Dev 133:1–10
25. Kampkötter A, Nkwonkam CG, Zurawski RF, Timpel C, Chovolou Y, Wätjen W, Kahl R (2007) Investigations of protective effects of the flavonoids quercetin and rutin on stress resistance in the model organism *Caenorhabditis elegans*. Toxicology 234:113–123
26. Kampkötter A, Timpel C, Zurawski RF, Ruhl S, Chovolou Y, Proksch P, Wätjen W (2008) Increase of stress resistance and lifespan of *Caenorhabditis elegans* by quercetin. Comp Biochem Physiol B: Biochem Mol Biol 149:314–323
27. Zarse K, Jabin S, Ristow M (2012) L-Theanine extends lifespan of adult *Caenorhabditis elegans*. Eur J Nutr 51:765–768
28. Yanase S, Yasuda K, Ishii N (2002) Adaptive responses to oxidative damage in three mutants of *Caenorhabditis elegans* (age-1, mev-1 and daf-16) that affect life span. Mech Ageing Dev 123:1579–1587
29. Abbas S, Wink M (2010) Epigallocatechin gallate inhibits beta amyloid oligomerization in *Caenorhabditis elegans* and affects the daf-2/insulin-like signaling pathway. Phytomedicine 17:902–909
30. Kawasaki I, Jeong MH, Oh BK, Shim YH (2010) Apigenin inhibits larval growth of *Caenorhabditis elegans* through DAF-16 activation. FEBS Lett 584:3587–3591
31. Lin K, Dorman JB, Rodan A, Kenyon C (1997) daf-16: an HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. Science 278:1319–1322
32. Cai WJ, Huang JH, Zhang SQ, Wu B, Kapahi P, Zhang XM, Shen ZY (2011) Icariin and its derivative icariside II extend healthspan via insulin/IGF-1 pathway in *C. elegans*. PLoS One 6:e28835
33. Havermann S, Chovolou Y, Humpf HU, Wätjen W (2014) Caffeic acid phenethylester increases stress resistance and enhances lifespan in *Caenorhabditis elegans* by modulation of the insulin-like DAF-16 signalling pathway. PLoS One 9(6):e100256
34. Ristow M, Schmeisser S (2011) Extending life span by increasing oxidative stress. Free Radic Biol Med 51:327–336
35. Miller RA (2009) Cell stress and aging: new emphasis on multiplex resistance mechanisms. J Gerontol A Biol Sci Med Sci 64:179–182
36. Sykiotis GP, Habeos IG, Samuelson AV, Bohmann D (2011) The role of the antioxidant and longevity-promoting Nrf2 pathway in metabolic regulation. Curr Opin Clin Nutr Metab Care 14:41–48
37. Possemiers S, Heyerick A, Robbens V, De Keukeleire D, Verstraete W (2005) Activation of proestrogens from hops (*Humulus lupulus* L.) by intestinal microbiota; conversion of isoxanthohumol into 8-prenylnaringenin. J Agric Food Chem 53:6281–6288