Effects of Fisetin on Fat Accumulation in *Caenorhabditis elegans*

An Honors Thesis

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1. **INTRODUCTION**

1.1 Obesity

Obesity, as characterized by The World Health Organization (WHO) considers a person with a BMI of ≥ 30 kg/m^2 as medically obese ("WHO | Obesity"). The prevalence of obesity was 39.8% and affected about 93.3million of US adults in 2015~2016 ("WHO | Obesity"). Obesity related conditions include heart disease, stroke, type 2 diabetes and certain types of cancer that are some of the leading causes of preventable, premature death (Must 1999). Obesity and being overweight are associated with an increased incidence of hypertension, stroke, dyslipidemia, osteoarthritis, and some cancers (Must 1999). It has also been suspected that obesity may be involved in causing a chronic and systemic inflammatory response that triggers metabolic disorders such as type 2 diabetes and cardiovascular disease (Hotamisligil 2006).

In addition to its serious health consequences, obesity has real economic costs that affect all of us. In 2017, the estimated annual health care costs of obesity-related illness are $190.2 billion which is 21% of annual medical spending in the United States (Tremmel 2017). In addition to growing health care costs attributed to obesity, the nation will incur higher costs for disability and unemployment benefits. Businesses in the US are losing $4.3 billion annually due to obesity-related job absentee (Tremmel 2017). As of 2020, no decreases in obesity have been reported, making obesity and overweight a still a highly relevant issue in the United States.

1.2 Properties of Fisetin

Fisetin, 2-(3,4-dihydroxyphenyl)-3,7-dihydroxy-4H-1-benzopyran-4-one, is a 7-hydroxyflavonol with additional hydroxyl groups at positions 3, 3' and 4 (Jung et al. 2013) (Fig. 1). It has a molecular weight of 286.2 and molecular formula of $C_{15}H_{10}O_6$ and a plant polyphenol that belongs to the flavonoid group (Fiorani & Accorsi 2005).

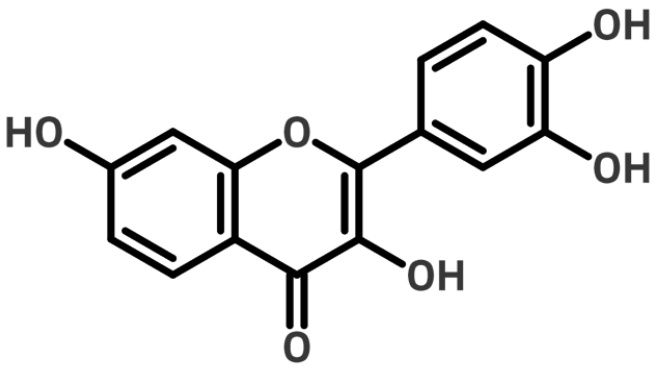

Figure 1: Chemical structure of fisetin

Fisetin is found in many plants, including Eudicotyledons, the honey locust, Quebracho Colorado, and species of the genus Rhus (Fiorani & Accorsi 2005). It can also be found in various fruits and vegetables, such as strawberries, apples, persimmons, grapes, onions, and cucumber at concentrations in the range of 2–160 μg/g (Khan et al. 2013). The relatively high concentrations of fisetin was found in strawberries (160 μg/g), in apple (26.9 μg/g), and persimmon (10.5 μg/g) (Khan et al. 2013).

Effects of fisetin against various diseases have previously been reported, including anticarcinogenic, anti-inflammatory, and neuron generation (Khan et al. 2013). Anti-cancer activity shows that fisetin blocks the phosphoinositide-3-kinase/ protein kinase B/ mammalian target of rapamycin pathway (PI3K/PKB/mTOR pathway) (Syed et al. 2013). In other studies, fisetin acts as an antiproliferative agent, interfering with the cell cycle in several ways (Gupta et al. 2014). In the US, fisetin is marketed as an dietary supplements in 100mg capsules or along with other natural products with health benefits of promoting cognition, brain health maintaining, and supporting healthy aging (Maher 2015).

1.3 Use of *Caenorhabditis elegans* as a Model Organism

The nematode *Caenorhabditis elegans* has been a significant experimental organism for almost 50 years in scientific research. The animals were first used as a research organism by Sydney Brenner over 40 years ago for studies of development and behavior (Marsh & May 2012). His work resulted in the Nobel Prize in Physiology or Medicine being awarded in 2002 (Marsh & May 2012). This nematode is a good candidate for scientific analysis, partly because of its rapid (3-day) life cycle, small size, and ease of laboratory cultivation (Riddle 1997). At adulthood, they are approximately 1 mm in length with a lifespan of around 21 days, reaching adulthood only 38 – 46 hours after hatching at 20°C (Marsh & May 2012).

The wild-type N2 strain has a growth and generational time of 3 days with broods of 300 per hermaphrodite and can be grown on agar plates using a non-pathogenic strain of OP50 *Escherichia coli* as a food source (Marsh & May 2012). Additionally, being transparent throughout its life allows scientists to follow the behavior of individual cells through their development (Marsh & May 2012).

The complete genome sequence of *C. elegans* was published in 1998. Since then, it has been discovered that the *C. elegans* genome is 100 million base pairs long (Marsh & May 2012). This is comparable to the number of human genes, which is about 20,500 genes (Marsh & May 2012). Although *C. elegans* is a relatively simple organism, a lot of the molecular signals responsible for its development can also be seen in organisms that are more complex, such as humans (Marsh & May 2012). The many mutants of *C. elegans* provide valuable models for many human diseases, including neurological disorders, congenital heart disease and kidney disease and can be used to screen potential drugs for significant diseases (Marsh & May 2012).

1.4 Use of *Caenorhabditis elegans* as a model for obesity research

C. elegans stores fat mainly within the hypodermal and intestinal cells (Zheng and Greenway 2012). To visualize these fat stores, lipid affinity dyes, such as Nile Red, Sudan black and Oil Red O, can be used to quantify using their transparent body by measuring the intensity of the accumulated dye (Zheng and Greenway 2012). Using these visualizing techniques, it is a relatively simple to identify compounds that modulate fat storage. Many core metabolic pathways found in mammals are well conserved in *C. elegans* (Mullaney and Ashrafi 2009). These include pathways for lipogenesis, elongation and desaturation, mitochondrial and peroxisomal β-oxidation of fatty acids, glycolysis, gluconeogenesis and amino acid metabolism (Mullaney and Ashrafi 2009). A part of several pathways responsible for regulating mammalian metabolism has also been identified to have highly conserved functions in *C. elegans* (Mullaney and Ashrafi 2009). Specifically, these include the fat and sugar transporters, nuclear hormone receptor and SREBP (sterol response element binding protein) transcriptional regulators, AMP-activated kinase (AMPK), and the target of rapamycin (TOR) kinase, as well as insulin and serotonin (Mullaney and Ashrafi 2009). Nonetheless, *C. elegans* lack certain key mammalian fat regulatory mechanisms, such as leptin, a hormone from mammalian adipocytes (Mullaney and Ashrafi 2009). However, the relative simplicity and efficiency of *C. elegans* make it an excellent transition from *in vitro* methods into higher animal models, further into humans in a more efficient and much less expensive manner (Marsh & May 2012).

From previous literature, *C. elegans* has shown to be successfully used in investigating the fat reduction qualities of a number of compounds. The treatment of green coffee bean extract was found to be reduced triglycerides level in *C. elegans* by 29% and 23% dependent on *sbp-1* (ortholog of mammalian sterol-regulatory-element-binding protein) and *daf-16* (ortholog of

mammalian Forkhead box O transcription factor) (Farias-Pereira et al. 2018). Furthermore, piceatannol at 50 and 100 μM significantly reduced fat accumulation of wild-type worms grown in normal and high-glucose conditions without affecting its pumping rates or locomotive activities (Shen et al. 2017). Cranberry water extract at 0.016% and 0.08% resulted in a dose-dependent reduction of fat accumulation by 43% and 74%, respectively, without affecting its pumping rates or locomotive activities (Sun et al. 2016). Since the cranberry water extract contained flavonoids, phenolic acids, and anthocyanidins as its active components, this finding is especially promising as fisetin is also a member of the flavonoid class of chemical compounds (Sun et al. 2016).

1.5 Research Study Indicates Fisetin's Obesity Regulatory Properties

A previous study reported whether fisetin inhibits mammalian target of rapamycin complex 1 (mTOR1) activity and assessed mTORC1 as a possible target for fat reduction (Jung et al. 2013). As a master growth regulator, mTORC1 is stimulated by nutrients and growth factors, and the evidence points to the notion that mTORC1 functions in the regulation of obesity (Jung et al. 2013). To evaluate whether fisetin regulates mTORC1 signaling, *in vitro* research was done to investigate the phosphorylation and kinase activity of the 70-kDa ribosomal protein S6 kinase 1 (S6K1) and mTORC1 in 3T3-L1 preadipocytes (Jung et al. 2013). They found that fisetin treatment of preadipocytes reduced the phosphorylation of S6K1 and mTORC1 in a time- and concentration-dependent manner (Jung et al. 2013). Their results suggest that inhibition of mTORC1 signaling by fisetin prevents adipocyte differentiation of 3T3-L1 preadipocytes (Jung et al. 2013). In an *in vivo* experiment, they found that mice fed a high-fat diet (HFD) supplemented with fisetin was able to reduce the HFD-induced increases in body weight (Jung et al. 2013). As a whole, their research indicates that fisetin inhibits adipocyte differentiation of

3T3-L1 preadipocytes and weight gain in HFD-fed mice. This therefore suggests that fisetin may be a useful phytochemical agent for attenuating diet-induced obesity (Jung et al. 2013).

2. METHODS

All procedures and techniques for raising, treating, and testing of the nematodes are as detailed in the study of cranberry product on fat accumulation in *C. elegans* published previously by Dr. Park's research group (Sun et al., 2016).

2.1 Materials and Resources

Fisetin (purity >96.0%) was obtained from Fisher Scientific (Logan, UT). N2 and mutant strains of *C. elegans* along with *Escherichia coli* OP50 were obtained from the *Caenorhabditis* Genetics Center in Minneapolis at the University of Minnesota. Triglyceride and protein quantity were determined using Thermo Scientific and Bio-Rad Co. kits, respectively. Fluorodeoxyuridine (FUDR) and carbenicillin were obtained from Sigma-Aldrich Co. and all other chemicals were purchased from Fisher Scientific unless stated otherwise. Household bleach (the Clorox Company, Oakland, CA) was used during bleaching of the eggs.

2.2 *C. elegans* Culture

The *C. elegans* strains used in this study were N2, Bristol (wild type) with all procedures for synchronization and triglyceride and protein measurements being the same for all strains. M9 buffer, S-complete used for *C. elegans* culture was prepared as described in Solis & Petrascheck 2011. A synchronous worm culture was obtained according to standard protocol (Solis & Petrascheck 2011). Treatments started with synchronized L1 worms in 12-well plates at 20°C. Treatments of fisetin was given at 100 and 200μM or control. After 48h of treatment, worms were collected for triglyceride and protein quantification.

2.3 Triglycerides and protein assay

Worms were collected and washed twice using 1 mL of double distilled water. Worms were collected and washed with M9 buffer to remove bacteria and S-complete media. *C. elegans* samples in 100 µL 0.05% Tween 20 solution were sonicated for 3 minutes (Sonicator 505, Fisher Scientific). After sonication, triglyceride and protein were quantified from these samples. The triglyceride assay was conducted with a commercial assay kit (Infinity Triglyceride Reagent; Thermo Scientific) and the protein content was measured with the Bio-Rad DC protein assay kit. Triglyceride contents for each sample was normalized with protein concentration.

2.4 Pharyngeal pumping rate and locomotion assay

Pumping rate, worm size, and locomotive behavior were measured after 48 hours of treatment. Food intake was measured by counting the number of pharyngeal contractions of 12 randomly selected nematodes and counted under the optic microscope (Olympus Corporation, Tokyo, Japan). Body length, body width, and movement speed were analyzed using the WormLab tracking system (MBF Bioscience, Williston, VT) as previously described (Shen et al., 2016). Locomotion behavior was analyzed by using the Wormlab tracking system (Allied Vision Technologies, Stadtroda, Germany, and Wormlab Software; MBF Bioscience, Williston, VT, USA). Ten recordings of 1 minute each (7 frames/second) were captured, and WormLab software was then used to analyze the body length, body width, and movement speed of over 50 nematodes per treatment group.

2.5 Statistical analysis

Data was expressed as means with standard errors and analyzed with the Statistical Analysis System (SAS Institute, Cary, NC, USA). One-way ANOVA and multiple comparisons

were performed using Prism7 from GraphPad. Statistically significant differences were accepted at $p<0.05$.

3. RESULTS

3.1 Fisetin's Effects on N2 *C. elegans*

Treatment of 100 and 200 µM fisetin resulted in significant reductions in fat accumulation of 19% and 28% of the control, respectively (Figure 2). To determine whether this reduction was due to effects of the compound on food intake, pumping rates as measured by pharyngeal contractions observation. Treatment of 200 µM fisetin resulted in a 22% increase of pumping rates compared to the control (Figure 3).

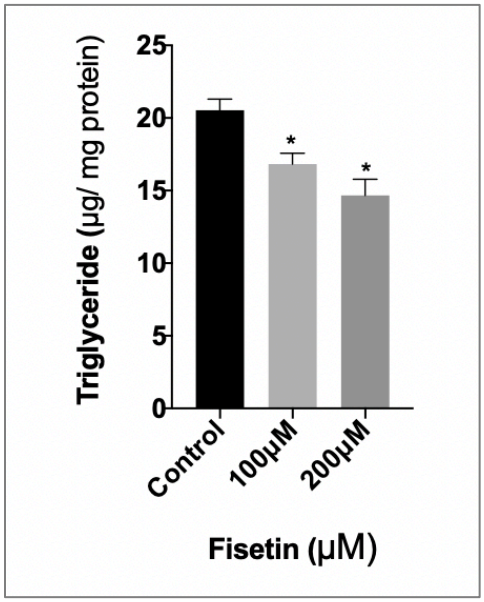

Figure 2: Effects of fisetin on fat accumulation in *C. elegans*. Numbers are mean ± S.E. (n = 4). * represents significant difference compared to the control at $p < 0.05$.

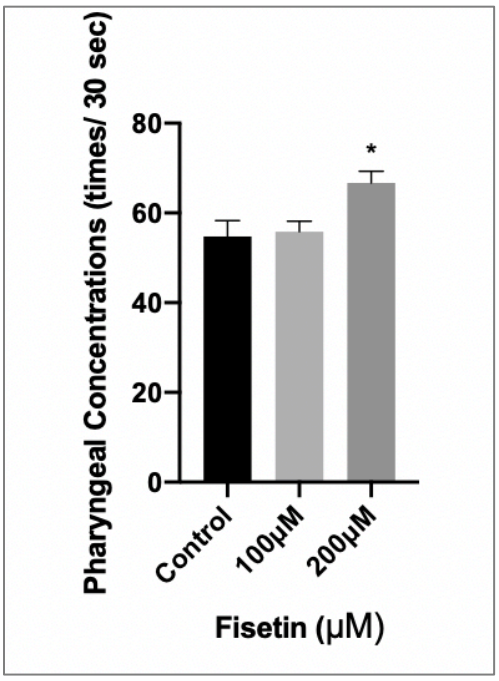

Figure 3: Effects of fisetin on pumping rates. Numbers represent mean values ± S.E. (n = 12). * represents significant difference compared to the control at $p < 0.05$.

Various other physiological effects of fisetin were tested by tracking *C. elegans* movement and measuring body length and width. No significant changes by fisetin in body length occurred (Figure 4). Average body width decreased by 200 μM fisetin by 5% compared to the control. A significant difference between 100 and 200 μM fisetin treatments on body width was also observed at $p < 0.05$. No significant changes in movement speed were observed between treatments.

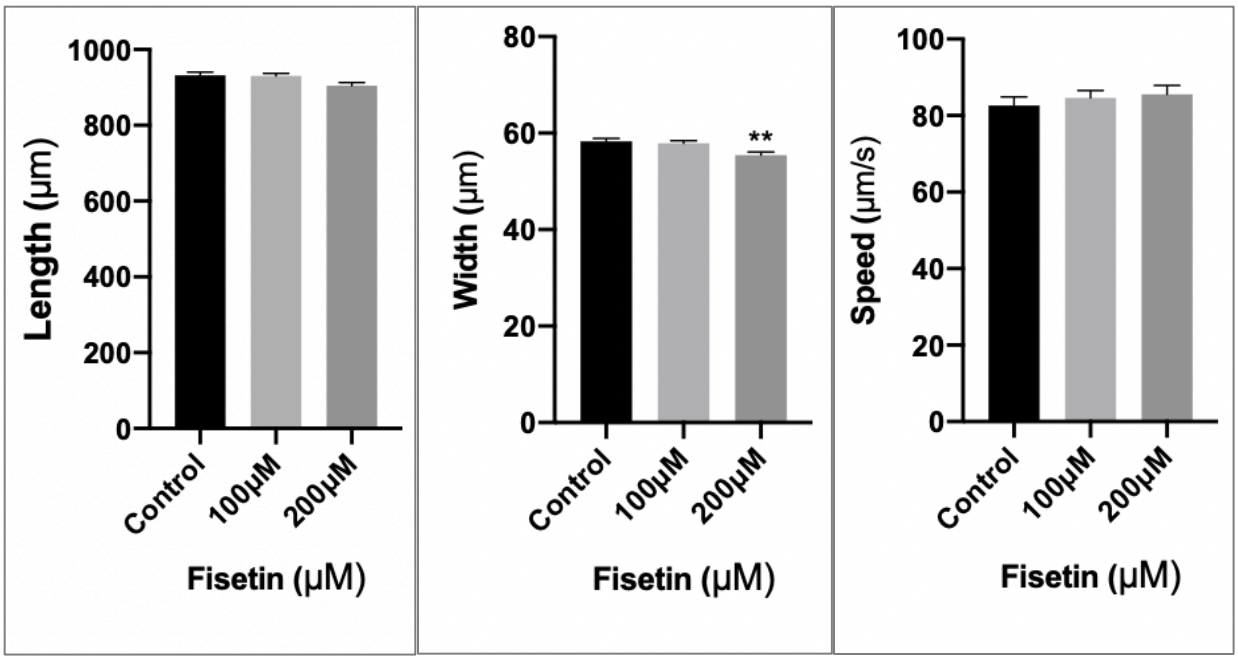

Figure 4: Effects of fisetin on body length, width and speed. Numbers represent mean values ± S.E. (n > 50). ** represents significant difference compared to the control at $p < 0.05$.

4. DISCUSSION

In this study, we observed that the food derived bioactive fisetin dose-dependently reduced triglyceride content in wild-type *C. elegans*. The current results are consistent to the previous *in vitro* studies on lipid accumulation with fisetin (Jung 2013). Fisetin treatment of 3T3-L1 preadipocytes had previously been found to inhibit mTORC1, reducing the levels of adipogenesis concentration-dependent manner (Jung et al., 2013). The main site of fat storage in *C. elegans* is in the intestinal cells. Even though *C. elegans* intestinal cells are multipurpose compared to the more dedicated mammalian adipocytes, the genetic markers are still highly conserved (Marsh & May 2012).

As a means for measuring food intake, pumping rates are often used in *C. elegans* (Zheng and Greenway 2012). Pharyngeal contractions were increased at 200 μM fisetin treatment in the current study. As this indicates increased food intake, this contradicts the observation that fisetin decreased triglyceride levels. From this result, we infer that fisetin decreased body fat independent to food intake, rather elicits its effect by other mechanisms. There have been researches that have observed similar contradictory effects as seen in the present study. Previously, *daf-7* TGF-B mutants decreased pharyngeal pumping even though there was an increase in fat stores (Watts 2009). Similarly, exogenous serotonin increased pharyngeal pumping rates, while decreasing fat stores (Lemieux and Ashrafi 2016). Thus, pumping rate alone may not be a good indicator to link with total body fat accumulation.

Fisetin at 200 μM reduced the width, likely due to reduced fat accumulation, without altering worm length. This reduction in body width were as expected due to the significant fat reduction in wild type *C. elegans*. This has been consistently observed by others in Shen et al. 2017, Farias-Pereira et al. (2020), and Liu et al. (2018). There were no significant changes in

movement speeds between each treatment group. This indicates that while fisetin may attenuate fat accumulation, there is no significant effect on the movement speed of these nematodes.

In conclusion, the current study concludes that fisetin (100 and 200 μM) can reduce fat accumulation *C. elegans*. The underlying mechanism of fisetin on fat reduction still needs to be determined in future. Given that fisetin exerts similar effects in both *C. elegans* and 3T3-L1 adipocytes, *C. elegans* can be a useful *in vivo* model to investigate any bioactives that may target lipid metabolism.

5. FUTURE DIRECTIONS

Based on current observations, further studies are necessary to continue understanding fisetin's genetic and enzymatic targets for attenuating fat accumulation. Next steps in this study are to test fisetin in knockout mutants related to energy metabolism and lipid metabolism, including mTORC1 and its targets as previously reported (Jung 2013). A possible target to investigate include glutamyl-prolyl tRNA synthetase (EPRS) which has been reported to be a mTORC1 target that contributes importantly to adiposity and aging (Arif et al. 2017). Another mTORC1 target that has been is identified is sterol regulatory element-binding proteins (SREBP), which has been shown to play a role in lipogenesis (Caron et al. 2015). With this research and the current research as a foundation, these mutants will further probe the specific genes and their signaling pathways. The current findings may provide evidence to promote the application of fisetin as a natural product in the prevention and treatment of obesity.

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