Morus alba leaf extract increases lifespan in Caenorhabditis elegans

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All parts of Morus alba offer great therapeutic potential, but are they beneficial to the whole organism, particularly with respect to aging? We evaluated the effect of an ethanol extract of mulberry leaves on the model organism Caenorhabditis elegans. The extract significantly extended mean lifespan by 17% from 17.6 to 20.6 days. This compares well to the effect of ethosuximide, a drug used to treat seizures in humans, which increased adult lifespan by 35%. This novel activity suggests that mulberry leaves have the potential to retard the aging process.

Key words: Morus alba, leaf extract, lifespan, C. elegans

INTRODUCTION

Functional compounds derived from Morus alba leaves, fruits, roots and stem bark have been studied from structural, biological, and pharmacological perspectives. A large number of active biomolecules and extracts show potential as antioxidants, antimicrobials, hyperglycemic treatments, atherosclerosis treatments, immuno-nutrients, neuroprotectants, and skin tone improvers (Butt et al., 2008; Kumar and Chauhan, 2008). Three flavonol glycosides (quercetin-3-[6-malonylglucoside], quercetin 3-rutinoside, and isoquercitrin [quercetin 3-glucoside]) in mulberry leaves are major LDL (low-density lipoprotein) antioxidants (Katsube et al., 2006). A single oral administration of mulberry 1-deoxynojirimycin (DNJ) to both normal rats and humans suppressed the elevation of postprandial blood glucose and the secretion of insulin (Miyahara et al., 2004; Kumar and Chauhan, 2008). Three flavonol glycosides (quercetin-3-[6-malonylglucoside], quercetin 3-rutinoside, and isoquercitrin [quercetin 3-glucoside]) in mulberry leaves are major LDL (low-density lipoprotein) antioxidants (Katsube et al., 2006). A single oral administration of mulberry 1-deoxynojirimycin (DNJ) to both normal rats and humans suppressed the elevation of postprandial blood glucose and the secretion of insulin (Miyahara et al., 2004; Kumar and Chauhan, 2008).

Kaempferol-3-O-(6-malonyl) glucoside, an antiamyloidalogenic substance from mulberry leaves, prevented the formation of amyloid β-peptide fibrils (Niidome et al., 2007; Khangkhan et al., 2009) and an ethanol extract of mulberry fruits protected dopaminergic neurons in toxin-induced Parkinson’s disease models (Kim et al., 2010). These results highlight the therapeutic worth of M. alba in neuroprotective functions.

Our group isolated 7,2′,4′,6′-tetrahydroxy-6-geranylflavanone, a prenylated flavanone, from ethyl acetate extracts of roots. This novel flavonoid killed rat hepatoma cells (Kofujita et al., 2004). We also suggested that a water-soluble glycoprotein-like complex from mulberry stem bark could boost in vitro immunity (Sillapakong et al., 2011). However, the therapeutic potential of each part of M. alba to promote longevity is not well documented, and is needed in order to validate the medicinal worth of M. alba. So we investigated whether mulberry leaves could extend lifespan in animals. We found that a mulberry leaf extract could indeed increase the mean lifespan of the nematode Caenorhabditis elegans, suggesting a new role for mulberry in human diet and as a herbal medicine.

MATERIALS AND METHODS

Reagents

We prepared DNJ by the modified method of Asano et al. (2001). We used ethosuximide (2-ethyl-methylsuccinimide; Sigma) as a positive control. Ethosuximide is a small heterocyclic ring compound that prevents seizures in humans by regulating neural activity (Evason et al., 2005).

Preparation of mulberry leaf extracts

(1) Dry mulberry leaves (Morus. alba L. cv. Aobanezumi) were extracted with 50% or 100% ethanol by the method of Miyahara et al. (2004). (2) Following the method of Piao et al. (2009) with modifications, dried leaves (100 g) were successively extracted with hexane (300 mL, 3 ×), ethyl acetate (300 mL, 2 ×), aqueous acetone (acetone/water = 7/3, 300 mL, 3 ×), and distilled water (400 mL, 2 ×). Each extraction was done at ambient temperature for 24 h. Each extract was centrifuged at 10,000 g for 10 min at 4°C. After evaporation, the fractions yielded 4.68 g hex-
ane extract, 1.19 g ethyl acetate extract, 4.32 g 70% acetone extract, and 6.50 g water extract. (3) Mulberry leaf powder (50 g) was mixed with 30 volumes (w/v) of Milli-Q water. The mixture was boiled at 60°C for 12 h and then filtered (No.2 filter, Advantec). The remains were reextracted and the combined filtrates were lyophilized and stored at −80°C until use. All seven preparations were used in the following assays.

Strain and culturing of *C. elegans*

*Caenorhabditis elegans* Bristol N2 was used as the wild-type strain. Nematodes were maintained at 20°C on a nematode growth agar medium with live *Escherichia coli* OP50 as a food source, following the method of Brenner (1974).

Life span assay

When the nematodes reached the fourth larval stage (just before adulthood), they were transferred to fresh plates and fed with mulberry leaf extracts. They were placed on fresh plates every other day. All nematodes in three or four independent experiments (*n* = 50-60 per treatment) were assessed daily throughout the assay; they were scored as alive or dead depending on whether or not they responded to touch stimuli and showed pharyngeal movements. We calculated age-specific mortality rates by dividing the number of dead nematodes on any day by the number that was alive the previous day.

Statistical analysis

JMP 8 (SAS Institute Inc.) statistical software was used for statistical analysis (Kaplan-Meier) and to determine survival means, maxima and percentiles. The log-rank (Mantel-Cox) test was used to test the hypothesis that the survival functions among groups were equal. *P* values were calculated for individual experiments, each consisting of control and treated nematodes examined at the same time.

**RESULTS AND DISCUSSION**

In a preliminary experiment, we ascertained that absolute ethanol, aqueous acetone, and hot water extracts of mulberry leaves increased lifespan, but DNJ derived largely from mulberry leaves did not have the same effect (data not shown). The extracts of hexane and ethyl acetate also

![Fig. 1.](image-url)

*Fig. 1.* An ethanol extract of mulberry leaves extends lifespan in *C. elegans*. Nematodes were placed on fresh plates on the day of their fourth larval stage molt (day 0) and fed with ethanol extract (2 mg/mL) or ethosuximide (2 mg/mL). Survival was scored every day. Survival rate is expressed as a percentage of 50 to 60 nematodes in 3 to 4 independent experiments in each treatment group.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Feed</th>
<th>Treatment</th>
<th>Mean lifespan ± SEM (days)</th>
<th>% Change</th>
<th>Maximum lifespan ± SEM (days)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>OP50</td>
<td>None</td>
<td>17.6 ± 1.6</td>
<td></td>
<td>24.9 ± 2.1</td>
<td>172 (4)</td>
</tr>
<tr>
<td>N2</td>
<td>OP50</td>
<td>Ethosuximide (2.0 mg/ml)</td>
<td>23.8 ± 1.1***</td>
<td>+35</td>
<td>32.0 ± 2.3***</td>
<td>142 (3)</td>
</tr>
<tr>
<td>N2</td>
<td>OP50</td>
<td>Ethanol extract of mulberry leaves (2.0 mg/ml)</td>
<td>20.6 ± 1.1***</td>
<td>+17</td>
<td>28.8 ± 2.3***</td>
<td>140 (3)</td>
</tr>
</tbody>
</table>

Significant lifespan extensions are denoted by *** *P* < 0.0001 and ** *P* < 0.01 (Kaplan-Meier analysis).

*N*: total number of worms. Parentheses: number of independent experiments.

See Fig. 1 for other details.
remained to be analyzed. However, as the absolute ethanol extract showed higher activity than the aqueous acetone extract, and the hot water extract had mucilaginous properties, we further investigated only the absolute ethanol extract for plant foods.

Adult wild-type nematodes grown under our standard laboratory conditions at 20°C had a mean lifespan of 17.6 days and average maximum lifespan of 24.9 days. On media containing either ethosuximide (2 mg/mL) as a positive control (Evason et al., 2005) or an ethanol extract of mulberry leaves (2 mg/mL), the mean lifespan was lengthened: to 23.8 or 20.6 days, respectively (P < 0.001, Fig. 1, Table 1). The maximum lifespan was also extended (P < 0.001 or P < 0.01, respectively).

As-yet unidentified chemicals present in the ethanol extract may protect against aging. Further studies of the effects of such chemicals in combination with genetic modifications will reveal the molecular mechanisms of the aging process, as reported already in relation to fruit polyphenols (Wilson et al., 2006) and trehalose (Honda et al., 2010).

Trehalose treatment of old adults is more effective in extending the lifespan than in young adults and lengthens the reproductive span (Honda et al., 2010). Resveratrol, a natural polyphenolic compound, also increases lifespan, not only in C. elegans (Parker et al., 2005), but also in vertebrates (Valenzano et al., 2006). The pharmacological effects of these compounds in food could be important in delaying senescence via the insulin-like signaling pathway or sirtuin-1-dependent induction (Kenyon, 2005; Lagouge et al., 2006). As-yet unidentified chemicals present in the ethanol extract may protect against aging. Further studies of the effects of such chemicals in combination with genetic modifications will reveal the molecular mechanisms of the aging process, as reported already in relation to fruit polyphenols (Wilson et al., 2006) and trehalose (Honda et al., 2010).

We did not examine dose-dependent effects of the ethanol extract on lifespan or on egg laying, locomotion, chemotaxis, stress response, or associative learning, which are well understood in C. elegans (Brown et al., 2006). Ethosuximide had the greatest effect, extending mean adult lifespan by 35% from 17.6 to 28.8 days at 2 mg/mL (Table 1). Our preliminary data, however, show that a major fraction of the ethanol extract separated by flash column chromatography dose-dependently had the specific activity by up to 10-fold at 0.20 mg/mL (data not shown). To our knowledge, this report is the first to demonstrate that a mulberry leaf extract increases the lifespan of C. elegans. Our finding may help to uncover the molecular mechanisms involved and the bioactive compounds in mulberry leaves.

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REFERENCES


