α-Mangostin Promotes DAF-16-Mediated Thermotolerance in *Caenorhabditis elegans*

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Abstract

Garcinia mangostana, commonly known as mangosteen, is a tropical fruit with a reddish-purple pericarp. In Southeast Asia, the pericarp has tradition-ally been used as a medicine to treat various diseases, including inflammation, wounds, and bacterial infections, as well as aging. *α*-mangostin is an abundant xanthone in the pericarp, and is thought to play a critical role in the medicinal effects of mangosteens. Previous studies have demonstrated numerous benefi-cial effects of *α*-mangostin, such as cytotoxicity in cancer cells. However, the effects of this xanthone in *in vivo* have not yet been studied. In the current study, *C. elegans* was used to test the *in vivo* effects of *α*-mangostin using sev-eral bioassays, including fat accumulation, pharyngeal movement (pumping) and heat-stress assays. Quantitative real time PCR (qRT-PCR) was also used to examine the expression of heat shock proteins. The results revealed that *α*-mangostin appeared to cause an increase in fat accumulation, which corre-lated with an increase in pharyngeal movement. The thrashing movement of the worms after heat stress also showed a correlation with an increase in heat shock protein mRNA expression.

Keywords

Mangosteen, *α*-Mangostin, Thermotolerance, *Caenorhabditis elegans*, DAF-16

1. Introduction

Garcinia mangostana Linn., commonly known as mangosteen, belongs to the Guttiferae family and is known as "the queen of fruits". It is native to and most cultivated in tropical areas, particularly in Southeast Asian countries such as In-donesia, Malaysia, The Philippines, and Thailand. In these countries, mangos-teens, especially the pericarp, have traditionally been used as a medicine for the treatment of diseases such as abdominal pain, urinary tract infections, and in-

fected wounds and suppuration, and is thought to have many other pharmaceutical effects [1]. Moreover, experimental studies have demonstrated that mangosteen pericarp extract comprises various xanthones, of which *α*-mangostin is the most abundant [2] [3]. Experimental studies have confirmed the therapeutic properties of *α*-mangostin, such as its antioxidant [4] [5], antitumoral [6], and anti-inflammatory [7] effects. However, although mangosteens are widely used to treat many health conditions, there is little scientific evidence to support their effectiveness in humans. At this time, *α*-mangostin has been extensively studied using *in vitro* models, such as cell culture, but *in vivo* studies using real animals have not been reported [8]. Therefore, the aim of the present study was to examine the physiological effects of *α*-mangostin using *in vivo* experiments.

Caenorhabditis elegans is a nematode that is used extensively in biological research. The worms are easy to cultivate due to their short lifespan and can be maintained under laboratory conditions. The genes that control *C. elegans* development have been conserved, resulting in similarities in form, function, and genetics to those of humans [9]. Moreover, the genome has also been well studied and a variety of mutants have been identified, therefore it is widely used to study genetic pathways. The *C. elegans* nervous system performs many of the same functions as that of higher organisms, and is therefore often studied to help understand the basic mechanisms that underlie more complex behaviors [10].

Despite the many advantages of using *C. elegans* as a model organism, the physiological effects of *α*-mangostin in this organism have not been extensively studied, and knowledge regarding the effectiveness of *α*-mangostin is scarce. Therefore, the present study sought to investigate the physiological effects of *α*-mangostin on *C. elegans,* as well as the pathways and genes that comprise the underlying mechanisms.

The results of our study revealed that after treatment with *α*-mangostin, *C. elegans* showed an increase in lipid accumulation within their bodies, and an increase in pharyngeal and thrashing movements. These effects were dose-dependent. Moreover, *daf*-16-defective organisms did not recover from these thrashing movements after treatment at 35˚C under any conditions, whereas *daf*-2-defective organisms showed a higher recovery rate compared to wild-type. This suggests that *daf*-16 is necessary for thermoregulation, which is consistent with previous studies [11] [12]. Furthermore, quantitative real time PCR showed a dose-dependent increase in the expression of genes related to heat stress resistance, such as *sod*-3 and *hsp*-12.6.

2. Materials and Methods

2.1. Model Organism

Wild-type *C. elegans* Bristol N2, daf-2 (e1370), and daf-16 (mgDf50) were provided by the Caenorhabditis Genetics Center (CGC, MN, USA). The worms were fed live *E. coli* (OP50 strain) on Nematode Growth Medium (NGM) plates and cultivated at 20˚C.

2.2. Age-Synchronization and Materials

After 5 - 7 days in culture, adult worms were treated with hypochlorous acid (20% NaClO solution) (NaClO: Haiter, KAO, Tokyo, Japan; NaOH: WAKO, Osaka, Japan) to extract the eggs from the body. The extracted eggs were cultivated in S-basal medium (0.1 M NaCl) for 18 hours to the larval hatching (L1) stage, before being transferred onto sample plates.

To prepare the sample plates, *α*-mangostin powder (WAKO, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO) and OP50 to generate the experimental concentrations (0, 0.1, 1, and 10 μg/mL), then 200 mL of each mixture was spread onto NGM plates. Larvae were cultivated for 96 hours before performing biological assays.

2.3. Fat Accumulation Assay

After 96 hours of cultivation, adult worms were dyed with Nile red (WAKO, Osaka, Japan), then paralyzed using 8% ethanol in S-basal medium. Observations were performed under a fluorescence microscope and the intensity was measured using ImageJ software (National Institutes of Health, USA, Maryland).

2.4. Pharyngeal Movement Assay

After 96 hours of cultivation, pharyngeal movement was observed for 15 seconds for each worm using a light microscope.

2.5. Heat Stress Assay (Thrashing Movement after Heat Stress)

After 96 hours of cultivation, adult worms were transferred to NGM plates without OP50 and incubated at 35˚C. After 4 hours, the movement of the worms under water (thrashing movement) was measured at 0, 12, and 24 hours (15 seconds per worm).

For daf-2, the thrashing movement was measured at 0, 3, and 6 hours, due to their high recovery rate.

2.6. Quantitative Polymerase Chain Reaction (qRT-PCR)

Age-synchronized L1 larvae were transferred onto sample plates and cultured for 4 days at 20˚C. RNA was purified from whole-cell extracts using RNAiso PLUS (Takara, Shiga, Japan). cDNA was synthesized using a PrimeScript® RT reagent kit with gDNA Eraser (Perfect Real Time; Takara). Amplification was performed using a Thermal Cycler Dice® Real Time System Lite (Takara) (cycling conditions: 95˚C/30 s, (95˚C/5 s, 60˚C/30 s) × 50 cycles) and Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). Actin was used as an internal control.

2.7. Statistical Analysis

Statistical significance was determined using a two-tailed t-test with the analysis software SPSS (IBM, NY, USA). Statistical differences are represented by *p ≤

0.05 and **p ≤ 0.001.

3. Results

3.1. *C. elegans* Accumulates More Lipid at High α-Mangostin Concentrations

Previous studies have shown that α-mangostin helps to reduce lipid accumulation in an *in vitro* system using an adipocyte cell line (Quan, 2012). Here, we investigated whether α-mangostin could decrease lipid content *in vivo* using *C. elegans* as a model organism. The results show that α-mangostin induced the lipid accumulation dose-dependently (Figure 1(a)).

3.2. *C. elegans* Appears to Consume More When Exposed to High α-Mangostin Concentrations

Since our results from the *in vivo* system did not agree with previous *in vitro* studies, we conducted a pumping or pharyngeal movement assay to examine the consumption rate of the worms. The results show that the worms tended to consume more when treated with α-mangostin compared to control (Figure 1(b)).

3.3. α-Mangostin Increases Heat Stress Tolerance through the daf-2 Pathway, Which Is Dependent on DAF-16

To observe the physiological effects of α-mangostin, we performed a heat stress assay to determine the recovery rate of the worms' movement after exposure to 35˚C heat stress for 4 hours. We also conducted a heat stress assay on mutant animals to identify the genes that contribute to the increase in thrashing movement
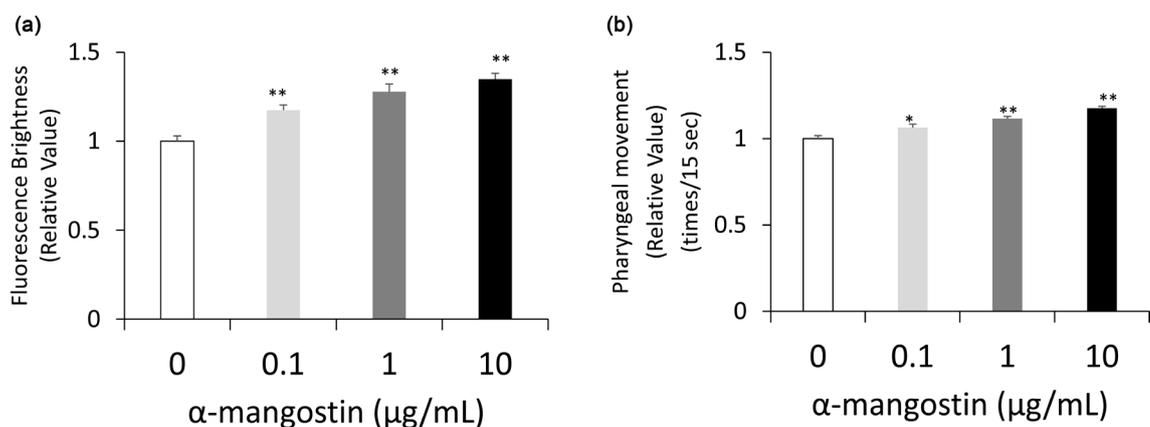
Figure 1. (a) Brightness of fluorescence of worms treated with α-mangostin. Age synchronized L1 larvae were cultured on α-mangostin plates (OP50 plus 0, 0.1, 1, or 10 μg/mL α-mangostin) at 20˚C for 96 hours. Adult worms were dyed with Nile red for 30 minutes and observed under a fluorescence microscope (n ≥ 20). (b) Pharyngeal movement of worms treated with α-mangostin. Age-synchronized L1 larvae were cultured on NGM plates with OP50 and 0, 0.1, 1, or 10 μg/mL α-mangostin at 20˚C for 96 hours. Adult worms were observed under a light microscope (n = 10) (times/15 sec). Mean ± SEM, *p ≤ 0.05, **p ≤ 0.001. Statistical significance was determined using a paired t-test.

speed after *α*-mangostin treatment. N2 animals showed an increase in thrashing movement speed in response to *α*-mangostin in a dose-dependent manner, after both 12 and 24 hours, although these changes were not significantly different (Figure 2). Mutant animals were used to investigate genes that may mediate the effects of *α*-mangostin. After exposure to 35˚C heat stress for 4 hours, we measured the thrashing movement of the animals at either 0, 6, and 12 hours, or 0, 3, and 6 hours for *daf*-16 and *daf*-2 mutant animals respectively. We found that *daf*-16 animals failed to recover at any of the timepoints (Figure 3), whereas *daf*-2 mutant animals showed an increased movement speed, which was dependent on the *α*-mangostin concentration (Figure 3). Furthermore, the movement speeds of *daf*-2 animals were higher than those of the N2 animals.
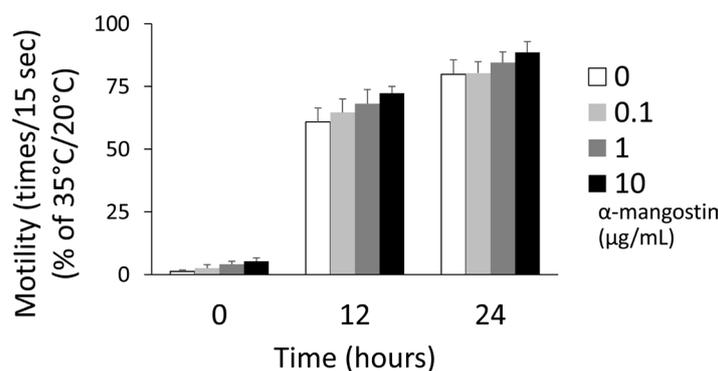
Figure 2. Thrashing movement of worms treated with *α*-mangostin after exposure to thermal stress (35˚C) for 4 hours. Age-synchronized L1 larvae were cultured on *α*-mangostin plates (OP50 and *α*-mangostin) at 20˚C for 96 hours. Adult worms were transferred to NGM plates (without food) and heated for 4 hours before being transferred back to *α*-mangostin plates. Thrashing movement was examined at 0, 12, and 24 hours (n = 10) (times/15 sec). *While this figure shows an increasing trend of trashing movement, there were no significant differences between experimental and control groups (p > 0.05).
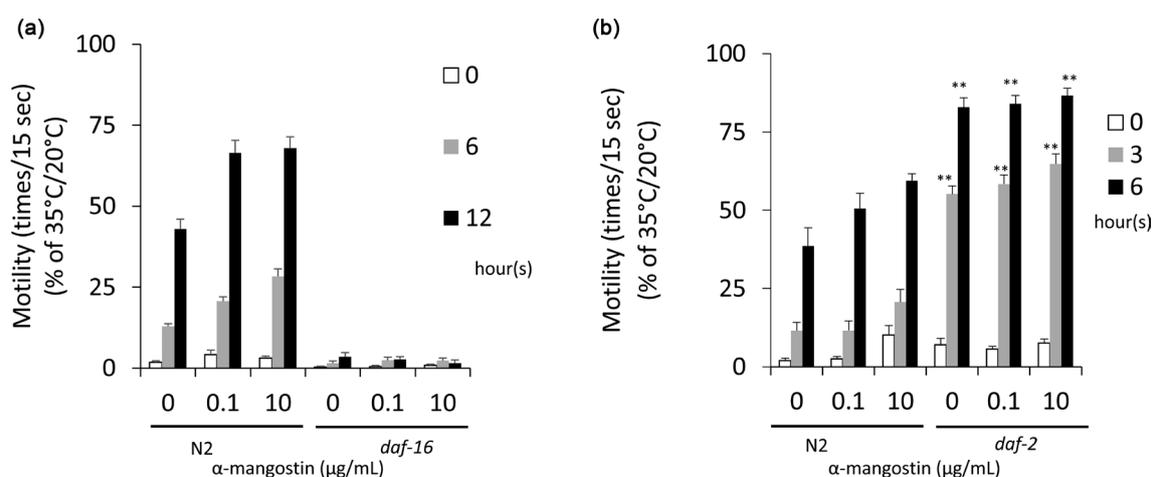
Figure 3. Thrashing movement of *daf*-16 and *daf*-2 mutants worms (N2) treated with *α*-mangostin after exposed to thermal stress (35˚C) for 4 hours. Age-synchronized L1 larvae were cultured on *α*-mangostin plates (OP50 and *α*-mangostin) at 20˚C for 96 hours. Adult worms were transferred to NGM plates (without food) and heated for 4 hours before being transferred back to *α*-mangostin plates. Thrashing movement of *daf*-16 and *daf*-2 mutants was examined at either 0, 6, and 12 hours or 0, 3, and 6 hours, respectively (n = 10) (times/15 sec). Mean ± SEM, *p ≤ 0.05, **p ≤ 0.001. Statistical significance was determined using a paired t-test.

3.4. *α*-Mangostin Increases the Expression of Genes Regulated by DAF-16

The results of the heat stress tolerance assay suggest that *α*-mangostin promotes thermotolerance through *daf*-16 (Figure 3). Therefore, we used quantitative real time PCR to examine *sod*-3 and *hsp*-12.6 expression, which are known to be downregulated by DAF-16. mRNA expression levels of both *sod*-3 and *hsp*-12.6 were increased according to *α*-mangostin concentration (Figure 4). However, at high concentrations (10 μg/mL), there was a slight decrease in mRNA expression of both *sod*-3 and *hsp*-12.6. We hypothesized that *α*-mangostin promotes thermotolerance in *C. elegans* through DAF-16 via downregulation of *sod*-3 and *hsp*-12.6.

We also analyzed mRNA expression of another important thermotolerance gene in *C. elegans*, HSF-1, which is a target of *hsp*-70 and *hsp*-16.2. Interestingly, *α*-mangostin had no effect on this gene (data not shown). We conclude that *α*-mangostin increases thermotolerance in *C. elegans* via DAF-16 but not HSF-1.

4. Discussion

Mangosteens are thought to have a number of health benefits [1]-[6]. In addition to general consumption, this fruit, particularly the pericarp, is widely used as a traditional remedy, particularly in Southeast Asian nations. Previous studies revealed that the major component of the pericarp is a xanthone called *α*-mangostin [3] [4]. Although *α*-mangostin has been extensively studied *in vitro*, *in vivo* experiments to date are insufficient. Therefore, the present study aimed to investigate the effects of *α*-mangostin using *C. elegans* as a model organism.

First, we performed a fat accumulation assay to examine the effect of *α*-mangostin on obesity. Our results revealed that fat accumulated inside the body of the worms, and increased with the concentration of *α*-mangostin. These findings contradict those from previous studies, both *in vitro* and *in vivo* [13] [14]. Therefore, we conducted a pharyngeal movement assay to examine the consumption rate of
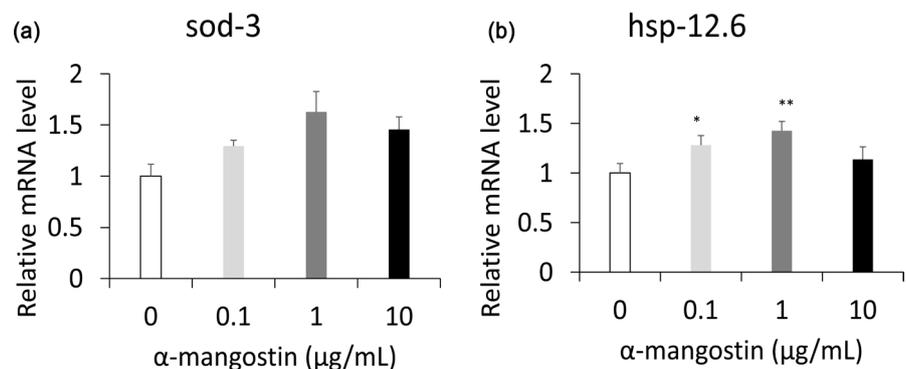
Figure 4. qRT-PCR analysis of mRNA expression in worms treated with *α*-mangostin. Age-synchronized L1 larvae were cultured on *α*-mangostin plates (OP50 and *α*-mangostin) at 20°C for 96 hours. cDNA was synthesized following RNA extraction from adult worms. mRNA expression was analyzed using quantitative real time PCR. Mean ± SEM, *p ≤ 0.05, *p ≤ 0.001. Statistical significance was determined using a paired t-test.

the worms. We found that the consumption rate increased dose-dependently with *α*-mangostin concentration, which could explain the increased lipid content in the animals. Unlike other *in vivo* studies, we did not restrict the amount of food that the experimental animals were allowed to consume. Therefore, the increase in consumption could be due to properties of *α*-mangostin, such as flavor, that are attractive to *C. elegans*, causing them to consume more, thus increasing their lipid content.

Our results show that *α*-mangostin increases the thrashing movement speed of N2 animals in a dose-dependent manner, suggesting that these animals have a higher thermotolerance at higher doses of *α*-mangostin. This confirms the analgesic effects of *α*-mangostin, which has been shown to extend the latency period of the hot-plate paw-licking response in mice [15] [16]. Therefore, we propose that a similar response could exist in *C. elegans*.

daf-16-depleted worms did not recover from the heat shock at any of the timepoints analyzed, implying that this gene is necessary for thermoregulation, which is consistent with previous studies [11] [12] [17]. Moreover, our results showed that *daf*-2 depleted worms had a higher thrashing movement speed compared to wild-type animals, suggesting that *α*-mangostin is more effective when the *daf*-2 gene is absent. Thus, it is possible that *α*-mangostin increases thermotolerance via the *daf*-2 pathway, and is dependent on DAF-16, which is known to play an important role in stress and lifespan extension [18].

We used quantitative PCR to analyze expression of the thermoregulating genes, *sod*-3 and *hsp*-12.6, *hsp*-70, and *hsp*-16.2, which are known to be downregulated by DAF-16 and HSF-1 [19] [20]. Our analysis revealed that *sod*-3 and *hsp*-12.6 expression increased with *α*-mangostin dose, but showed a slight decrease at higher concentrations ([Figure 4](#)), whereas *hsp*-70 and *hsp*-16.2 expression was not affected by *α*-mangostin (data not shown). Thus, our study concluded that *α*-mangostin increases thermotolerance in the animals via DAF-16 but not HSF-1. However, according to previous studies on the toxicity of the xanthone, high concentrations of *α*-mangostin can lead to cell death in *in vivo* [21] [22] and is thought to be toxic to living matter [23]. Toxicity could, therefore be the reason for the slight decrease in *sod*-3 and *hsp*-12.6 mRNA expression in high concentration (10 μg/mL). Additionally, we found no difference in the lifespan of N2 worms after treatment with 0, 0.1, or 1 μg/mL *α*-mangostin, while 10 μg/mL caused a significant decrease in lifespan, supporting the notion that high concentrations of *α*-mangostin are potentially toxic to *C. elegans* (data not shown).

Furthermore, due to the lack of materials such as primers, our laboratory could not conduct an experiment to examine other thermoregulating genes. Therefore, investigating other gene expression of additional thermoregulating genes could be a next challenge for this the subject of future research. In addition, examining whether the action of *α*-mangostin is passive (do animals consume this xanthone directly), or active (do bacteria convert this xanthone into

other metabolites) could be important for understanding how to effectively utilize α-mangostin as a treatment in human as well [24] [25].

Acknowledgements

Conflict of Interest Statement

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

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