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Original Research Article

Effect of Hawthorn on *Drosophila Melanogaster* Antioxidant-Related Gene Expression

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Abstract

Purpose: To study the effects of various doses of hawthorn extract on Drosophila lifespan, antioxidant enzyme activity and expression of antioxidant-related regulation genes.
Methods: Experiments with Drosophila as an animal model were conducted. The effects of hawthorn on Drosophila melanogaster antioxidant related gene expression were investigated by lifespan tests of Drosophila, antioxidant enzyme activity assay of Drosophila, and mRNA expression of antioxidant genes by real time-PCR assay.
Results: The results indicate that hawthorn extract prolonged the life span of Drosophila, with 50 % survival time of 0.8 and 4 mg/mL groups being increased from 52 days (control) to 56 and 62 days, respectively. Addition of 0.8 mg/mL hawthorn extract increased CuZn-SOD enzyme activity significantly ($p < 0.05$); the 4 mg/mL extract significantly increased CuZn-SOD enzyme ($p < 0.01$) and CAT enzyme activity ($p < 0.05$), but decreased MDA levels. Real time-PCR results show that the 4 mg/mL extract significantly improved the expression levels of CuZn-SOD and CAT mRNA ($p < 0.05$); on the other hand, both extract concentrations improved PHGSH-Px mRNA level significantly compared with that of control group ($p < 0.05$).
Conclusion: The antioxidant activity of hawthorn *in vivo* may be achieved by increasing endogenous antioxidant enzymes.

Keywords: *Drosophila melanogaster*, Hawthorn, Lifespan, Enzyme, Gene expression, Phospholipid hydroperoxide glutathione peroxidase, Superoxide dismutase, Catalase

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INTRODUCTION

Hawthorn is a kind of traditional medicinal and edible plant, rich in flavonoids, anthocyanins, ursolic acid, saponins, carotene and other active ingredients [1]. Toxicological studies have shown that long-term consumption of hawthorn is beneficial to health, with almost no side effects [2]. Pharmacological studies also show that the active ingredients of hawthorn increased blood antioxidant activity but lowered LDL cholesterol [3]. As a kind of mature anti-aging animal model, Drosophila's aging gene is highly similar to humans [4,5].

The objective of this study was to investigate to study the effect of hawthorn extract on the life span, internal antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) activity, and the oxidation product,

malonaldehyde (MDA) content of Drosophila. Besides, the effect of hawthorn on the expression levels of antioxidant-related regulatory genes such as SOD, CAT, phospholipid hydroperoxide glutathione peroxidase (PHGSH-Px) and regulatory particle non-ATPase 11 (Rpn11) mRNA were examined at the molecular level.

EXPERIMENTAL

Materials and chemicals

Hawthorn fruits were collected at the end of September to about the middle of November 2012 from the local market in Tianjin, China. The fruit material was authenticated by Professor Hao Wang, College of Food Engineering and Biotechnology, Tianjin University of Science and Technology. A voucher specimen (JF-UC206714) has been kept in the herbarium of Tianjin Jian Feng Natural Products Company, China for future reference. Other chemicals used were of analytical grade.

Extraction procedure

Hawthorn extracts were produced with help of Jian Feng Natural Products Company. Specific extraction process was as follows: The seeds were removed and then the fresh fruit was freeze-dried and ground into powder in a coffee grinder. The powdered fruit (1.5 kg) was extracted three times with 80 % ethanol (5 L) for 24 h at room temperature. The pooled ethanol filtrates were concentrated using a vacuum rotary evaporator, extracted with ethyl acetate, and after purification with resin (AB-8), the products were lyophilized into powder.

HPLC analysis of the active ingredients of extract

The chromatographic conditions used are as follows [6]. Column: Hypersil ODS-2 (250 × 4.6 mm, 5 μm); mobile phase A: 5% acetonitrile (25 μmol/L NaH_2PO_4); mobile phase B: 25 % acetonitrile (25 mmol/L NaH_2PO_4); gradient elution (min/B%): 0/10, 20/80, 55/80, 60/10; flow rate of 1.0 mL/min; detection wavelength: 280 nm and 360 nm; injection volume: 20 μL; column temperature: 30 ^oC.

Life span tests

Two-days-old adult Drosophila flies (600 males) were collected (Drosophila donee with Chinese University of Hong Kong and expanded reproduction)and randomly divided into 3 groups (control group and two test groups of 0.8 and 4 mg/mL hawthorn extract), 10 tubes were assigned to each group with 20 Drosophila flies in each tube. They were cultured in a constant temperature and humidity incubator at 25 ^oC/50 %RH. During the test, the medium was replaced every 3 days with a fresh one and life span data were recorded until all the flies were dead. The experiment carried was out in duplicate. The medium used for culturing [6] consisted of yeast 10g, distilled water 750 mL, agar 6g, glucose 72 g, corn flour 72 g, and preservative 40 mL (1 % ethyl hydroxybenzoate); the extract was incorporated in the medium for the hawthorn test groups.

Antioxidant enzyme activity assay

Six hundred 2-days-old male adult Drosophila flies were collected and randomly divided into 3 groups (control group and two test groups) as described above. After culturing for 30 days, the Drosophila were anaesthetized with CO_2 at -80 ^oC, divided into 10 tubes and reserved for further use. The fruit flies (5*)* were homogenized in 1 mL of cold saline on ice, then centrifuged at 4 ^oC/2500 rev/min for 20 min, and the supernatants used for enzyme activity measurements. In brief, the assay for SOD was based on its ability to inhibit the oxidation of oxymine by the xanthine–xanthine oxidase system. The hydroxylamine nitrite produced by the oxidation of oxymine has an absorbance peak at 550 nm. One unit of SOD was defined as amount of enzyme causing 50 % inhibition in the NBT reduction rate. Catalase activity was determined using the manufacturer's kit, in which the disappearance of H_2O_2 is followed spectrophotometrically at 240 nm. Enzyme units are expressed as nM/min/mg of the sample protein. The thiobarbituric acid reaction (TBAR) method[6] was used to determine MDA (detected at 532 nm). MDA content was expressed as nM/mg of the sample protein.

mRNA expression of antioxidant genes by Real time-PCR assay

In brief, total RNA was extracted using the commercial extraction agent TRIzol (Takara,Dalian , China). Fruit flies (5 homogenates) were homogenized in 1mL of TRIzol solution, and then centrifuged at 12,000g at 4 °C for 5 min. The supernatant was transferred to another new tube containing 160 μL chloroform. The mixture was then subjected to centrifugation at 12,000 g at 4 °C for 15 min. The upper layer was mixed with 400 μL isopropanol. After 10 min of incubation at room temperature, the samples were centrifuged at 12,000 g at 4 °C for 10 min, and the pellet was

saved and washed in 1 ml of 75 % ethanol followed by re-centrifugation. Finally, 20µL DEPC water was employed to re-suspend the RNA pellet. The concentration and purity of RNA obtained were determined by measuring their absorbance at 260 nm and 280 nm. High capacity cDNA was obtained by reverse transcription and then stored at -80 ^{o}C pending further use. Real time-PCR assay was applied for the determination of the mRNA expression levels of antioxidant related genes. Relevant information on the gene primers are listed in Table 1.

Statistical analysis

Data are expressed as mean ± SD. Significant difference between samples was assessed using a Student t-test. Differences were considered significant when $p < 0.05$. Statistical Package for the Social Sciences (SPSS) version 17.0 software (SPSS Inc, Chicago, IL, USA) was used for the analysis.

RESULTS

Active ingredients of hawthorn

HPLC analysis showed that the hawthorn extract contained 19.86 % proanthocyanidins B_2, 15.27 % epicatechin, 3.10 % chlorogenic acid, 2.91 % hyperoside and 1.34 % iso quercetin. The main active ingredients are shown in Figure 1.

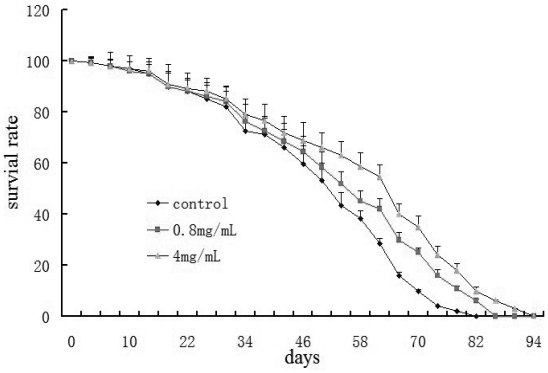

Figure 1: Life span of *Drosophila melanogaster* treated with 0 (control), 0.8 and 4 mg/mL of hawthorn fruit extract

Effect of hawthorn extract on lifespan of Drosophila

The results (Figure 1) show that hawthorn extract extended the lifespan of Drosophila, compared with control flies with 50 % survival time rising from 52 (control) to 56 ($p < 0.05$) and 62 ($p < 0.01$) days following treatment with 0.8 and 4 mg/mL, respectively, of the treatment.

Effect of hawthorn extract on enzyme activity of Drosophila

After feeding Drosophila with hawthorn extract for 30 days, CuZn-SOD activity of Drosophila improved significantly ($p < 0.05$ and $p < 0.01$) for 0.8 and 4.0 mg/mL, respectively. The effect on

Table 1: Real time PCR primers used to measure *Drosophila melanogaster* antioxidant mRNA gene expression

Gene	Accession no.	Forward primer 5, -3,	Reverse primer5, -3,
RP49	NM_079843.2	CTTCATCCGCCACCAGTC	GCACCAGGAACTTCTTGAATC
Cu-Zn-SOD	NM_057387.3	GCGGCGTTATTGGCATTG	ACTAACAGACCACAGGCTATG
Mn-SOD	NM_057577.3	CACATCAACCACACCATCTTC	CGTCTTCCACTGCGACTC
CAT	NM_080483.3	TGAACTTCCTGGATGAGATGTC	TCTTGGCGGCACAATACTG
Rpn11	NM_079843.2	TGGTCGTCGGCTGGTATC	GGATTGCTGCGTATTGATGTC
PHGSH-Px	NM_168025.2	ATGTCTGCTAACGGAGATTAC	CACTTGGAGGCGATGTTC

Table 2: The mean activity of protein in *Drosophila melanogaster*

Enzyme activity	Howthorn fruit extract		
	Control	*0.8mg/mL*	*4mg/mL*
Cu-Zn-SOD(U/mg prot)	65.34±2.74	87.94±10.21*	95.94±11.69**
Mn-SOD(U/mg prot)	20.21±2.12	20.35±2.78	20.52±2.39
CAT(U/mg prot)	17.55±1.47	18.68±2.22	20.35±1.62*
MDA(nmol/mg pro)	0.52±0.07	0.49±0.07	0.44±0.05

*$p < 0.05$; **$p < 0.01$

mRNA expression levels of antioxidant genes

CuZn-SOD, CAT mRNA expression levels significantly increased ($p < 0.05$) following treatment with 4 mg/mL, but the effect on Mn-SOD mRNA expression levels was not significant ($p > 0.05$). The results showed that Rpn11 expression level which is consistent with previous results for blueberry extracts in Drosophila [8]. PHGSH-Px inhibited peroxidation of membrane phospholipid; mRNA level of phospholipid glutathione peroxidase in Drosophila was significantly increased by 0.8 and 4 mg/mL extrac ($p < 0.05$ and $p < 0.01$), respectively(Table 3).

Table 3: The levels of *Drosophila melanogaster* antioxidant mRNA gene expression

RNA expression	Hawthorn fruit extract		
	Control	*0.8mg/mL*	*4mg/mL*
Cu-Zn-SOD	1±0.16	0.99±0.31	1.33±0.35*
Mn-SOD	1±0.17	1.13±0.20	1.09±0.31
CAT	1±0.28	1.28±0.38	1.42±0.30*
Mth	1±0.08	0.94±0.25	1.37±0.36*
RP-n11	1±0.17	1.08±0.29	1.14±0.32
GSH-PX	1±0.16	1.26±0.12*	1.75±0.27**

**p < 0.05; **p < 0.01; data were normalized with RP49; values are expressed as mean ± SD. with those for control being arbitrarily taken as 1*

DISCUSSION

According to the free radical theory of aging, it is unavoidable that living creature in aerobic conditions would produce reactive oxygen species (ROS), and there are free radical scavenging systems in the body. *In vivo*, antioxidants and endogenous antioxidant enzymes can remove free radicals to maintain homeostasis, in which SOD catalyzes O_2^- into H_2O_2, and CAT and GSH-Px change H_2O_2 to H_2O. However, excessive free radicals (OH^-, O^-_2 and H_2O_2) could be beyond the scavenging capacity of the body, and thus could attack intracellular proteins, lipids, DNA, and ultimately lead to oxidative damage [8,9].

MDA is an end-product of lipid peroxidation after free radical attack, and may produce crosslinks between macromolecules such as proteins and nucleic acids, leading to cytotoxicity. At present, although the relationship between oxidative damage and aging/life span is not yet clear, most studies have shown that excessive accumulation of oxides in different tissues can cause aging [10].

Hawthorn is rich in flavonoids and other active ingredients. Some researchers have shown that hawthorn extract removes free radicals such as OH^-, O^-_2 and H_2O_2, and possesses high antioxidant activity both *in vivo* and *in vitro* [11,12]. Hao *et al* [6] has proved that hawthorn extracts protects the body from Cu^{2+}-mediated

LDL oxidative damage in the SAM rat model. Drosophila is a typical anti-oxidation and anti-aging animal model and the experimental results show that hawthorn extract can prolong the life span of Drosophila. Overexpression of Rpn11 could inhibit the 26S proteasome activity which is associated with aging, thus prolonging the life span of Drosophila [13]. In the current experimental settings, Drosophila showed an up-regulation of Cu-Zn-SOD, CAT, Mth andGSH-Px mRNA expression, which were consistent with the results of Shanthi that taking hawthorn could prevent antioxidant levels such as glutathione and vitamin E from dropping, and maintain the activities of antioxidant enzymes in the liver, aorta and heart [14].

CONCLUSION

Hawthon extract elevates the expression of antioxidant genes, such as SOD, CAT, Rpn11 and PHGSH-Px. Therefore, dietary consumption of hawthorn extracts could have beneficial impact on endogenous antioxidant enzymes.

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