European Journal of Nutrition (2020) 59:137–150
https://doi.org/10.1007/s00394-019-01894-w

ORIGINAL CONTRIBUTION

Hibiscus sabdariffa L. extract prolongs lifespan and protects against amyloid-β toxicity in *Caenorhabditis elegans*: involvement of the FoxO and Nrf2 orthologues DAF-16 and SKN-1

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Received: 20 September 2018 / Accepted: 5 January 2019 / Published online: 1 February 2019

Abstract

Purpose *Hibiscus sabdariffa* L. is commonly used as an ingredient for herbal teas and food supplements. Several studies demonstrated the beneficial effects of *Hibiscus sabdariffa* L. extracts (HSE); however, the bioactive components and their mode of action still remain unclear. *Caenorhabditis elegans* (*C. elegans*) was used to study health-related effects and the underlying molecular mechanisms of HSE in this model organism as well as effects of hydroxycitric acid (HCA), a main compound of HSE, and its structural analogue isocitric acid (ICA).

Methods Survival and locomotion were detected by touch-provoked movement. Thermotolerance was analysed using the nucleic acid stain SYTOX green, and intracellular ROS accumulation was measured via oxidation of H_2DCF. Localisation of the transcription factors DAF-16 and SKN-1 was analysed in transgenic strains (DAF-16::GFP, SKN-1::GFP). The involvement of DAF-16 and SKN-1 was further investigated using loss-of-function strains as well as gene silencing by feeding RNAi-inducing bacteria. Protection against amyloid-β toxicity was analysed using a transgenic strain with an inducible expression of human amyloid-β peptides in body wall muscle cells (paralysis assay).

Results HSE treatment resulted in a prominent extension of lifespan (up to 24%) and a reduction of the age-dependent decline in locomotion. HCA, a main compound of HSE increased lifespan too, but to a lesser extent (6%) while ICA was not effective. HSE and HCA did not modulate resistance against thermal stress conditions and did not exert antioxidative effects: HSE rather increased intracellular ROS levels, suggesting a pro-oxidative effect of the extract in vivo. HSE and HCA increased the nuclear localisation of the pivotal transcription factors DAF-16 and SKN-1 indicating an activation of these factors. Consistent with this result, lifespan prolongation by HSE was dependent on both transcription factors. In addition to the positive effect on lifespan, HSE treatment also elicited a (strong) protection against amyloid-ß induced toxicity in *C. elegans* in a DAF-16- and SKN-1-dependent manner.

Conclusion Our results demonstrate that HSE increases lifespan and protects against amyloid-β toxicity in the model organism *C. elegans*. These effects were mediated, at least in parts via modulation of pathways leading to activation/nuclear localisation of DAF-16 and SKN-1. Since HCA, a main component of HSE causes only minor effects, additional bioactive compounds like flavonoids or anthocyanins as well as synergistic effects of these compounds should be investigated.

Keywords *Hibiscus sabdariffa* L. · *Caenorhabditis elegans* · Amyloid-β · Plant extract · Alzheimer's disease · Hydroxycitric acid

Abbreviations

daf-16	Abnormal dauer formation-16 (FoxO orthologue)
DCF	2′,7′-Dichlorofluorescein
FoxO	Forkhead box O
GFP	Green fluorescent protein
HCA	Hydroxycitric acid
HSE	*Hibiscus sabdariffa* L. extract
ICA	Isocitric acid
lof	Loss of function
NGM	Nematode growth medium

Karoline Koch and Nora Weldle contributed equally.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00394-019-01894-w) contains supplementary material, which is available to authorized users.

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ROS Reactive oxygen species
skn-1 Skinhead-1 (Nrf2-orthologue)

Introduction

Hibiscus sabdariffa L. (*H. sabdariffa*) belongs to the family of *Malvaceae* and originates from tropical and subtropical regions of the world. Dried calyces of the plant are used as food supplement, beverage and as natural colouring agent in the food and cosmetic industries [1]. *H. sabdariffa* flowers are also traditionally used as a medicinal herb. Antihypertensive and antihyperlipidemic effects were shown in clinical trials which qualifies the plant as a functional food [2, 3]. Moreover, animal models and cell culture studies indicate antioxidative, anti-inflammatory, antidiabetic as well as anticancerogenic effects of *H. sabdariffa* extracts [4]. Recently it was shown that oral *H. sabdariffa* supplements (combination with collagen peptides, vitamin C, and *Aristotelia chilensis* extract) reduce markers of dermal skin ageing in female patients indicating a protective effect against age-related degenerative processes [5]. Nade et al. showed that *H. rosa sinensis* improved short-term and long-term memory deficits in aged swiss albino mice (32 weeks old) in a way that these animals were comparable to young untreated animals (8 weeks old) [6]. Neuroprotective effects of *H. sabdariffa* (ethyl acetate fraction) were also reported in streptozotocin-(STZ) induced diabetic mice [7]. The authors suggested the reduction in oxidative stress markers as well as acetylcholinesterase activity as possible modes of action. Neuromodulatory effects were also detectable in swiss albino mice treated with *H. rosa sinensis* as anxiolytic effects mediated by ionotropic GABA receptors were observed [8]. *H. sabdariffa* extracts were shown to inhibit dopaminergic cell death in primary midbrain cultures treated with rotenone and furthermore, a *H. asper* leaves extract increased antioxidant enzyme activity in a 6-hydroxydopamine-lesioned rat model of Parkinson's disease [9, 10].

Hibiscus sabdariffa contains various bioactive compounds, e.g. organic acids such as hydroxycitric acid (HCA), ascorbic acid and hibiscus acid, anthocyanins like delphinidin-3-sambubioside (hibiscin) and cyanidin-3-sambubioside (gossypicyanin), polysaccharides, as well as flavonoids and phenolic acids [1]. The protective effects of the plant are mostly attributed to the anthocyanins and flavonoids because of their multiple biological actions, e.g. antioxidative effects or interaction with enzymes and signalling pathways [4, 11, 12]. However, there is only limited knowledge about the mode of action of other components such as HCA.

We used the model organism *Caenorhabditis elegans* in order to study protective effects of a *Hibiscus sabdariffa* L. water extract (HSE) in vivo. The nematode serves as model to study ageing as well as age-related diseases on a molecular basis. For various human genes, orthologues are known in *C. elegans* enabling researchers to study evolutionary conserved signalling pathways and molecular processes involved in human diseases [13, 14]: More than 50% (10,678/20,310) of the human protein-coding genome has recognizable orthologues in the nematode supported by current versions of orthology-prediction methods. Several transgenic strains have been engineered to study human neurodegenerative diseases in the nematode [15]. Additionally, *C. elegans* is used to investigate the biological functions of food compounds [16, 17]. Using this model system, Fitzenberger et al. already showed that HSE was able to protect against stress induced by high glucose concentrations [18]. They concluded that delphinidin-3-sambubioside and cyanidin-3-sambubioside were not responsible for this effect, which indicates that other constituents may be active. We investigated the effect of HSE as well as the main compound HCA on ageing and age-related parameters in *C. elegans* (antioxidative effects in vivo, stress resistance, protection against Aβ-toxicity). To identify specific effects of the HSE compound HCA, the structural analogue isocitric acid (ICA) was also investigated.

Materials and methods

Materials

SYTOX Green was obtained from Molecular Probes (Eugene, OR, USA) and Levamisole hydrochloride, Tetracycline hydrochloride, Streptomycin sulphate from AppliChem (Darmstadt, Germany). *Hibiscus sabdariffa* L. dry extract (HSE) (100% native) was provided by Plantextrakt GmbH & Co. KG (Vestenbergsgreuth, Germany). All other chemicals were of analytical grade and purchased from SIGMA (Deisenhofen, Germany).

C. elegans strains maintenance and treatment

Caenorhabditis elegans strains used in this study (wild-type N2 var. Bristol, CF1038 [*daf-16(mu86) I.]*, EU1 [*skn-1(zu67) IV/nT1 [unc-?(n754) let-*?] (IV;V).], TJ356 [*zIs356 IV (pdaf-16-daf-16::gfp; rol-6)*], LD001 [*ldIs007 pskn-1::skn-1b/c::gfp; rol-6*]) and CL4176 [*(pAF29)myo-3p::A-beta (1–42)::let-? 3'UTR) + pRF4 rol-6 (su1006)*] and OP50/OP50-1 bacterial strains were provided by the *Caenorhabditis* Genetics Centre (CGC) and RNAi strains were from the *C. elegans* RNAi collection from Ahringer. Except for CL4176, which was kept at 16 °C, all other strains were maintained at 20 °C on 94 mm nematode growth medium (NGM) agar plates containing a lawn of *E. coli* var. OP50. For the CL4176 and EU1 strains synchronisation was performed by egg laying and for all other

strains by bleaching according to standard protocols [19]. Compound treatment of *C. elegans* was conducted in liquid NGM as described earlier [20, 21] with minor modifications: HSE, ICA and HCA stock solutions were prepared in liquid nematode growth media (NGM) and added to the incubation media (NGM, 50 µg/ml streptomycin and 10^9/ml OP50-1 *E. coli*) in the desired final concentration. The control group received the respective amount NGM. The worms were incubated with the extract, ICA or HCA together with OP50-1 *E. coli*. It was verified that HSE does not change the bacterial growth rate and does not change the pharyngeal pumping rate, e.g. that the effects were mediated indirectly by caloric restriction (Supplementary Fig. 1). Furthermore, we verified that HSE has no impact on the nematode size (length and area) as well as number of offspring (Fig. 1e). However, it cannot be excluded that the HSE-mediated effects were mediated by compounds generated by autoxidation or bacterial metabolism.

Stress resistance

Synchronized L4 larvae and young adult animals (N2) were treated with concentrations of HSE ranging from 0.25 to 1 mg/ml, 2 mM HCE or ICA in the incubation medium for 48 h at 20 °C. Animals were washed in PBST and single nematodes were transferred into wells of a 384-well microtiter plate containing 1 µM SYTOX Green nucleic acid stain, then thermal stress (37 °C) was applied. Fluorescence was measured in a plate reader (Synergy MX, BioTek Instruments, Inc.) every 15 min for 12 h. When the fluorescence values (excitation: 485 nm; emission: 535 nm) of individual nematodes exceeded a defined cut-off value, the animals were scored as dead.

Measurement of ROS accumulation

Synchronized L4 larvae and young adults (N2) were treated with different HSE concentrations or 2 mM HCA or ICA for 48 h in the incubation medium [2.2] at 20 °C, washed in PBST and transferred into a 384-well microtiter plate. Subsequently, 50 µM H_2DCF-DA-solution was added to each well and fluorescence intensities (excitation: 485 nm; emission: 535 nm) were recorded every 15 min for 12 h under thermal stress (37 °C) in a plate reader (Synergy MX, BioTek Instruments, Inc.).

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

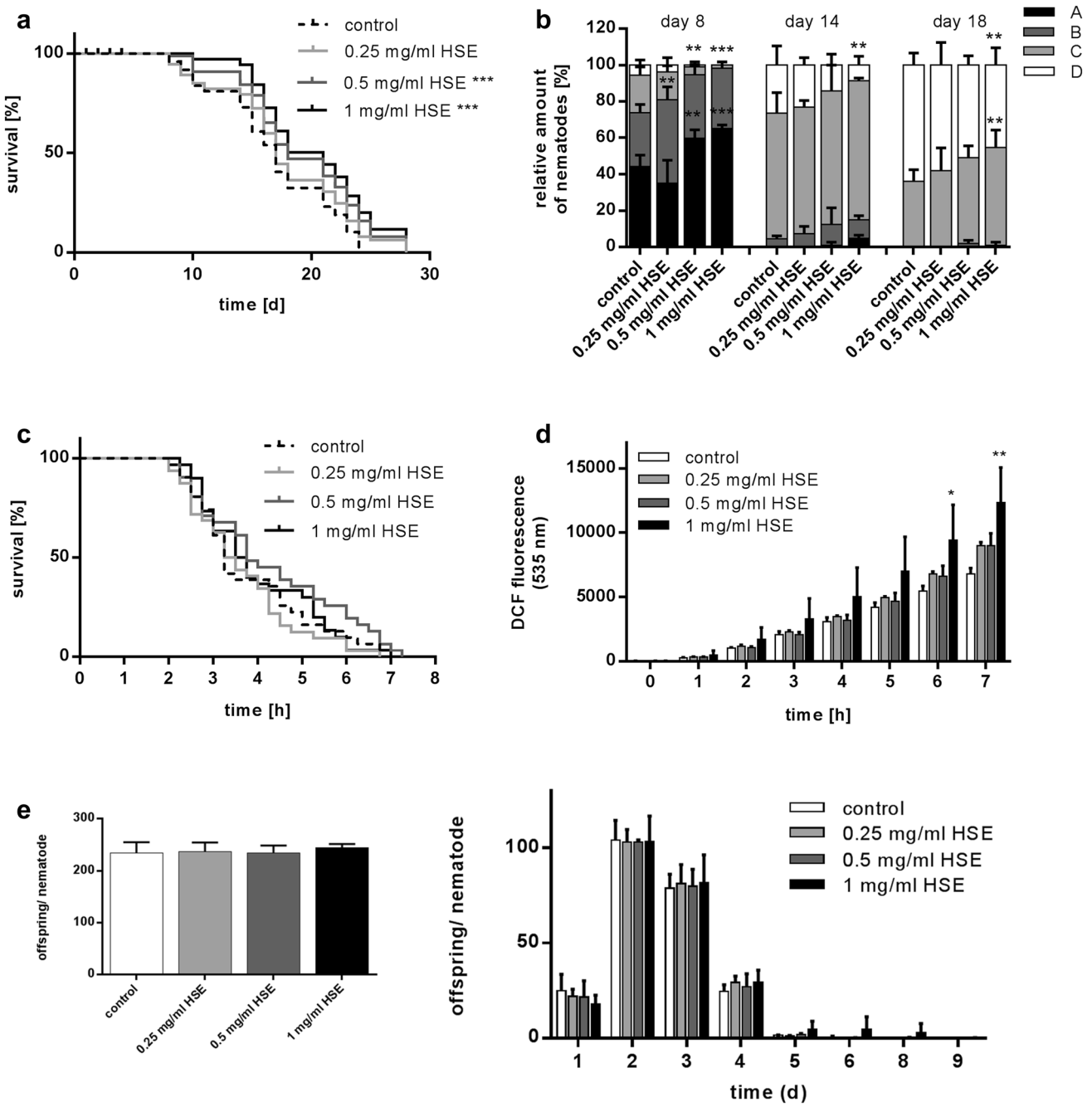
Intracellular localisation of DAF-16 and SKN-1 was detected using the transgenic strains TJ356 and LD001. Synchronized L4 larvae and young adult animals of the corresponding strains were treated with different HSE concentrations or 2 mM HCA or ICA for 1 h in the incubation medium at 20 °C, respectively. Nematodes were transferred to a microscope slide, sedated with levamisole and cellular localisation of DAF-16::GFP/SKN-1::GFP was detected by means of a fluorescence microscope (Eclipse Ni with Intensilight C-HGFI, Nikon) equipped with a GFP filter. As a positive control a short heat stress (37 °C 5 min) was conducted to induce nuclear DAF-16::GFP localisation in the TJ356 nematodes. For the nuclear translocation of SKN-1::GFP the LD001 nematodes were treated with the compounds or 1 mM H_2O_2 (positive control) for 1 h. Only in the SKN-1::GPF-translocation experiment, heat-inactivated (30 min 65 °C) OP50-1 *E. coli* were used to inactivate bacterial catalase and allow H_2O_2 to induce SKN-1 translocation in the nematode.

Lifespan and locomotion

For the analysis of the lifespan at 25 °C the wild-type strain N2 and the loss-of-function mutants CF1038 and EU1 were used. 40 synchronized L4 larvae and young adult animals were transferred into liquid media (incubation medium: 2.2) with different HSE concentrations or 2 mM HCA or ICA (day 0 of the lifespan). During the first 9 days, medium contained 120 µM 5-fluoro-2-deoxyuridine (FUDR) to prevent the hatching of viable progeny. The media were exchanged 5 days a week and the survival of the animals was measured by touch-provoked movement. Lost or ruptured animals were censored. Locomotion analyses were conducted simultaneously at days 8, 14 and 18 for the wild-type strain N2 and only at day 8 for the mutant strains due to a shorter lifespan. Briefly, nematodes were classified according to their mobility after shaking the petri dish: (A) nematodes moved freely, (B) nematodes moved after prodding with a platinum wire, (C) nematodes moved scarcely their head or tail after prodding and (D) nematodes showed no movement (dead).

Aβ-paralysis

The transgenic *C. elegans* strain CL4176 with temperature-inducible expression of the human $A\beta_{3-42}$ was used to analyse Aβ toxicity in *C. elegans*. Synchronized eggs were incubated in liquid media with different HSE concentrations or 1 mM caffeine as a positive control at 16 °C. Following 64 h of treatment, 40 larvae were transferred to 60 mm NGM agar plates with a lawn of OP50 *E. coli* and set at 25 °C to induce transgene expression. Body movement of the nematodes was analysed every other hour 26 h after the temperature up-shift. Nematodes that showed no movement or only moved the head after prodding with a platinum wire were considered as paralysed. Lost or ruptured animals were censored. For RNAi experiments *E. coli* var. HT115 expressing *daf-16, skn-1* dsRNA or empty vector were applied and



50 µg/ml ampicillin, 12 µg/ml tetracycline and 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) were added to the incubation media. Eggs of the CL4176 strain were treated for 40 h at 16 °C with different HSE concentrations and the hatched larvae were transferred to 60 mm RNAi plates (NGM plates with 50 µg/ml ampicillin, 12 µg/ml tetracycline, 1 mM IPTG) seeded with HT115 *E. coli* expressing the respective dsRNA. Paralysis was tested according to the standard experiment. In order to validate the efficiency of the *daf-16* knockdown, eggs of TJ356 nematodes were collected and incubated with *daf-16* RNAi or empty vector HT115 *E. coli* in the RNAi incubation media at 20° for 40 h. Subsequently, nematodes were analysed by means of a fluorescence microscope (Nikon) equipped with a GFP filter. Images of 30 larvae were taken with a connected camera and the optical density (OD) of the animals was measured with ImageJ. *Skn-1* knock-down efficiency was verified by measuring the reproduction rate of CL4176 nematodes. Briefly, eggs of CL4176 nematodes were exposed to empty vector or *skn-1* RNAi expressing bacteria in liquid RNAi incubation media at 16 °C for 40 h. Afterwards 10 larvae were transferred to 60 mm RNAi plates. After the end of the

◂**Fig. 1** Effects of HSE on lifespan, locomotion and stress resistance in *C. elegans*. **a** HSE prolongs lifespan dose-dependently. Wild-type L4 larvae were treated with different concentrations of HSE or the solvent control NGM at 25 °C. Media change was conducted 5 times a week and survival of the nematodes was examined by touch-provoked movement simultaneously, $n=3$ (40 individuals per group), Kaplan–Meier survival analysis with Log-Rank (Mantel-Cox)-test, $^{***}p \leq 0.001$ vs. control. **b** HSE prevented age-associated decline in locomotion. Mobility was analysed by prodding the nematodes with a platinum wire at days 8, 14 and 18 of their lifespans. The individuals were classified according to the movement: A—free movement, B—movement after prodding, C—weak movement after prodding, D—dead, $n=3$ (40 nematodes per group), two-way ANOVA with Tukey's multiple comparisons test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. **c** HSE did not influence thermal stress resistance. Wild-type L4 *C. elegans* were pretreated with different concentrations of HSE or the solvent control NGM for 48 h at 20 °C before thermal stress (37 °C) was applied. Individual virtual times of death were determined by measuring the fluorescence of the nucleic acid stain SYTOX®Green. Kaplan–Meier statistics was used for the comparison of the survival curves, $n=3$ (8 individuals per group), Kaplan–Meier survival analysis with Log-Rank (Mantel-Cox)-test. **d** HSE increases thermally induced ROS accumulation. The fluorescence of DCF as an indicator for intracellular ROS was measured in the pretreated wild-type nematodes after thermal stress (37 °C) was applied, $n=3$ (8 individuals per group), one-way ANOVA with Dunnett's multiple comparisons test, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ vs. control. **e** Treatment with HSE does not influence progeny of *C. elegans*. Left figure: Total progeny, $n=3$ (10 individuals/group and trial), one-way ANOVA with Dunnett's multiple comparisons test. Right figure: daily progeny, $n=3$ (10 individuals/group and trial), two-way ANOVA with Tukey's multiple comparisons test

reproductive period the nematodes were removed, and the living progeny and non-hatched eggs were counted.

Statistics

Statistical significance was determined by one-way ANOVA with Dunnett's post-hoc test or two-way ANOVA with Tukey's post-hoc test while lifespan analyses, thermal stress and Aβ paralysis assays were calculated using Kaplan–Meier survival analyses with log-rank test (PASW Statistics for Windows, SPSS Inc.; Chicago, USA; GraphPad Prism 6, La Jolla, USA).

Results

Effects of HSE on lifespan and stress resistance

HSE increased the lifespan of the nematode dose-dependently: the median lifespan was enhanced by 24% and 6% after incubation with 1.0 and 0.5 mg/ml HSE, respectively. The lowest concentration (0.25 mg/ml) was not able to increase the lifespan significantly (Fig. 1a; Table 1). The concentrations used for our studies (0.25, 0.5 and 1 mg/ml) are only slightly higher than concentrations used for studies in mice: Seung et al. used the ethyl acetate fraction from *Hibiscus sabdariffa* L. in a concentration of 100 and 200 mg/kg of body weight [7]. HSE was used up to 1 mg/ml due to acidification of the incubation medium (< pH 6.0) at higher concentrations. In addition to prolongation of the lifespan, incubation with HSE prevented the age-related decline of locomotion of the nematodes. As shown in Fig. 1b, the amount of agile and freely moving nematodes diminishes dramatically with time: While at day 8 approximately 45% of the control nematodes move freely (category A), at day 14 no nematodes in this category are detectable. However, after an incubation with HSE (0.5 mg/ml and 1 mg/ml), the amount of agile nematodes is significantly increased (60% and 65% category A) at day 8 and the amount of dead nematodes is significantly decreased at days 14 and 18. The highest HSE concentration (1 mg/ml) also significantly prevents locomotion decline at day 18 (category C). We next investigated if the nematodes are also more resistant against thermal stress (SYTOX assay), but this was not the case (Fig. 1c). Congruent with this result, no antioxidative effects of HSE were detected in *C. elegans* under thermal stress conditions (Fig. 1d). On the contrary, HSE (1 mg/ml) even increases the accumulation of ROS in the animals, suggesting a pro-oxidative effect of the extract. Since the antioxidant compound N-acetylcysteine was able to diminish the HSE-mediated effects (increased nuclear localisation of SKN-1 and DAF-16; Supplementary Fig. 3), we conclude that HSE mediated its effects at least in parts, via generation of oxidative stress. However, using paraquat as a redox-cycling agent to produce superoxide radicals continuously or using the *mev-1* strain generating higher amounts of ROS, HSE was able to protect against this stressor suggesting also antioxidative properties (Supplementary Fig. 3). Furthermore, HSE was able to decrease the accumulation of the age-pigment lipofuscin (Supplementary Fig. 3). However, the amount of offspring was not changed by HSE (Fig. 1e).

Effects of HCA/ICA on lifespan and stress resistance

Since HSE contains approx. 40% HCA, we investigated if incubation with the respective amount of HCA generates effects comparable to those detected with 1 mg/ml HSE. Similar to HSE, 2 mM HCA increased the median lifespan significantly, but to a lesser extent (6%). ICA, which was used as internal control (comparable structure to HCA, but not a component of HSE) had no effects on the lifespan of the nematodes (Fig. 2a). In contrast to HSE, neither ICA nor HCA affected the locomotion of the animals (Fig. 2b). In analogy to HSE, neither increase in survival (Fig. 2c) nor antioxidative effects (Fig. 2d) were caused by the compounds in response to thermal stress conditions. However, no pro-oxidative effect (increase in ROS accumulation) was detectable as seen after incubation with the highest concentration of HSE.

Table 1 Summary of lifespan data—HSE and HCA prolong lifespan of *C. elegans*

Treatment	Adult lifespan (days)		*n* (censored)	*p* value vs. DMSO (log-rank)
	Mean (±SEM)	Median (±SEM)		
N2 (wild-type)				
Control	17.2 ± 0.45	17 ± 0.37	120 (10)	–
0.25 mg/ml HSE	18.2 ± 0.48	18 ± 0.46	120 (14)	ns
0.5 mg/ml HSE	19.4 ± 0.46	18 ± 1.02	120 (15)	< 0.001
1 mg/ml HSE	20.3 ± 0.44	21 ± 1.00	120 (22)	< 0.001
2 mM HCA	18.4 ± 0.50	18 ± 0.90	120 (18)	0.022
2 mM ICA	17.5 ± 0.50	17 ± 0.58	120 (11)	ns
CF1038 (Δ*daf-16* mutant)				
Control	11.2 ± 0.31	10 ± 0.34	120 (5)	–
0.5 mg/ml HSE	11.3 ± 0.34	11 ± 0.45	120 (10)	ns
1 mg/ml HSE	11.2 ± 0.32	10 ± 0.59	120 (8)	ns
Control	10.8 ± 0.32	10 ± 0.37	120 (8)	–
2 mM HCA	10.4 ± 0.30	10 ± 0.36	120 (8)	ns
EU1 (Δ*skn-1* mutant)				
Control	13.5 ± 0.47	14 ± 0.99	120 (31)	–
0.5 mg/ml HSE	13.9 ± 0.39	14 ± 0.75	120 (16)	ns
1 mg/ml HSE	14.4 ± 0.39	15 ± 0.77	120 (23)	ns
2 mM HCA	12.6 ± 0.35	11 ± 0.36	120 (10)	ns

Lifespan prolongation by HSE and HCA is mediated via intracellular signalling pathways

We analysed which intracellular signalling pathways are involved in the life prolongation caused by HSE/HCA. First, we focused on the insulin-like signalling pathway investigating the effects on the DAF-16 transcription factor. The nuclear localisation of DAF-16 is strongly enhanced by HSE: 0.25 mg/ml HSE more than doubles the number of nematodes with a mainly nuclear phenotype (29% vs. 11%), while 1 mg/ml HSE induced a sixfold increase (60%). A similar effect was caused by an incubation with 2 mM of the main HSE component HCA, while 2 mM of the chemical analogue ICA showed no significant effect (Fig. 3a). We used DAF-16 deficient nematodes to investigate if the life-prolonging effects of HSE/HCA are dependent on functional DAF-16. Repeating the experiment from Fig. 1a using DAF-16 deficient nematodes, no prolongation of lifespan was detectable (0.5 and 1 mg/ml HSE as well as 2 mM HCA) (Fig. 3b; Table 1). Mobility analysis at day 8 of the lifespan showed that 1 mg/ml HSE additionally impaired locomotion (category C) in the DAF-16 mutant animals while HCA shows no effect (congruent with the wild-type nematodes) (Fig. 3c). Next, we investigated the requirement of SKN-1 (Nrf2-orthologue), another pivotal transcription factor for the effects of HSE and HCA. Comparable to the results of DAF-16, incubation of *C. elegans* with HSE (0.5 and 1 mg/ml) also increases the number of nematodes with a mainly nuclear phenotype of SKN-1 (48% and 49% vs 13%, respectively) (Fig. 4a). However, HCA treatment (2 mM) resulted in no significant change in SKN-1 localisation compared to the control animals (Fig. 4a). Using SKN-1 deficient nematodes, no increase in lifespan was detectable for HSE (0.5 and 1 mg/ml) as well as 2 mM HCA (Fig. 4c, d; Table 1). Furthermore, no increase in locomotion was detectable in the HSE or HCA-incubated animals (Fig. 4e, f).

HSE protects against amyloid-β toxicity via DAF-16 and SKN-1

Due to some reports showing neuroprotective effects of HSE, we investigated if the extract causes protective effects in a simple *C. elegans* model of Alzheimer's disease. Using transgenic nematodes which paralyse after temperature-induced expression of the human amyloid-β peptide, we could show that HSE (0.5 and 1.0 mg/ml) caused a delayed onset of paralysis in comparison to the control nematodes (Fig. 5a) implying a protective effect against amyloid-β-toxicity. The median time span until onset of paralysis was delayed by 2 h in both treatment groups (Table 2). Using RNA-interference technique, we investigated if the pivotal transcription factors DAF-16 and SKN-1 that are involved in the anti-ageing effects of HSE are necessary for this effect. In this modified experimental system, we applied a different *E. coli* strain (HT115) which expresses dsRNA of the gene of interest. Treatment with 0.5 mg/ml HSE delayed onset of paralysis in the amyloid-β-strain fed with the control vector bacteria while no effect was seen in the nematodes exposed

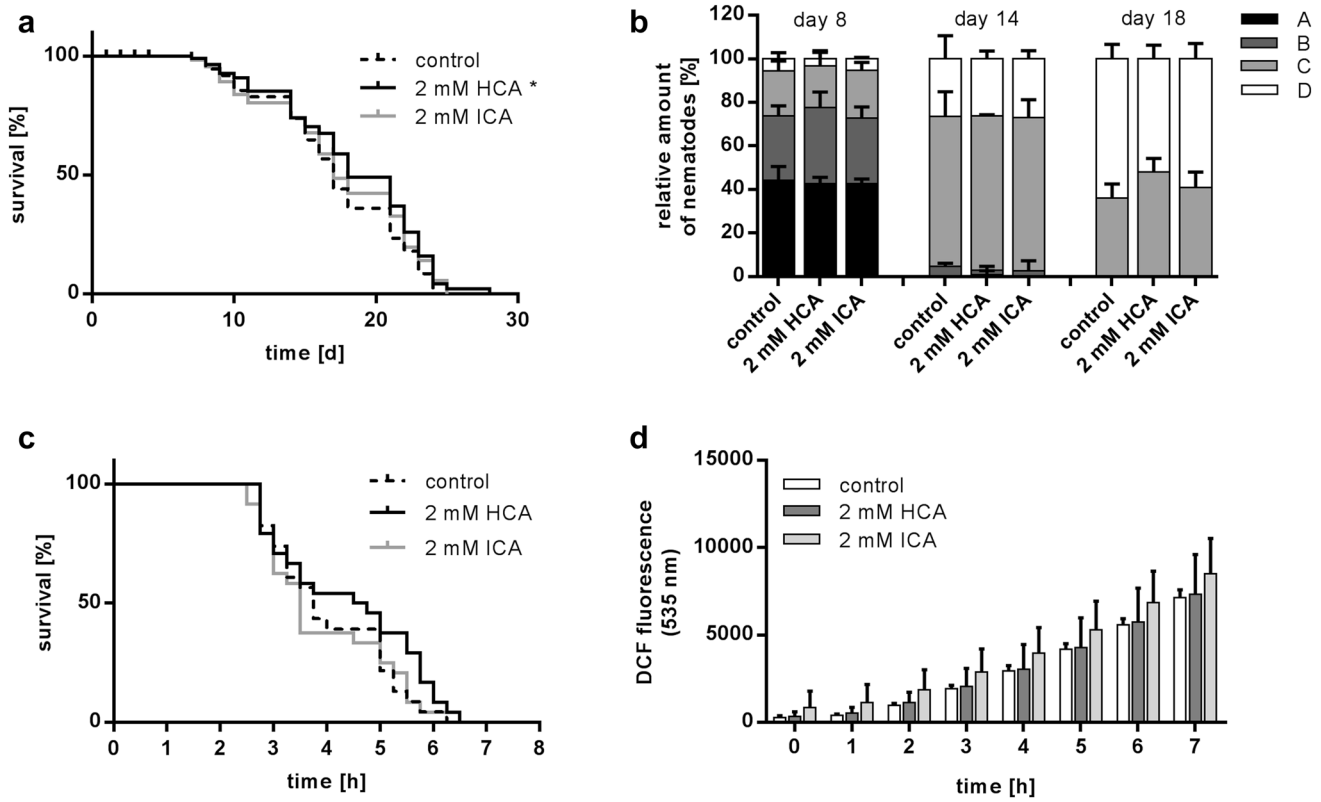


Fig. 2 Effects of HCA/ICA on lifespan, locomotion and stress resistance in *C. elegans*. **a** HCA prolongs lifespan. Wild-type L4 larvae were treated with 2 mM HCA/ICA or the solvent control NGM at 25 °C. Media change was conducted 5 times a week and survival of the nematodes was examined by touch-provoked movement simultaneously, $n=3$ (40 individuals per group), Kaplan–Meier survival analysis with Log-Rank (Mantel-Cox)-test, $*p \leq 0.05$ vs. control. **b** Age-associated decline in locomotion was analysed by prodding the nematodes with a platinum wire at days 8, 14 and 18 of their lifespans. The individuals were classified according to the movement: A—free movement, B—movement after prodding, C—weak movement after prodding, D—dead, $n=3$ (40 nematodes per group), two-way ANOVA with Tukey's multiple comparisons test. **c** HCA/ICA did not influence thermal stress resistance. Wild-type L4 *C. elegans* were pretreated with 2 mM HCA/ICA or the solvent control NGM for 48 h at 20 °C before thermal stress (37 °C) was applied. Individual virtual times of death were determined by measuring the fluorescence of the nucleic acid stain SYTOX®Green. Kaplan–Meier statistics was used for the comparison of the survival curves, $n=3$ (8 individuals per group), Kaplan–Meier survival analysis with Log-Rank (Mantel-Cox)-test. **d** HCA/ICA did not modulate thermally induced ROS accumulation. The fluorescence of DCF as an indicator for intracellular ROS was measured in the petreated wild-type nematodes after thermal stress (37 °C) was applied, $n=3$ (8 individuals per group), one-way ANOVA with Dunnett's multiple comparisons test

to *daf-16* RNAi (Fig. 5b). A similar effect was visible when nematodes were exposed to *skn-1* RNAi bacteria indicating that both DAF-16 and SKN-1 are involved in the protection against amyloid-β toxicity (Fig. 5b). Using the highest amount of HSE (1 mg/ml), the protective effect was also abolished in control vector-fed nematodes (Online Resource Suppl. Fig. 3). Additionally, another effect occurred in the nematodes with SKN-1-knock-down: Compared to the nematodes receiving bacteria with control plasmid, the bacteria expressing the SKN-1-interfering RNA were more resistant against toxic amyloid-β, even if no HSE is added (Fig. 5b, Online Resource Suppl. Fig. 3). However, this protection was abolished when using a double knock-down model system (DAF-16 and SKN-1 simultaneously) (Fig. 5b). Additionally, treatment with 0.5 mg/ml of HSE was not able to cause a protective effect in this system reconfirming the requirement of both transcription factors.

Discussion

H. sabdariffa preparations possess interesting pharmacological properties in mammals, e.g. antidiabetic, antihypertensive and anticancerogenic effects. Due to its common use as tea and food additive, HSE is considered to be safe and well tolerable for humans [4]. Recent in vitro and in vivo studies indicate anti-ageing and neuroprotective effects of HSE [5–10]. However, the bioactive compounds as well as the mode of action of HSE are poorly understood.

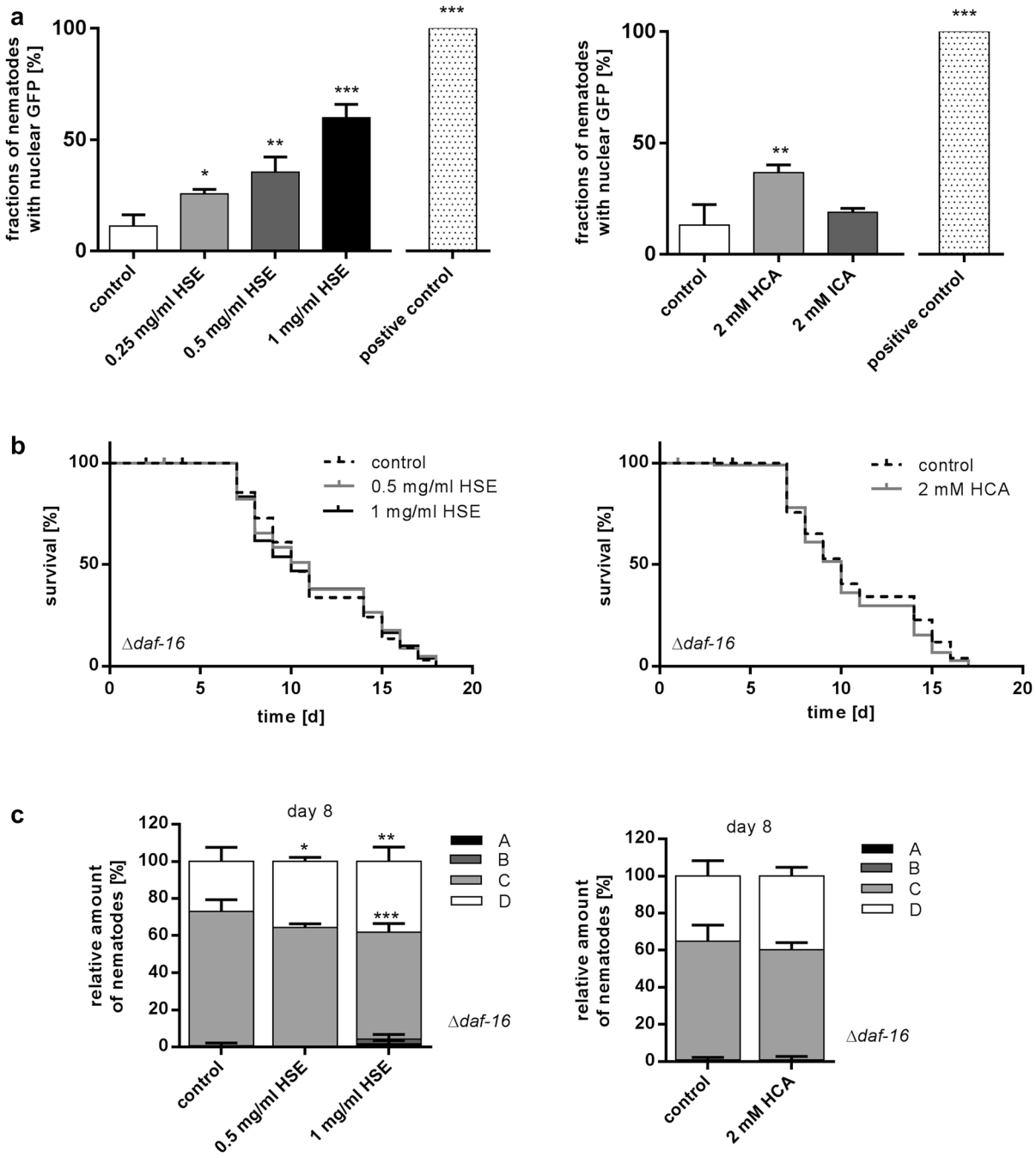


Fig. 3 DAF-16 is essential for lifespan prolongation by HSE and HCA. **a** HSE (left) and HCA (right) induce nuclear accumulation of DAF-16. L4 nematodes (TJ356) were treated with different concentrations of the compounds or the solvent control NGM for 1 h and subsequently analysed by means of fluorescence microscopy. Nematodes with nuclear localisation of DAF-16 were counted and a short heat stress (5 min 37 °C) was used as a positive control, $n=3$ (30 individuals per group), one-way ANOVA with Dunnett's multiple comparisons test, ***$p \le 0.001$ **$p \le 0.01$, *$p \le 0.05$ vs. control. **b** L4 staged *daf-16* lof nematodes (CF1038) were treated with 0.5 and 1 mg/ml HSE (left), 2 mM HCA (right) or the solvent control NGM at 25 °C. Media change was conducted 5 times a week and survival of the nematodes was examined by touch-provoked movement simultaneously, $n=3$ (40 individuals per group), Kaplan–Meier survival analysis with Log-Rank (Mantel-Cox)-test. **c** HSE (left) and HCA (right) do not modulate locomotion in *daf-16* lof nematodes (CF1038). Locomotion was analysed by prodding the nematodes with a platinum wire at day 8 of their lifespans. The individuals were classified according to the movement: A—free movement, B—movement after prodding, C—weak movement after prodding, D—dead, $n=3$ (40 nematodes per group), two-way ANOVA with Tukey's multiple comparisons test

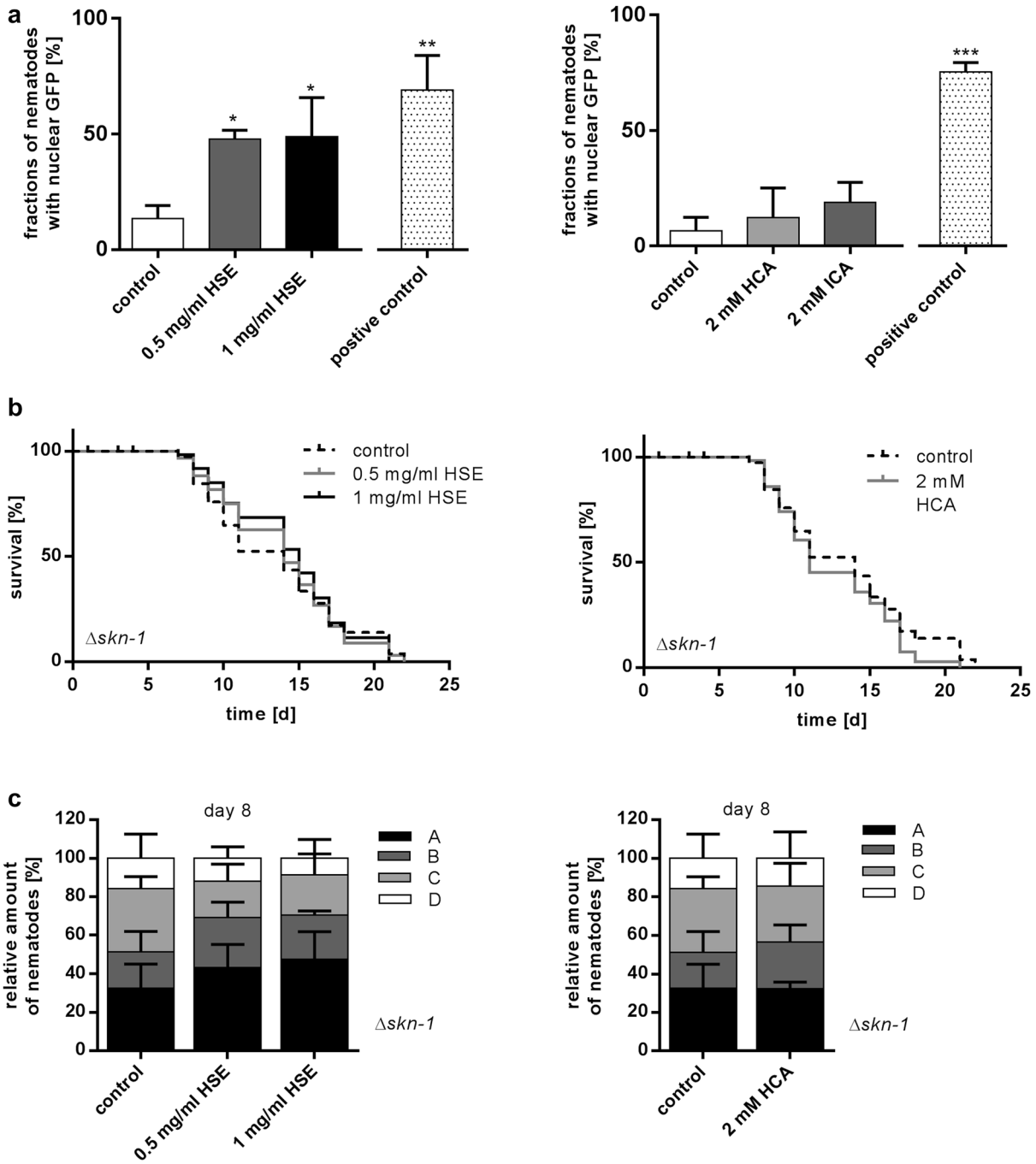


Fig. 4 SKN-1 is essential for lifespan prolongation by HSE and HCA. **a** HSE (left), but not HCA (right), induces nuclear accumulation of SKN-1. L4 nematodes (LD001) were treated with different concentrations of the compounds or the solvent control NGM for 1 h and subsequently analysed by means of fluorescence microscopy. Nematodes with nuclear localisation of SKN-1 in the intestinal cells were counted and 10 mM H_2O_2 was used as a positive control, $n=3$ (30 individuals per group), one-way ANOVA with Dunnett's multiple comparisons test, $^{***}p \leq 0.001$ $^{**}p \leq 0.01$, $^{*}p \leq 0.05$ vs. control. **b** L4 staged *skn-1* lof nematodes (EU1) were treated with 0.5 and 1 mg/ml HSE (left), 2 mM HCA (right) or the solvent control NGM at 25 °C. Media change was conducted 5 times a week and survival of the nematodes was examined by touch-provoked movement simultaneously, $n=3$ (40 individuals per group), Kaplan–Meier survival analysis with Log-Rank (Mantel-Cox)-test. **c** HSE (left) and HCA (right) do not modulate locomotion in *skn-1* lof nematodes (EU1). Locomotion was analysed by prodding the nematodes with a platinum wire at day 8 of their lifespan. The individuals were classified according to the movement: A—free movement, B—movement after prodding, C—weak movement after prodding, D—dead, $n=3$ (40 nematodes per group), two-way ANOVA with Tukey's multiple comparisons test

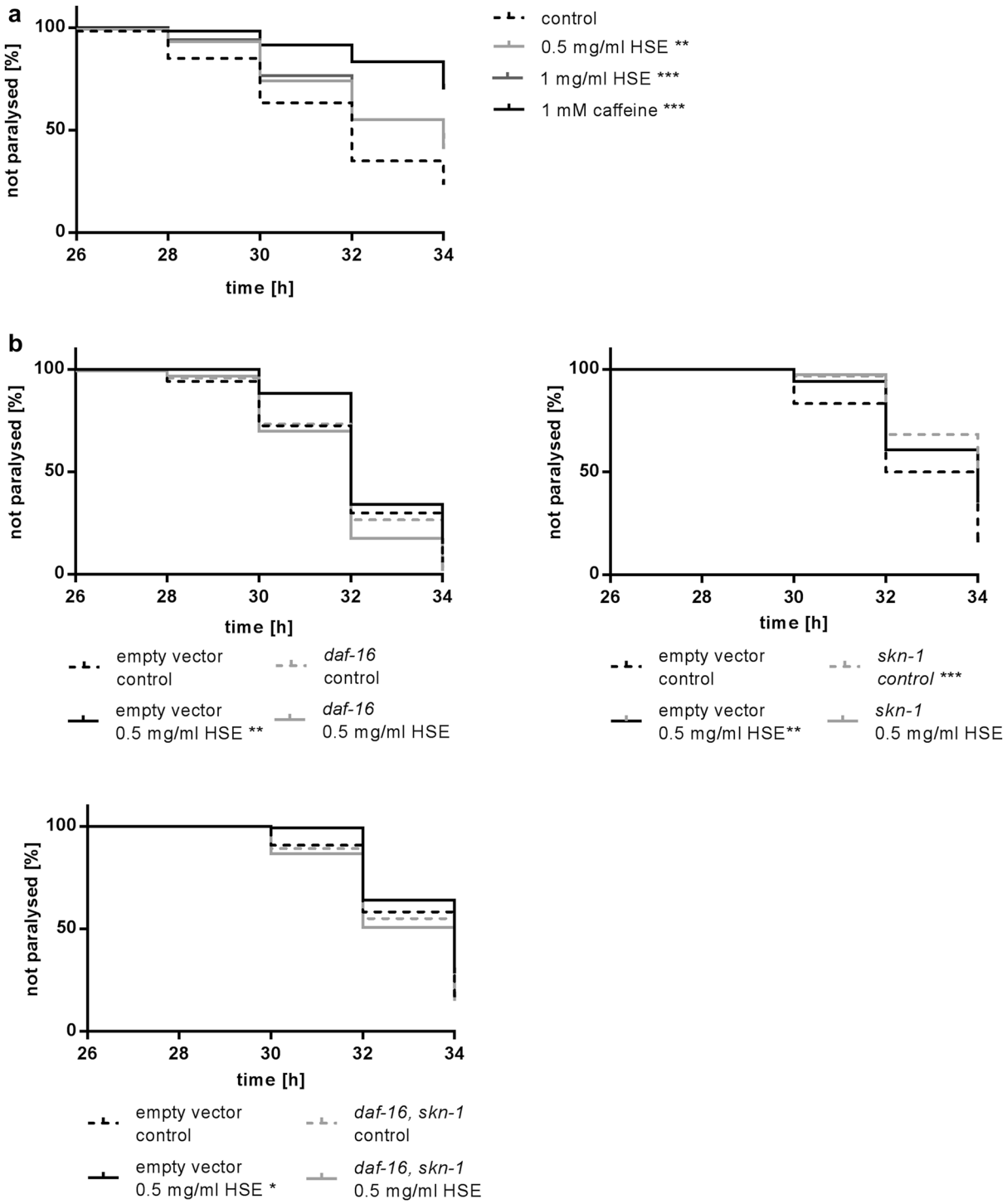


Fig. 5 HSE protects against Aβ-induced toxicity in *C. elegans* via DAF-16 and SKN-1. Paralysis curves of pretreated *C. elegans* strain CL4176 expressing temperature-inducible human Aβ peptide in body wall muscle cells measured every other hour 26 h after temperature up-shift. **a** Eggs of CL4176 were treated with different concentrations of HSE or the solvent control NGM for 64 h at 16 °C before temperature was shifted to 25 °C, $n = 3$ (40 individuals per group), Kaplan–Meier survival analysis with Log-Rank (Mantel-Cox)-test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ vs. control. **b** Eggs of CL4176 nematodes fed with HT115 *E. coli* expressing *daf-16* dsRNA (left), *skn-1* dsRNA (right), *daf-16* and *skn-1* dsRNA (bottom) or empty vector were treated with 0.5 mg/ml HSE or the solvent control NGMk for 40 h at 16 °C before temperature was shifted to 25 °C, $n = 3$ (40 individuals per group), Kaplan–Meier survival analysis with Log-Rank (Mantel-Cox)-test, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ vs. empty vector control

Table 2 Summary of Aβ paralysis data—HSE protects against Aβ toxicity in *C. elegans* via DAF-16 and SKN-1

Treatment	Onset of paralysis (h)		*n* (censored)	*p* value vs. DMSO (log-rank)
	Mean (±SEM)	Median (±SEM)		
N2 (wild type)				
Control	31.6±0.20	32±0.31	120 (28)	–
0.5 mg/ml HSE	32.4±0.18	34±0.63	120 (49)	0.002
1 mg/ml HSE	32.5±0.18	34	120 (55)	<0.001
1 mM caffeine	33.5±0.12	–	120 (84)	<0.001
daf-16 RNAi				
Empty vector control	31.9±0.16	32±0.20	120 (7)	–
Empty vector 0.5 mg/ml HSE	32.5±0.12	32±0.16	120 (18)	0.008
Empty vector 1 mg/ml HSE	31.8±0.14	32±0.16	120 (12)	ns
daf-16 control	31.9±0.15	32±0.17	120 (4)	–
daf-16 0.5 mg/ml HSE	31.7±0.14	32±0.13	120 (2)	ns
daf-16 1 mg/ml HSE	31.5±0.14	32±0.14	120 (6)	ns
skn-1 RNAi				
Empty vector control	32.7±0.14	32±0.27	120 (19)	–
Empty vector 0.5 mg/ml HSE	33.1±0.11	34±0.30	120 (39)	0.002
Empty vector 1 mg/ml HSE	33.0±0.11	34±0.25	120 (29)	ns
skn-1 control	33.3±0.10	34±0.30	120 (46)	–
skn-1 0.5 mg/ml HSE	33.2±0.11	34±0.31	120 (40)	ns
skn-1 1 mg/ml HSE	33.1±0.08	34±0.27	120 (33)	ns
daf-16, skn-1 RNAi				
Empty vector control	33.0±0.12	34±0.16	120 (20)	–
Empty vector 0.5 mg/ml HSE	33.3±0.09	34±0.23	120 (34)	0.026
daf-16, skn-1 control	32.9±0.13	34±0.16	120 (18)	–
daf-16, skn-1 0.5 mg/ml HSE	33.0±0.06	34±0.10	120 (25)	ns

Here we investigated the effects of HSE on ageing and Aβ-induced toxicity in the model organism *C. elegans*. HSE treatment resulted in a prominent lifespan extension of *C. elegans* in a dose-dependent manner. Mobility declines with increasing age as a consequence of sarcopenia and loss of neuromuscular function in the nematode [22]. We categorized the nematodes according to their locomotion states. This behavioural assay has been previously used to analyse the health span (adult lifespan with no physical impairment) of *C. elegans* [23]. HSE (0.5 and 1 mg/ml) treatment significantly increased the amount of *C. elegans* without physical impairment indicating a slowed-ageing phenotype. The anti-ageing effect of HSE was not based on caloric restriction since HSE (1 mg/ml) neither influenced pharyngeal pumping as a marker for food intake nor bacterial growth of OP50-1 *E.coli* as a marker for food availability (Online Resource 1, Fig. 1a). Moreover, developmental growth as well as adult size of *C. elegans* was not modulated by treatment with HSE (Online resource 1, Fig. 1b, c).

Long-lived mutants often show an increased stress tolerance, an effect that can be simulated by plant compounds like the flavonoids baicalein and myricetin [16, 20]. Fitzenberger et al. showed that HSE ameliorates glucose-impaired thermal stress resistance in *C. elegans* [18], but no data on effects of HSE without glucose were available in this study. However, we showed that HSE did not influence thermotolerance in *C. elegans* under basal conditions. During these thermal stress conditions, the extract even induced a prooxidative effect as intracellular ROS levels increased. In contrast to the ROS induced by thermal stress, HSE treatment was able to increase survival under paraquat-induced oxidative stress (Online resource 1, Fig. 3) indicating a protective effect against oxidative insults. However, both kinds of stress (thermal-induced vs. paraquat-induced) are not directly comparable: On the one hand, during paraquat-induced oxidative stress mainly superoxide radical anions are produced over a relatively long time period (days), while on the other hand, during lethal thermal stress conditions (besides other effects like induction of heat-shock proteins, effects on membrane fluidity…) a rapid increase in ROS production occurs and the nematode dies within some hours. Therefore, it is not contradictory that HSE is able to protect against the mild oxidative stress while the extract is not protective during thermal stress conditions. Protective effects of HSE were found in various animal models, e.g. of diabetes, hepatotoxicity, nephrotoxicity and brain injury: Treatment with

H. sabdariffa extracts ameliorated oxidative stress in these models by increasing antioxidative enzyme activity [24–27].

Since *H. sabdariffa* was reported to possess antioxidative as well as anti-diabetic properties in mammals, we analysed if HSE modulates DAF-16 (orthologue of mammalian FOXO) and SKN-1 (orthologue of mammalian Nrf2), key transcription factors regulating glucose metabolism, oxidative stress and longevity in *C. elegans* [28, 29]. We demonstrated that the anti-ageing effect of HSE was at least partially mediated by these redox-active transcription factors: Extension of lifespan and health span by HSE was abolished in mutant strains lacking functional DAF-16 or SKN-1. Additionally, HSE induced a nuclear translocation of both transcription factors in *C. elegans*. Recently, an upregulation of FoxO, the mammalian DAF-16-orthologue by a *Hibiscus rosa sinensis* extract was demonstrated by Pillai and Mini [30] using pancreatic ß-cells as model.

Flowers of *H. sabdariffa* consist of multiple bioactive compounds like flavonoids and anthocyanins, which are likely to contribute to these effects. For example, the flavonoids quercetin and myricetin as well as anthocyanin-rich plant extracts were shown to modulate the DAF-16 or the SKN-1-dependent signalling pathways in *C. elegans* [16, 31–34]. In addition to the polyphenolic compounds, HSE contains HCA, which is the major organic acid in the water extracts of *H. sabdariffa* calyces [1]. HCA is thought to induce the anti-obesity and anti-diabetic effects of HSE [35, 36]. Therefore, we investigated if the HSE-mediated effects on longevity are also mediated by the main HSE compound HCA. This was the case: HCA treatment was able to increase lifespan of *C. elegans* and this effect was dependent on the presence of DAF-16 and SKN-1. However, the anti-ageing effect of HCA alone was weaker than the respective amount of HSE (1 mg/ml) which indicates that HCA may act synergistically with other components of HSE. Organic acids like fumarate, malate and oxaloacetate were shown to increase lifespan of *C. elegans* in a DAF-16 dependent manner [37, 38]. Therefore, we investigated if the anti-ageing effect of HCA was a more general effect of organic acids rather than a specific effect of HCA. We analysed the effect of the structural derivative ICA, which lacks the C3 hydroxyl moiety compared to HCA. Treatment with ICA neither modulated lifespan nor nuclear DAF-16 translocation in the nematodes suggesting a specific effect for HCA.

Since HSE showed prominent anti-ageing properties, we analysed if HSE also protects against one of the most frequently occurring age-associated diseases. Alzheimer's disease is the most common form of dementia which affects mostly people exceeding the age of 65 years [39]. A hallmark of Alzheimer's disease is the accumulation of amyloid-β peptides (Aβ) in the brains of the persons. Overexpression of these Aβ peptides induces neurotoxic effects in different cell lines and animal models [40, 41]. Since extracts of different *Hibiscus* species showed prominent neuroprotective effects in aged and diabetic mice, we investigated if HSE also protects against Aβ toxicity in vivo [6, 7]. We used a transgenic *C. elegans* strain expressing $A\beta_{3-42}$ in the body wall muscle cells resulting in a time-dependent paralysis of the body [42]. Treatment with 0.5 mg/ml HSE resulted in a protection against amyloid-β toxicity in *C. elegans*, which was dependent on DAF-16 and SKN-1. Surprisingly, the highest concentration of HSE (1 mg/ml) was not effective when nematodes were fed with *E. coli* strain HT115. Shifting *C. elegans* from OP50 *E. coli* to HT115 *E. coli* results in distinct metabolic changes, e.g. a decrease in triglyceride levels and an increase in lifespan [43]. Therefore, the sort of diet and hence the metabolic state may influence the effect of HSE in vivo. Moreover, *skn-1* knock-down resulted in a protection against Aβ toxicity in *C. elegans*. Slightly lower paralysis rates upon *skn-1* knock-down have been observed in *C. elegans* by several groups [44, 45]. However, down-regulation of *skn-1* also results in increased Aβ accumulation in the nematode [44]. We think, that knock-down of *skn-1* by RNAi may stimulate compensatory mechanisms leading to decreased paralysis rates. Here we could show that double knock-down of *skn-1* and *daf-16* reversed the protective effects of *skn-1* knock-down.

In conclusion, we demonstrated that HSE is able to prolong the lifespan of the model organism *Caenorhabditis elegans* in a concentration-dependent manner. Since HCA, the main component of HSE, but not its structural derivative ICA, also caused a prolongation of lifespan, this component seems to be, at least in parts, responsible for the effect on longevity. Since the effect is less pronounced, other components of HSE seem to be involved. The effect on lifespan was dependent on the presence of the transcription factors DAF-16 and SKN-1 showing the importance of these pivotal molecules. Moreover, we demonstrated that HSE ameliorates amyloid-β toxicity in *C. elegans* via DAF-16 and SKN-1 indicating a therapeutic role for HSE in Alzheimer's disease.

Acknowledgements The nematode strains used in this work were provided by the Caenorhabditis Genetics Centre, which is funded by the NIH National Centre for Research Resources (NCRR). This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. We thank Dr. Sebastian Honnen for helpful discussions.

Author contributions NW, KK: performed experiments, KK, CB: supervision of experiments, WW: coordination of experiments, WW, KK: preparation of manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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