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# Anthocyanin-rich Purple Wheat Prolongs the Life Span of *Caenorhabditis elegans* Probably by Activating the DAF-16/FOXO Transcription Factor

Wei Chen,[†] Dolores Müller,[‡] Elke Richling,[‡] and Michael Wink*[,†]

[†]Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany

[‡]Department of Food Chemistry and Toxicology, Molecular Nutrition, University of Kaiserslautern, Erwin-Schroedinger-Strasse 52, D-67663 Kaiserslautern, Germany

**ABSTRACT:** Colored cereals attract public attention due to their potential antioxidant properties and corresponding health benefits. Purple wheat, rich in anthocyanins, is one of the newly developed cereals on the market. Cyanidin-3-*O*-glucoside (42.6%) is the predominant anthocyanin in purple wheat, followed by peonidin-3-*O*-glucoside (39.9%) and malvidin-3-*O*-galactoside (17.4%). To investigate the potential antiaging and antioxidant properties of purple wheat, the nematode *Caenorhabditis elegans* was chosen as an experimental model organism. It was found that an anthocyanin-rich methanolic extract of purple wheat extended the mean life span of wild type worms and of *mev-1(hn1)* mutants, which are sensitive to oxidative stress, by 10.5 and 9.2%, respectively. Life span extension depends on the transcription factor DAF-16; no significant increase of longevity was seen in *daf-16 (mgDf50)* mutant worms. Translocation of DAF-16/FOXO to the nucleus implies that the transcription factor DAF-16/FOXO was activated under purple wheat treatment by inhibition of the insulin/IGF-1-like signaling pathway which includes the insulin receptor DAF-2. Moreover, purple wheat increased stress response in *C. elegans* as well as reduced oxidative stress. Anthocyanins of purple wheat apparently exhibit beneficial effects in *C. elegans*. They may exert similar properties in humans, which is an issue to be explored in future studies.

**KEYWORDS:** *Caenorhabditis elegans, purple wheat, anthocyanins, polyphenols, life span, DAF-16*

## INTRODUCTION

Higher plants produce a diversity of natural products usually termed secondary metabolites that function as defense compounds against herbivores and microbial pathogens.[1] Due to their properties as active agents, many secondary metabolites serve an important role as therapeutics against human diseases and health disorders.

Polyphenols, which occur in most plants, are one of the largest classes of secondary metabolites. They are constituents in many extracts used in phytotherapy or in fruits, vegetables, and juices of the human diet. The potential beneficial properties of polyphenols are attributed to their pronounced antioxidant activities, that is, scavenging of free radicals and reactive oxygen species (ROS). The dynamic balance between antioxidants and reactive oxygen species is a critical key to maintaining a healthy metabolic environment. Deactivation of ROS by polyphenols depends on the donation of hydrogen radicals from phenolic OH groups.[2] In addition, polyphenols can dissociate under physiological conditions. The resulting phenolate ions can form noncovalent ion bonds with side chains of charged basic amino acid residues of proteins. This binding reduces the flexibility of protein−ligand or protein−protein interactions, hence influencing the function of enzymes, transporters, ion channels, structural proteins, and transcription factors.[1,3]

Colored cereal grains are increasingly attracting interest from grain producers and customers alike due to their potential health-promoting properties. Yellow-colored grains are characteristic for Einkorn wheat due to the high content of carotenoids, especially lutein, zeaxanthin, and $\beta$-carotene, with beneficial antioxidant properties.[4] Other major crops, such as potatoes with red- and purple-colored flesh of tubers, are cultured and appreciated for their high content of anthocyanins with antioxidant properties beneficial for human health.[5] Purple wheat (*Triticum aestivum*), which has been cultivated and consumed in New Zealand since 1980, owes its unusual color to anthocyanins in the pericarp.[6,7] Anthocyanins, the glycosides of anthocyanidins (Figure 1), are water-soluble pigments and are widely found in higher plants. They are responsible for many kinds of colors such as red, blue, yellow, or purple in flowers, vegetables, fruits, and cereal grains. Consequently, they constitute an important source of antioxidants in the human diet in addition to other polyphenols. Moreover, with increased health concerns regarding synthetic food dyes, anthocyanins have a promising potential as a natural food color supplement. Anthocyanins are powerful antioxidants and are believed to exert many beneficial properties, including the prevention of cancer and cardiovascular disorders.[8,9]

*Caenorhabditis elegans*, a small free-living soil nematode, plays an established role in aging and medicinal research. Various natural compounds and drug studies related to aging and aging-affiliated pathology were conducted with this model organism.

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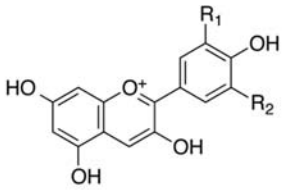


| | $R_1$ | $R_2$ |
|---|---|---|
| Pelargonidin | H | H |
| Cyanidin | OH | H |
| Delphinidin | OH | OH |
| Malvidin | $OCH_3$ | $OCH_3$ |
| Peonidin | $OCH_3$ | H |
| Petunidin | $OCH_3$ | OH |

**Figure 1.** Chemical structures of anthocyanidins.

The multitude of mutant strains available through the *Caenorhabditis* Genetics Center (CGC) offers a convenient possibility to study signaling pathway dependent mechanisms. In addition, the GFP reporter gene easily visualizes gene expression and signaling pathways in mutant worm strains.

The insulin/insulin-like growth factor (IGF-1) pathway is one of the best-understood regulatory signaling pathways influencing aging in animals. In *C. elegans*, a loss of function mutation of *daf-2*, encoding an endocrine receptor like the insulin/IGF-1 receptor, extends the life span to more than twice the normal.[10,11] In addition, DAF-16, a member of the FOXO transcription factor family, and other transcription factors such as heat shock factor HSF-1 and SKN-1 are also required for life span extension. DAF-16 is under control of DAF-2. Binding of insulin-like molecules to the insulin/IGF-1 receptor (DAF-2) (which carries a tyrosine kinase activity) initiates a series of kinase cascades resulting in the phosphorylation and thus inactivation of the DAF-16/FOXO transcription factor.[12,13] Phosphorylated, inactivated DAF-16 stays in the cytoplasm under normal conditions. Accumulation of DAF-16 in the nucleus can be seen in mutants that carry a DAF-16 variant which cannot be phosphorylated. When DAF-16 remains nonphosphorylated in normal worms, due to a reduced DAF-2 signal or a mutated kinase cascade, DAF-16 accumulates in the nucleus, triggering the expression of stress-related genes such as antioxidant genes, small heat shock genes, and antimicrobial and metabolic genes.[14−16] An active FOXO gene is apparently directly related with longevity in *Hydra*, a member of the phylum Cnidaria, and possibly in humans.[17] The evolutionary conserved insulin/IGF-1 signaling pathway plays a role in the aging process not only of nematodes but also of fruit flies and mice, in which reduced insulin-like signals extend longevity.[18−20]

In this study, anthocyanins of purple wheat, which are located in the pericarp, were isolated and used to investigate potential beneficial effects such as stress resistance and antioxidant and antiaging mechanisms in *C. elegans*. This study provides evidence that anthocyanin-rich purple wheat can increase the stress resistance in *C. elegans* as well as significantly extend the life span by activating the transcription factor DAF-16.

## MATERIALS AND METHODS

**Plant Material and Preparation of Anthocyanins.** Anthocyanins were extracted from purple wheat (obtained from Erik von Baer, Semillas Baer, Temuco, Chile) according to a previously reported method.[21] Three grams of the whole grain was mixed with 24 mL of methanol acidified with 1.0 M HCl (85:15, v/v) and stirred overnight. The crude extract was then stored at 4 °C for 2 days to precipitate large molecules. After centrifugation at 4000*g* for 30 min, the colored supernatant was concentrated under a stream of nitrogen.

**High-Performance Liquid Chromatography (HPLC) of Purple Wheat Extract.** HPLC-UV−vis analysis was carried out according to the method described by Muller et al.,[22] using the Jasco HPLC-UV system (Jasco, Gross-Umstadt, Germany) equipped with a Jasco PU-2080 HPLC pump and a Jasco UV-2075 plus intelligent UV−vis detector. A Luna 3 μm C18(2), 250 × 4.6 mm, column (Phenomenex, Aschaffenburg, Germany) was used for separation. Gradient solvent A was acetonitrile/water/formic acid (87:3:10, v/v/v) and solvent B, acetonitrile/water/formic acid (50:40:10, v/v/v), and the elution gradient used was as follows: 2−14% B (0−20 min); held at 14% B (20−40 min); increased to 15% B (40−50 min); raised to 20% B (50−56 min); washed with 2% B (65−110 min). The detection wavelength was 520 nm at room temperature. A standard mixture of 15 authentic reference compounds, with a purity of ≥95%, obtained from Extrasynthese (Genay, France) or Polyphenols Laboratories AS (Sandes, Norway) or isolated individually by Michael Kraus (University of Wuerzburg, Germany), was used for quantitation. Peaks were identified by comparing retention times and UV−vis spectra with those of authentic standards. For quantitation, 100 μg/mL of internal standard (IS) (delphinidin-3,5-diglucoside, delphin) was added in a 1:10 ratio to the sample (final concentration of IS was 10 μg/mL). The internal calibration was performed with the plotting of the ratio of the area of the peak of anthocyanin to the IS against the ratio of the concentration of anthocyanin to the IS concentration.

**DPPH• Free Radical Scavenging Activity of Anthocyanin-rich Purple Wheat.** The free radical scavenging activity of the extract was measured using DPPH• according to the method of Brand-Williams.[23] Freshly made 0.2 mM DPPH• in methanol (100 μL) and different concentrations of purple wheat extract (100 μL) were mixed in 96-well plates. After 30 min of incubation at room temperature, the absorbance was measured at 517 nm using a Safire2 microplate reader (Tecan AG, Männedorf, Switzerland) and subsequently calculated using the following formula: inhibition % = $(1 - A_{substance}/A_{control}) \times 100\%$ ($A_{substance}$, absorbance of substance, $A_{control}$, absorbance of control). A 0.2 mM DPPH• solution (100 μL) plus 100 μL of methol was used as negative control.

***C. elegans* Strains and Maintenance Conditions.** The wild type strain N2 and mutant strains TK22 [*mev-1(kn1)*], TJ375 [*hsp-16.2::GFP(gpIs1)*], and TJ356 [*daf-16::daf-16-gfp; rol-6*] were obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, USA). GR1307 [*daf-16 (mgDf50)*] was kindly provided by Dr. Nadine Saul (Humboldt University of Berlin). All of the strains were maintained at 20 °C on a standard nematode growth medium (NGM) with living bacteria *Escherichia coli* OP50 as a food source.[24]

**Quantitation of *hsp-16.2* Expression via Fluorescence Microscopy.** In the *hsp-16.2/GFP* strain, which contains a promoter of *hsp-16.2* in combination with the gene for green fluorescence protein (GFP), GFP is locally expressed in the pharynx of *C. elegans* after either treatment with heat shock or oxidative stress such as exposure to the pro-oxidant naphthoquinone juglone. Age-synchronized worms were treated with the purple wheat extract on the first day after hatching for 48 h and were then exposed to 20 μM juglone for 24 h. After induction, the expression of *hsp-16.2* was measured directly through measuring the fluorescence intensity of the reporter protein GFP by fluorescence microscopy with an Eclipse 90i(2) at the Nikon Imageing Center, Heidelberg University.

**Intracellular ROS Level in *C. elegans*.** Intracellular ROS levels in *C. elegans* were measured using the fluorogenic probe 2,7-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA).[25] Synchronized worms were treated with anthocyanin-rich purple wheat extract (10, 50, 100 μg/mL) on day 1 for 72 h and then collected into 100 μL of

PBS with 1% Tween-20. Then the nematodes were sonicated and pipetted into 96-well plates containing 50 $\mu$M $H_2$DCF-DA at 37 °C. The fluorescence was recorded in a Safire2 microplate reader (Tecan AG, Männedorf, Switzerland).

**Life Span Assay.** Synchronized young adult worms (3 days after hatching) were grown in S-basal medium with living *E. coli* OP50 containing purple wheat extract during the whole life span of the nematodes. Wild type N2 and mutant strains TK22 [*mev-1(kn1)*] and GR1307 [*daf-16 (mgDf50)*] were employed in the life span assay. During the first 3−10 days the worms were transferred to a new medium (plus ingredients) daily to separate different generations; after that, they were transferred every second day. Death was scored if worms failed to respond to gentle physical touch.

**Subcellular DAF-16 Location.** To further investigate how purple wheat extended the longevity of *C. elegans*, the transgenic strain TJ356, which expresses a DAF-16::GFP fusion protein, was selected. Synchronized L2 larval stage worms, cultivated in S-medium with living *E. coli* OP50, were treated with purple wheat extract for 1 h versus a heat shock treatment (37 °C for 15 min) inducing a rapid nuclear localization of DAF-16 as a positive control.[26] Subsequently, living worms were placed on microscope slides and paralyzed with a drop of 10 mM sodium azide. GFP signals were visualized by fluorescence microscopy with an Eclipse 90i(2) at the Nikon Imageing Center, Heidelberg University.

**Statistical Analyses.** All data represent means of three independent experiments unless mentioned. Continuous variables were presented as the mean ± SD. A two-tailed unpaired Student's *t* test was performed to compare two groups, whereas comparisons between multiple groups were executed by one-way analysis of variance (ANOVA) followed by post hoc analysis with Tukey's test (Prism, GraphPad Software, San Diego, CA, USA). The life span curves were estimated according to Kaplan−Meier survival analysis, and the log-rank test for comparison used StatView 5.0 software (SAS).

## RESULTS AND DISCUSSION

HPLC-UV−vis chromatograms of the purple wheat extract recorded at 520 nm are given in Figure 2. Quantitative data are shown in Table 1. The total anthocyanin content of purple wheat extract was 218 $\mu$g/mL. Cyanidin-3-*O*-glucoside (42.6%) is the predominant anthocyanin, which is consistent with previous research,[27] followed by peonidin-3-*O*-glucoside (39.9%) and malvidin-3-*O*-galactoside (17.4%).

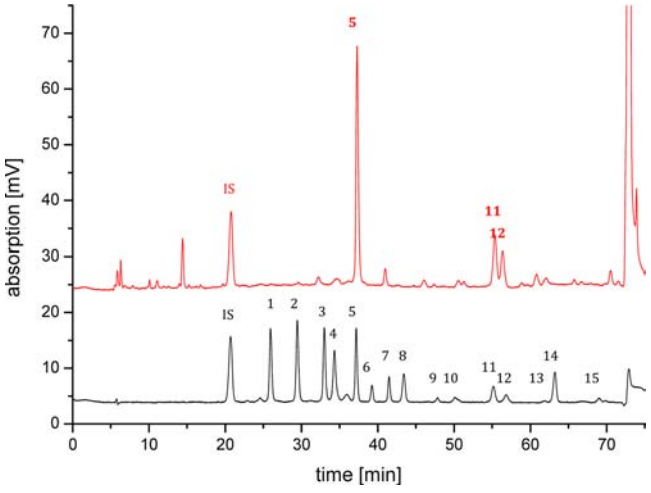


**Figure 2.** HPLC-UV−vis chromatograms of a methanolic extract of purple wheat and a standard mixture of 15 anthocyanins (peaks 1−15): (1) delphidin-3-*O*-galactoside; (2) delphidin-3-*O*-glucoside; (3) cyanidin-3-*O*-galactoside; (4) delphidin-3-*O*-arabinoside; (5) cyanidin-3-*O*-glucoside; (6) petunidin-3-*O*-galactoside; (7) cyanidin-3-*O*-arabinoside; (8) petunidin-3-*O*-glucoside; (9) petunidin-3-*O*-galactoside; (10) petunidin-3-*O*-arabinoside; (11) peonidin-3-*O*-glucoside; (12) malvidin-3-*O*-galactoside; (13) peonidin-3-*O*-arabinoside; (14) malvidin-3-*O*-glucoside; (15) malvidin-3-*O*-arabinoside; (IS) Internal standard (delphin).

**Table 1. Anthocyanin Content and Composition in Purple Wheat**

| peak | anthocyanin | retention time (min) | content ($\mu$g anthocyanins/mL extract solution) | anthocyanin amount (%) |
|---|---|---|---|---|
| 5 | cyanidin-3-*O*-glucoside | 37.3 | 92.9 ± 5.8 | 42.6 ± 2.6 |
| 11 | peonidin-3-*O*-glucoside | 55.4 | 87.1 ± 4.9 | 39.9 ± 2.0 |
| 12 | malvidin-3-*O*-galactoside | 56.4 | 38.0 ± 2.3 | 17.4 ± 1.0 |
| | total | | 218.0 ± 12.6 | 100.0 |

Extracts of purple wheat display free radical scavenging activity in vitro using DPPH$^\bullet$ ($IC_{50}$ of anthocyanins = 331.31 ± 2.75 $\mu$g/mL, compared to that of L-ascorbic acid, a strong antioxidant, 5.73 ± 0.39 $\mu$g/mL) (Figure 3). This result is consistent with previous studies of anthocyanins from blue wheat and black soybean seed.[28,29]

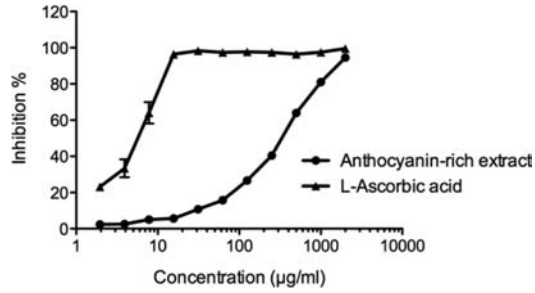


**Figure 3.** Dose dependence of DPPH$^\bullet$ antioxidant activity of purple wheat. L-Ascorbic acid was used as positive control. Data were from three independent experiments. Data are shown as the mean ± SD.

Aging is a complicated progress and, up till now, still a mystery to scientists. The public worries about aging because it is related to several health disorders including diabetes, cancer, and Alzheimer's and other neurodegenerative diseases. Scientists have been trying to understand the underlying mechanisms of aging and the possibilities to combat these diseases by targeting aging. *C. elegans* is increasingly becoming a model organism in aging research. The mechanisms of aging, especially the regulatory signaling pathways, and corresponding genes are mostly conserved in *C. elegans* and mammals during evolution.

To investigate possible antiaging effects of purple wheat, we performed life span assays. Figure 4A demonstrates that exposure of worms to 100 $\mu$g/mL purple wheat extract significantly extends the mean life span of wild type worms by 10.5% ($P < 0.01$) (Table 2). To test the protective property against oxidative stress, we used the *mev-1(hn1)* mutant strain, in which overproduced free radicals lead to a shortened life span.[30] The result showed that anthocyanins can significantly prolong the mean life span of *mev-1(hn1)* by 9.2% ($P < 0.05$)


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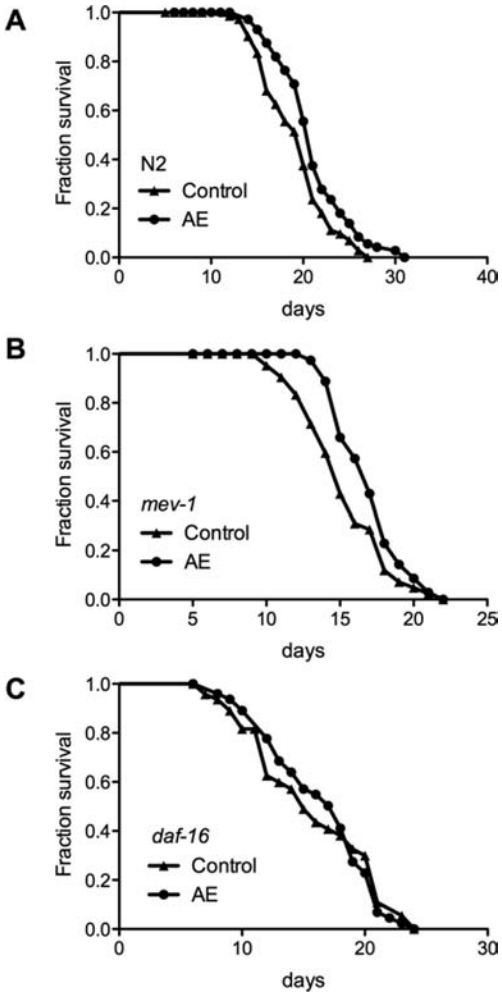


**Figure 4.** Effects of the anthocyanins of purple wheat extract on the life span of *C. elegans*. The anthocyanin-rich extract (AE) (100 μg/mL) significantly extended the mean life span of wild type N2 (A) (control, 19.27 ± 0.43 days; AE, 21.29 ± 0.45 days; **, $P < 0.01$) and *mev-1* worms (B) (control, 15.35 ± 0.44 days; AE, 16.76 ± 0.42 days; *, $P < 0.05$), but not in *daf-16* strain (C) (control, 15.66 ± 0.80 days; AE, 16.45 ± 0.64 days; $P = 0.98$). See Table 2 for statistical analysis and life span data.

(Figure 4B and Table 2). However, there is no corresponding extension of life span in *daf-16* mutants *(mgDf50)*, which do not produce the DAF-16/FOXO transcription factor (Figure 4C and Table 2). Therefore, the longevity-promoting effect of purple wheat is probably mediated through a DAF-16-dependent pathway. To confirm the molecular model of longevity in *C. elegans*, the transgenic mutant TJ356 was utilized. In this strain, a DAF-16::GFP fusion protein is constructed as a reporter to reveal the subcellular localization of DAF-16 as shown in Figure 5B. The anthocyanin treatment resulted in the translocation of transcription factor DAF-16 from the cytosol to the nucleus (Figure 5C), indicating that the anthocyanins from purple wheat activate the DAF-16 transcription factor by inhibition of proteins in the insulin/IGF-1 signaling pathway, which is regulated by DAF-2. As mentioned above, polyphenols interact with proteins, changing their flexibility and three-dimensional structure; this could be the case for any of the proteins of the insulin/IGF-like signaling pathway or even other pathways. Furthermore, a synergistic interaction between anthocyanins with other polyphenols present, such as ferulic acid, coumaric acid, and caffeic acid, might be possible.[31] Therefore, our explanation for the DAF-16 effects is plausible but certainly not exclusive. It has been reported that the anthocyanins of blueberry, similar to those of purple wheat, and EGCG from green tea extend the life span of *C. elegans*.[32,33]

In the mutant strain TJ375 [*hsp-16.2::GFP(gpIs1)*], the *hsp16.2* promoter coupled with a GFP reporter provides stress-related information of the worms.[34] Juglone, being a ROS generator, activates the *hsp16.2* expression, as indicated by a strong GFP fluorescence in the pharynx of the worms (Figure 6). The expression of *hsp16.2* under juglone-induced oxidative stress was attenuated by 34.4% ($P < 0.05$) in the group of worms pretreated with purple wheat extract (Figure 6). This suggests that purple wheat can diminish oxidative stress and ROS levels in vivo, similar to the effect of L-ascorbic acid or epigallocatechin gallate (EGCG). It has been reported that anthocyanins from black soybean activate the PI3 kinase/AKT pathway to inhibit ROS production.[35]

Furthermore, wild type N2 worms were treated with 10, 50, and 100 μg/mL anthocyanins, respectively, from L1 stage for 72 h; after that, the intracellular level of ROS (especially $H_2O_2$) was measured by $H_2$DCF-DA, a nonfluorescent compound converted to fluorescent DCF through esterase activity and oxidation. High fluorescence levels indicate high ROS levels inside cells or organisms. These results showed that the fluorescence level was significantly diminished after anthocyanin treatment by up to 39.0% compared to the control group (Figure 7).

Oxidative stress, associated with increased ROS production or the decline of antioxidant defense systems, is thought to play a role in many age-related diseases, for instance,, neurodegenerative diseases, diabetes, cardiovascular disease, and even cancer.[36−38] Growing evidence from epidemiology suggests that antioxidants reduce the level of oxidative stress. Their possible use as health-promoting agents is a logical consequence.[39,40] Our results demonstrate that anthocyanins are taken up by the worms and that they exert antioxidant

**Table 2. Effects of Anthocyanin-rich Purple Wheat Extract on the Life Span of *Caenorhabditis elegans***

| strain | treatment | *N* | mean ± SE (days) | extension % | | log-rank test |
|---|---|---|---|---|---|---|
| wild type | control | 100 | 19.27 ± 0.43 | | | |
| | anthocyanin-rich extract | 100 | 21.29 ± 0.45 | 10.5 | ** | <0.01 |
| *mev-1 (hn1)* | control | 90 | 15.35 ± 0.44 | | | |
| | anthocyanin-rich extract | 90 | 16.76 ± 0.42 | 9.2 | * | <0.05 |
| *daf-16 (mgDf50)* | control | 50 | 15.66 ± 0.80 | | | |
| | anthocyanin-rich extract | 50 | 16.45 ± 0.64 | 5.0 | | 0.98 |

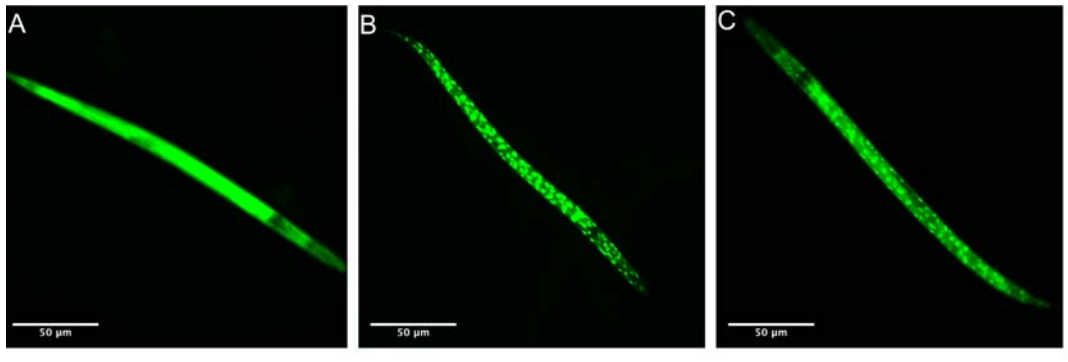


**Figure 5.** Effects of purple wheat and thermal stress on nuclear DAF-16 localization in TJ356: (A) negative control, without any treatment; (B) heat shock treatment at 37 °C for 15 min as a positive control; (C) DAF-16 nuclear localization induced by 100 μg/mL purple wheat treatment. Three independent experiments with at least 20 worms per group were performed. Scale bar represents 50 μm.

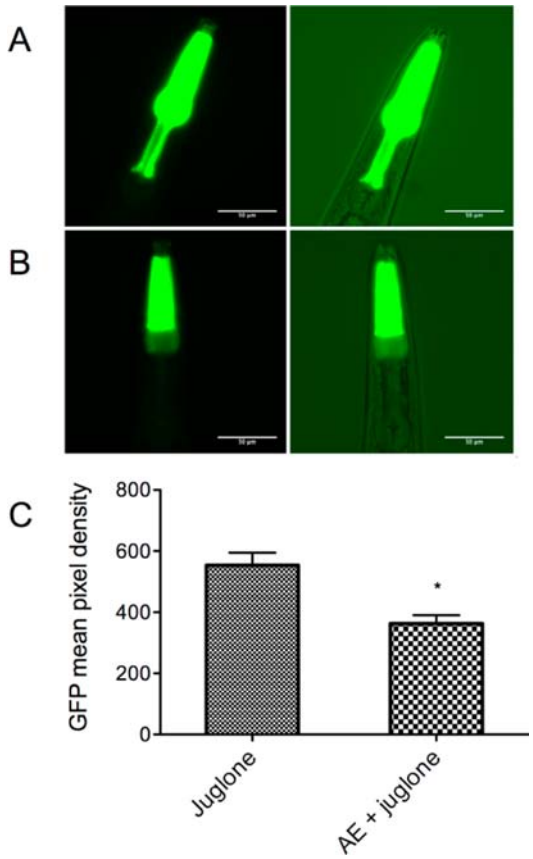


**Figure 6.** Inhibition of *hsp 16.2* expression through anthocyanins under oxidative stress in transgenic *C. elegans* TJ375: (A) images of worms treated with MeOH solvent as negative control, 24 h exposure to juglone; (B) images of worms with 100 μg/mL anthocyanin-rich extract (AE), 24 h exposure to juglone; (C) quantification of GFP density in the worms' pharynx from three independent experiments with 25 worms in one group; mean ± SD; *, $P < 0.05$.

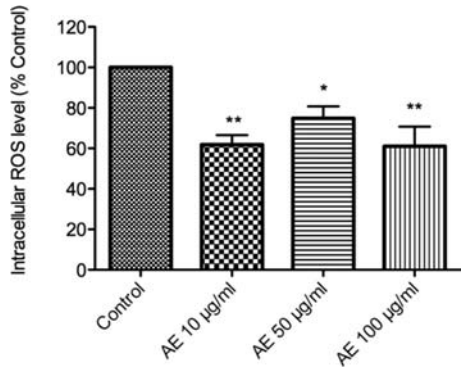


**Figure 7.** Decrease of ROS levels through purple wheat extract: wild type worms treated with anthocyanin-rich extract (AE) (10, 50, 100 μg/mL) from L1 stage for 72 h. The ROS level was measured by DCF-DA assay, 20 worms per group. Results are expressed as a percentage of fluorescence (%DCF) relative to control. Data are from three independent experiments. Data are shown as the mean ± SD; *, $P < 0.05$; **, $P < 0.01$.

protective activity within the worms. In previous studies the bioavailability of anthocyanins from red wine has been documented.[41] Similar results have been obtained with EGCG, a main active polyphenol of green tea, and also with anthocyanins from bilberries.[32,42]

White- and red-grained types of wheat are a common choice for producers and customers, whereas purple-grained wheat is currently receiving intensive attention due to its health potential. Apart from dietary fibers, proteins, tocopherol, and carotenoids, purple wheat contains anthocyanins in the pericarp, which produce its purple color. Because anthocyanins are potent antioxidants, possible beneficial effects in the human diet have been suggested.[27,43,44] Anthocyanins can differ in their pronounced oxygen radical scavenging capacity. Cyanidin-3-glucoside, the main anthocyanin in purple wheat, displayed the highest antioxidant activity among anthocyanins, which was even higher than that of Trolox (a vitamin E analogue).[45] A decrease in oxidative damage under the influence of anthocyanins and an increase in antioxidant capacity of the serum after consumption of anthocyanins have been observed.[46,47] Moreover, cyanidin-3-glucosides have been reported to be stable in cell culture environments because of their conjugated sugar moieties, which contributes to their anti-inflammatory effects in vitro.[48]

In conclusion, these results show that exposure of worms to anthocyanins leads to a translocation of DAF-16 to the nucleus, where it stimulates the expression of stress resistance and longevity-related genes. The lack of any significant extension of life span in the *daf-16 (mgDf50)* mutant is indicative of a DAF-16-dependent mechanism. Because of the complexity of pathways involved, our explanation of DAF-16 effects is plausible but certainly not exclusive. Due to this property of its rich anthocyanin content, we suggest purple wheat as a potential candidate for food supplementation and as a functional food that could help improve human health.


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However, dietary intervention studies are required to translate the results from *C. elegans* to humans.

## AUTHOR INFORMATION


### Corresponding Author

*Phone: +49 6221 544881. Fax: +49 6221 544884. E-mail: wink@uni-hd.de.



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### Notes

The authors declare no competing financial interest.

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