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Fat Lowering Effects of Fisetin in *Caenorhabditis elegans*

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FAT LOWERING EFFECTS OF Fisetin in *Caenorhabditis elegans*

A Thesis Presented

by

NIKOLAS J. RODRIGUEZ

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
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ABSTRACT

FAT LOWERING EFFECTS OF Fisetin IN *Caenorhabditis elegans*

May 2021

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Fisetin, a flavanol with anti-inflammatory, anti-cancer, and anti-aging properties, has shown promise for reducing fat accumulation in tissue culture and animal models. This plant sourced compound has limited studies supporting its effects on fat accumulation. Therefore, this study was completed to determine fisetin's role in fat reduction along with its mechanism of action using *Caenorhabditis elegans*. *C. elegans* is a small roundworm with roughly 65% of its genes being conserved in humans related to disease. In this study, 100 and 200 μM fisetin has shown to reduce fat accumulation in wild-type worms. Body size, locomotion, and pumping rate were assessed in wild-type worms to determine if fisetin modified worm size, speed, and feed behavior, respectively. Mutant strains were tested to elucidate a potential pathway, of which *tub-1* knockout mutants failed to reduce fat accumulation after fisetin treatment, suggesting this gene's involvement. Gene expression of *tub-1* was not altered by fisetin treatment, suggesting potential post-transcriptional regulation of fisetin. This study serves as an introduction to fisetin's fat reducing effects via a *tub-1* dependent mechanism.

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CHAPTER 1

INTRODUCTION

Obesity is rapidly increasing in children and adults globally.^{1,2} The United States is one of the most obese populations, having more than 1/3 of its population being clinically obese at a rate that is steadily rising.³ Obesity has been linked to higher rates of diabetes and cardiovascular disease, therefore, there is great effort to find methods for reducing its prevalence.⁴⁻⁷ One such intervention would be using bioactive compounds with anti-obesity effects. For example, compounds such as cafestol, epigallocatechin-3-gallate, and butein have shown to reduce fat accumulation in animal studies.⁸⁻¹⁰

Fisetin is a flavanol found from several plants and fruits such as acacia trees, honey locust, strawberries, and grapes.¹¹ The compound has shown to have anti-inflammatory¹²⁻¹⁸, anti-cancer¹⁹⁻²⁶, and anti-aging properties.²⁷⁻³⁰ However, research on the compound's anti-obesity effects are limited.³¹⁻³³

Caenorhabditis elegans, a small, transparent nematode found in soil and decomposing matter, was the first organism to have its whole genome sequenced in 1998.^{34,35} First proposed by Dr. Sydney Brenner in his scientific article *The Genetics of Caenorhabditis elegans* in 1974, the worm has been used as a staple in biological studies.³⁵⁻³⁸ *C. elegans* has shown to be an effective model in studying obesity, with up to 65% of its genes being conserved in humans related to disease.³⁷ The worm is not only cost effective, but is easily grown and exhibits identical progeny, allowing rapid and accurate analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 *Caenorhabditis elegans* background

2.1.1 Introduction

C. elegans is a eukaryotic organism found in several geographical locations such as Northern Africa, North America, Asia, etc.³⁹ Primarily found in soil and decomposed organic matter, the worm naturally consumes many species of bacteria. Among them, *Escherichia coli* OP50 is traditionally chosen as feed in laboratories for *C. elegans*. Nematode Growth Media (NGM), a media commonly used for *C. elegans* in laboratories, has limited quantities of uracil; and since *E. Coli* OP50 requires uracil for growth but cannot synthesize it, this strain is used as the standard diet for *C. elegans*.^{35,40-42} Growth temperature for *C. elegans* in laboratory settings ranges from 15°C - 25°C, depending on the desired rate of growth⁴³, agreeing with their wild counterparts who predominate in temperate climates.⁴⁴

2.1.2 Life Cycle

C. elegans has larval developmental stages (L1-L4) prior to adulthood, which can take up to 3 days' time at 20°C (Fig. 1.1).⁴⁵ *C. elegans* are unique in that they are either male (XO chromosome, roughly 0.1-0.2% of progeny) or hermaphrodites (XX chromosome), which can self-fertilize.⁴⁶ Once the worm reaches the adult stage, eggs are laid for mainly 3 days in hermaphrodites or longer if through mating.⁴⁶ *C. elegans* prodigies are genetically identical to their parent, and exhibit a total of 959 somatic cells

with 302 neurons in adult hermaphrodites. Adult males have an additional 79 neurons primarily for mating behavior.⁴⁷

Once eggs are laid, embryogenesis can take upwards of 16 hours at 20°C (Fig. 1.1).⁴⁶ The L1 to L2 stage can take ~16 hours and each stage following takes ~12 hours.⁴⁶ Between stages, the worm exhibits lethargus where a new cuticle forms and is shed through molting.⁴⁶ The growth rate of each stage is temperature-dependent where marginal decreases in temperature (e.g. 15°C) delay growth and marginal increases in temperature exhibit accelerated growth (e.g. 25°C).⁴⁵ If worms succumb to low food, inadequate temperatures, or environmental stresses (e.g. worm population density), they can transition to a dauer phase as early as their second molt.⁴⁸ The dauer phase is a state of arrested development, which can be induced by pheromones, such as the potent ascaroside, (-)-6-(3,5-dihydroxy-6-methyltetrahydropyran-2-yl)oxy heptanoic acid, as a result of the above-mentioned variables.⁴⁸ The cuticle strengthens with alae, exterior pores close, specific gravity increases, pharyngeal pumping hinders, and movement halts.⁴⁸⁻⁵⁰ Recovery can occur when favorable environmental conditions are introduced.⁴⁸⁻

50

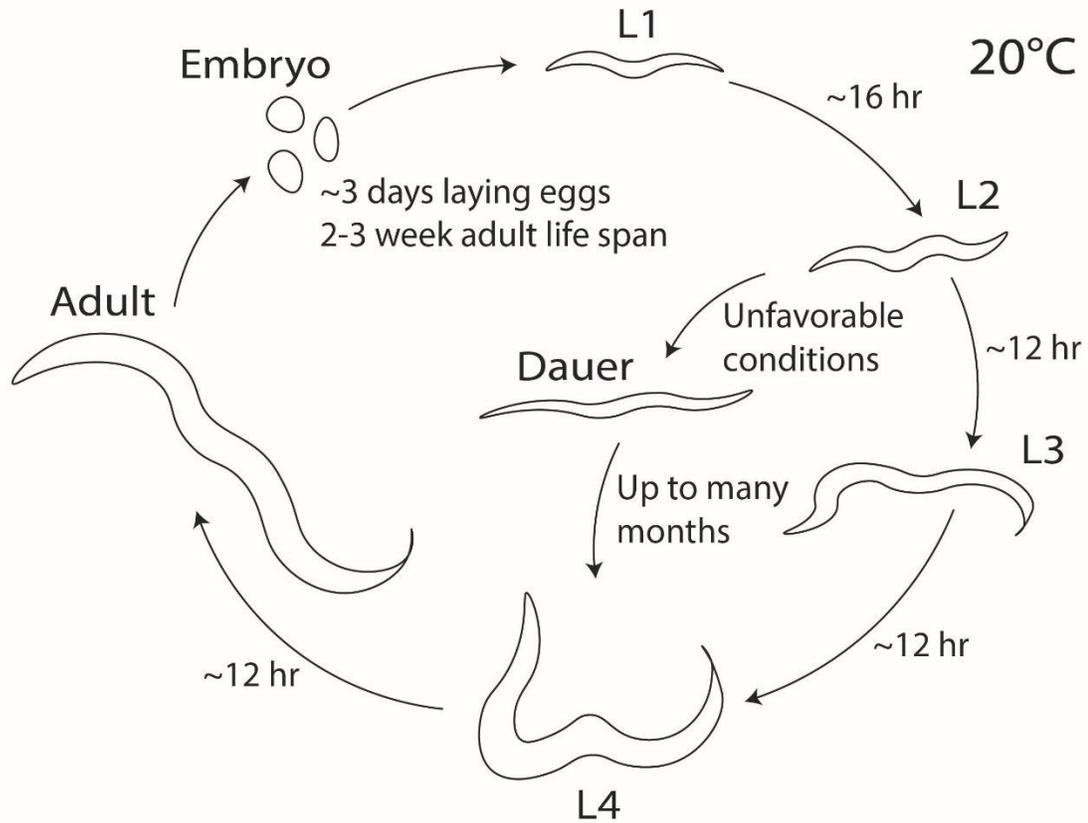


Figure 2.1 *Caenorhabditis elegans* life cycle at 20°C. Information taken from Corsi et al.⁴⁶

2.1.3 Compound Intake

C. elegans intakes compounds via the skin or pharynx using sensory cilia to register nutrient presence.⁵¹⁻⁵⁵ The skin is covered by a tough exoskeleton known as the cuticle, roughly 0.5 μm thick and 80% collagen, which allows for the cylindrical shape of the animal.^{51,52} The ridged surface, referred to as the annulus, contains sublayers, including the cortical, medial, and basal membrane, followed by the hypodermis.^{51,52} The pharynx is a muscular tube totaling sixty-two cells, twenty of which encompass the pharyngeal nervous system, that uses rhythmic contractions to move ingested food along to the lumen for grinding.⁵³ The cell makeup includes epithelial, gland, marginal, muscle, and neuron cells.⁵³ Zheng et al.⁵⁶ showcased the varied degrees in which researchers can

deliver various drugs to *C. elegans* in laboratory settings, called the NGM live and dead methods, using either live or dead *E. coli* OP50 after heating it for 30 minutes at 65°C.⁵⁶ This is because absorption efficiency may differ between the two methods as *E. coli* OP50 may consume the drug.⁵⁶ Similarly, the liquid LB medium can use either live or dead *E. coli* OP50.⁵⁶ Zheng et al.⁵⁶ suggested that the delivery of compounds on the plate with dead *E. coli* OP50 serves both an economic advantage and minimizes the potential loss of compounds due to the metabolism of *E. coli* OP50.⁵⁶

2.1.4 Locomotion

C. elegans moves via undulatory propulsion, similar to other nematodes.^{57,58} Seventy-five motoneurons innervate the worms body wall muscles to allow for movement.⁵⁹ Twenty-eight motoneurons innervate the neck and head, providing the ability for steering.⁵⁹ Ninety-five body wall muscle cells, a pair on each side of the dorsal and ventral nerve cords, respectively, repeat from head to tail along the worm.⁵⁹ They can crawl on solid surfaces or swim in aqueous solutions, producing short-waved and crescent-like body movements, respectively.⁶⁰ Interestingly, confined spaces allow for greater swimming speeds of the worm due to its ability to utilize surrounding obstacles for propulsion.⁶⁰ Pierce-Shimomura et al.⁶¹ showed that *che-3* mutants, sensory deficient phenotype, exhibit lower bending frequencies when crawling and suddenly changes between swimming and crawling behaviors (as characterized by bending patterns) while in liquid media.⁶¹ Along with similar, yet less expressive changes in *osm-3* mutants, which are sensory deficient, Pierce-Shimomura et al.⁶¹ believe *C. elegans* movement

behaviors are in part dictated by their ability to sense their surroundings as supported by the organism's chemotaxis behavior.^{61,62}

2.1.5 Application of *Caenorhabditis elegans* in Life Science Research

C. elegans has shown to be an effective model in characterizing diseases, particularly diseases related to aging and obesity.³⁷ With roughly 65% of *C. elegans* genes related to genes in human disease, the model exhibits a diverse and cost-effective way to perform disease analysis in a relatively short time.³⁷ For example, *C. elegans* models have been developed to express human amyloid β or tau, which are peptides forming a plaque or a protein aggregate, respectively, which are characteristics of Alzheimer's disease.^{38,63} In addition, *C. elegans* models expressing α -synuclein, which is believed to play a major role in dopamine metabolism and neurotransmission, has been developed to study Parkinson's disease.^{38,64} Lastly, *C. elegans* has similar lipid metabolism and insulin signaling pathway to mammals, which makes this model effective to study obesity and diabetes related research.⁶⁵ For example, *C. elegans* has conserved pathways for lipogenesis and fat oxidation, including *sbp-1*, *fat-6*, *fat-7*, and *nhr-49*.⁶⁵ The *daf-2* gene, an ortholog to the insulin/insulin-like growth factor receptor in humans, plays a role in the production of fat and glycogen, where downstream targets such as *daf-16* play a critical role in insulin signaling pathways.⁶⁵

2.2 Fisetin Background

2.2.1 Introduction

Fisetin (3,3',4',7-tetrahydroxyflavone, Fig. 2.2) is a yellow pigmented flavanol, a subclass of flavonoids⁶⁶ found in several plant sources, such as strawberries (*Fragaria* sp.), cucumbers (*Cucumis* sp.), peach (*Prunus* sp.), honey locust (*Gleditsia triacanthos*), etc.^{11,67,68} Fisetin is found in cucumbers and tomatoes in amounts as low as 0.1 µg/g, while strawberries contain up to 160 µg/g.^{11,67-69} Similar to other flavanols, *in vitro* and *in vivo* model studies have shown fisetin to exhibit anti-inflammatory¹²⁻¹⁸, anti-cancer¹⁹⁻²⁶, and anti-aging properties.²⁷⁻³⁰ As obesity is known to be associated with underlying inflammatory responses, the anti-inflammatory effects of fisetin may also contribute its anti-obesity effects,⁷⁰ although effects of fisetin on obesity are currently limited.³¹⁻³³

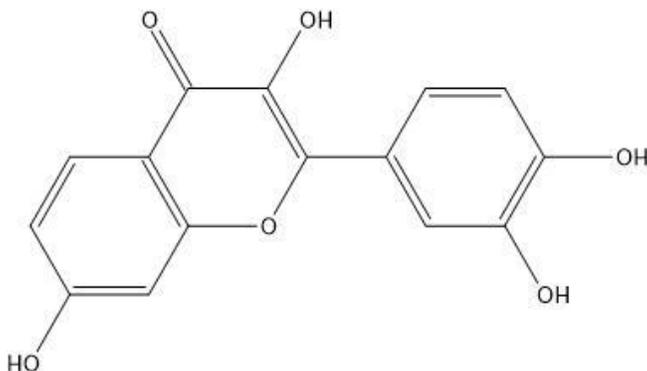


Figure 2.2 Structure of fisetin.

2.2.2 Anti-Inflammatory Effects of Fisetin

Similar to other flavanols, fisetin exhibits anti-inflammatory properties.⁷¹ Farsad-Naeimi et al.¹² explored fisetin's effects on inflammatory markers in colorectal cancer using a double-blind, placebo-controlled study with 37 males averaging 55 years old for

one year.¹² Fisetin significantly reduced plasma levels of interleukin-8 (IL-8), an inflammatory marker shown to contribute to tumor progression.¹²

Yang et al.¹³ investigated fisetin's effects on neuroinflammation due to lead contamination in mice.¹³ Both dosages of fisetin (25 and 50 mg/kg bodyweight) for four weeks reduced activation of nuclear factor kappa-light-chain-enhancer of activated B cells p65, tumor necrosis factor alpha (TNF- α), IL-6, toll-like receptor 4, and myeloid differentiation primary response 88; all well-known inflammatory markers in the brain.¹³ Others similarly reported that pre-treatment with fisetin reduced gene expressions of TNF- α , IL-6, interleukin-1- β (IL-1 β), and monocyte chemoattractant protein-1, as well as decreased protein expressions of TNF- α , IL-6, IL-1 β , cyclooxygenase-2, inducible nitric oxide synthase, and high-mobility group protein 1 compared to lipopolysaccharide treated controls.¹⁴ Others have also shown reductions in TNF- α and IL-1 β , inflammatory factors associated with atrial fibrillation (a risk maker associated with myocardial infarction) in Sprague-Dawley rats post-myocardial infarction, in male Wistar rats chronically exposed to cigarette smoke (also showing reductions in granulocyte macrophage colony-stimulating factor, IL-4, and IL-10), and in diabetic male Wistar rates (also showing reductions in IL-6).¹⁵⁻¹⁷ Huang et al.¹⁸ observed a reduction in several inflammatory cytokines, including IL-1 β , TNF- α , IL-2, IL-4, IL-5, and IL-18, in asthma-induced mice treated with fisetin, suggesting fisetin regulated airway hyperresponsiveness via the Myd88- and NF- κ B- dependent signaling pathways.¹⁸

2.2.3 Anti-Cancer Effects of Fisetin

Fisetin has shown anti-cancer effects in several types of cancers, including: bladder¹⁹, breast²⁰, cervical²¹, colorectal²², leukemia²³, lung²⁴, pancreatic²⁵, and skin²⁶ (Summarized in Table 2.1).

Table 2.1 *In vitro* and *In vivo* studies on fisetin's anti-cancer properties

Model	Observation ^a	Suggested Mechanism ^b	Concentration /Dose of Fisetin	Treatment Time	Reference
<i>In vitro</i>					
MDA-MB-231 (breast/mammary)	↓ Proliferation, migration, invasion	↓ N-cadherin, Vimentin, Snail, p-Akt, p-GSK-3β; ↑ E-cadherin, Claudin, PTEN	10 -100 μM	72 h	20
BT549 (breast/mammary)					
HeLa (cervical cancer) cells	↓ Cell viability; ↑ Apoptosis	↑ caspase-8, caspase-3, cleaved-PARP, ERK1/2	0-80 μM	24 and/or 48 h	21
PIK3CA-mutant HCT116 & HT29 (colorectal cancer)	↓ Cell viability; ↑ Apoptosis	↓ mTOR, Raptor, Rictor, GβL, p-PRAS40, 4EBP1, eIF4E, p70S6K; ↑ AMPKα	30 - 90 μM	48 h	22
Human K562 leukemia	↓ Cell viability, topo I activity, topo II activity	N/A	1 – 174 μM	0.5 to 24 h or 5 days	23
PANC-1 (pancreatic cancer)	↓ Cell viability; ↑ Apoptosis, autophagic flux	↓ p-p70s6k; ↑ p-AMPK, p8, PERK, ATF6, ATF4, PINK1, p-p53, p-PKC-α, p21	25 - 400 μM	24 and/or 48 h	25
RPMI-7951 & A375 (melanoma)	↓ Melanoma cell invasion	↓ p-MEK1/2, p-ERK1/2, p-IκBα, IKKα, N-cadherin, vimentin, snail, fibronectin; ↑ E-cadherin, desmoglein	5-20 μM	24 h	26
<i>In vivo</i>					
N-methyl-N-nitrosourea treated female Wistar rats	↓ % of total rats with tumor growth, carcinoma, TUNEL-positive cells	↓ PCNA, Bcl-2, cyclin D1, p-IκB-α, IKKβ; NFκB; ↑ p21, p53, Bax, IκBα, p19 ^{ARF}	200 mg/kg, once a week	18 weeks	19

MDA-MB231 xenograft female mice	↓ Tumor size	↓ N-cadherin, Vimentin, Snail, p-Akt, p-GSK-3 β ; ↑ E-cadherin, Claudin, PTEN	100 mg/kg, every three days	4 weeks	²⁰
HeLa tumor xenograft male mice	↓ Tumor growth rate	N/A	2 and 4 mg/kg, twice per week	35 days	²¹
FC ^{13K} 1Apc ^{Min/+} mice	↓ % of total rats with tumor growth	N/A	1 mg, twice per week	3 weeks	²²
Benzo(a)pyrene treated male Swiss Albino mice	↓ Lung weight, alveolar damage; ↑ BW	↓ SOD, CAT, GPx, GR, GST, PCNA; ↑ GSH, Vit. E and Vit. C	25 mg/kg body twice per week	16 weeks	²⁴
PANC-1-Luciferase xenograft female mice	↓ Tumor growth	↓ PCNA, Ki67, p-H3; ↑ LC3B, Parkin, ATF4, ATF6	300 mg/kg, every other day	20 days	²⁵

a. topo I, type I topoisomerase; topo II, type II topoisomerase; BW, bodyweight

b. AMPK α , AMP-activated protein kinase-alpha; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; Bax, BCL2 associated X; Bcl-2, B-cell lymphoma 2; CAT, catalase; eIF4E, eukaryotic translation initiation factor 4E; ERK1/2, Extracellular signal-regulated kinases 1/2; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione-S-transferase; G β L, G-protein beta-subunit-like protein; I κ B α , nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IKK α , inhibitor of nuclear factor kappa-B kinase subunit alpha; IKK β , inhibitor of nuclear factor kappa-B kinase subunit beta; LC3B, light chain 3B; mTOR, mechanistic target of rapamycin; NF κ B; nuclear factor kappa-light-chain-enhancer of activated B cells; p-Akt, phosphorylated Akt; p-AMPK, phosphorylated activated protein kinase; PARP, poly (ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; PERK, protein kinase R like endoplasmic reticulum kinase; p-ERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; p-GSK-3 β , phosphorylated glycogen synthase kinase-3 beta; p-H3; phosphorylated histone H3; PINK1, PTEN-induced kinase 1; p-I κ B α , phosphorylated nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; p-MEK1/2, phosphorylated dual specificity mitogen-activated protein kinase kinase 1/2; p-PKC- α , phosphorylated protein kinase c alpha; p-PRAS40, phosphorylated proline-rich Akt substrate of 40 kDa; p-p53, phosphorylated tumor protein p53; p-p70S6K, phosphorylated ribosomal S6 kinase; PTEN, phosphatase and tensin homolog; p21; cyclin-dependent kinase inhibitor 1; p53, tumor protein p53; p70S6K, ribosomal S6 kinase; SOD; super oxide dismutase; Vit. C, vitamin C; Vit. E, vitamin E; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1

2.2.4 Fisetin and Aging

Yousefzadeh et al.²⁷ reported the effects of fisetin treatment lowered senescence-associated markers, β -galactosidase (SA- β -gal) and p16^{Ink4a} expressions, in mouse fibroblasts.²⁷ This was further confirmed that fisetin treatment for 5 days reduced senescent cell count in white adipose tissue and p16^{Ink4a} expression to similar levels of younger mice.²⁷ SA- β -gal activity was also significantly reduced in the omental adipose tissue treated with 20 μ M fisetin post-surgical removal for 48 hours.²⁷ Others have also reported fisetin's senescent reducing properties *in vitro*.²⁸

Currais et al.²⁹ explored fisetin's effects on behavior associated with aging. Animals treated with 500 mg fisetin/kg body weight for 7 months not only maintained their ability to relearn object location in the Barnes maze test, but also maintained short-term recognition of familiar items in the recognition test compared to control animals.²⁹ Interestingly, it was also reported that protein associated with synaptic functions (activity-regulated cytoskeleton-associated protein, Homer, and synapse-associated protein 102) were preserved in older mice treated in fisetin.²⁹ In addition, fisetin supplementation to improve spatial learning in older mice using the Morris water maze test, as well as reduce levels of malondialdehyde in the brain, showed a decline in oxidative stress in older mice.³⁰

2.2.5 Effects of Fisetin on Fat Accumulation

Jung et al.³¹ showed that male C57BL/6J mice fed a high fat-diet and treated with 0.5% (w/w) fisetin for 10 weeks experienced a significant reduction in body weight and both epididymal and retroperitoneal fat pad weights compared to the control.³¹ Similarly,

others reported that intraperitoneal injection of 20 mg fisetin treatment/kg body weight twice per week for 10 weeks significantly reduced body weight to 4.62 ± 1.25 g compared to the control (9.55 ± 1.38 g) in male C57BL/6J mice fed a high fat-diet.³²

Along with reduced body and adipose tissue weight, reduced adipocyte cell sizes with fisetin treatment were reported in *in vivo* animal studies.³¹⁻³³ Consistently, a study reported that 50 μ M fisetin significantly decreased preadipocyte differentiation in 3T3-L1 preadipocytes.³¹

As an underlying mechanism of fisetin on fat reduction, Jung et al.³¹ investigated fisetin's impact on phosphorylation levels of mammalian target of rapamycin complex (mTORC1) and its target, ribosomal protein S6 kinase beta-1 (S6K1), both of which were down-regulated by fisetin.³¹ Others reported that treatment of fisetin was associated with decreased gene expression of peroxisome proliferator-activated receptor gamma (PPAR γ), sterol regulatory element-binding protein 1 (SREBP1), fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD1), and upregulated carnitine palmitoyltransferase I (CPTI), carnitine palmitoyltransferase II (CPT2), and cytochrome C oxidase subunit 8 β (COX8 β).³³ Overall, these reports, although limited, support fisetin's ability to reduce fat accumulation.

2.2.6 Studies of Fisetin in *Caenorhabditis elegans*

Two studies have explored fisetin's effects in *C. elegans*.^{72,73} Kampkötter et al.⁷² found that fisetin increased worm survivability under heat stress, decreased oxidative stress and lipofuscin, and increased localization of the *daf-16* gene to the nucleus, which plays a critical role in several signaling pathways.⁷² Others reported that fisetin's effects

survival and reproduction capacity with co-treatment of cyclophosphamide, a chemotherapy drug.⁷³ Treatment of cyclophosphamide decreases *C. elegans* survivability (suggested to be due to its effects on attenuating immunity), while fisetin improved survivability and fertility.⁷³ They further concluded that fisetin is involved with insulin/IGF-like signaling, as evident of the increased gene expression of *egl-17* and attenuated *daf-2* expression.⁷³ This is further supported by others, *egl-17* has shown to repress *daf-2* receptors, thus allowing *daf-16* activation and improving worm longevity under stress conditions.^{73,74}

2.3 Conclusion

Bioactives found in natural food sources may serve as potent therapeutics for attenuating obesity. Fisetin, a natural flavanol found in several plant sources, has shown many beneficial effects including anti-inflammation, anti-cancer, and anti-aging. Although fisetin has exhibited anti-obesity properties, the studies are currently limited. Thus, we used *C. elegans*, a small yet powerful model for obesity research, to determine the effects of fisetin on lipid metabolism.

CHAPTER 3

METHODS AND MATERIALS

3.1 Materials

Fisetin (purity >96.0%, batch: 6CZFM-CP) and 2'-deoxy-5-fluorouridine (FUDR) were purchased from TCI America (Portland, OR, USA). Infinity™ Triglycerides Liquid Stable Reagent was purchased from Fisher Diagnostics (Middletown, VA, USA). Pierce™ BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Middletown, VA, USA). Ampicillin was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Carbenicillin was purchased from Fisher Bioreagents (Pittsburgh, PA, USA). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA). *C. elegans* wild-type (N2), *C. elegans* mutants (*tub-1*, *nhr-49*, *sbp-1*, *sir-2.1*, *daf-12*, *daf-16*, and *daf-2*), and *E. coli* OP50 were purchased from the Caenorhabditis Genetics Center (CGC, Minneapolis, MN, USA).

3.2 Maintenance and Preparation

C. elegans were maintained on NGM plates as previously described.⁸ Worms were fed as needed with live *E. Coli* OP50.⁴² To obtain a synchronized worm population, eggs were collected by dissolving collected gravid worms from NGM plates in a bleaching solution.^{37,42} After bleaching, worms were washed with M9 buffer and then S-complete (both prepared as previously described⁷⁵). L1 synchronized worms were fed with live *E. coli* OP50 until L4, where worms were washed of live *E. coli* OP50 with M9 and S-complete, then transferred to a 12-well plate. From this, worms were fed dead *E. coli* OP50, heat killed for 30 minutes at 65°C⁵⁶, with ampicillin (100 µg/mL) and

carbenicillin (50 µg/mL), as well as FUDR to prevent hatching of eggs.⁸ Once worms were grown to the adult stage, they were treated with fisetin. Fisetin was prepared at 100 mM and 200 mM stock solution using dimethyl sulfoxide (≥99.9%, DMSO) as a vehicle. Adult nematodes were treated with 0.1% DMSO (control), 100 µM, or 200 µM fisetin as a final concentration.

3.3 Triglyceride and Protein Quantification

Post treatment, worms were transferred to centrifuge tubes. After washing worms with water five times, 0.05% Tween 20 was added and sonicated prior to triglyceride (TG) and protein quantification.^{8,76} TG was measured using Infinity™ Triglycerides Reagent with glycerol as a standard.⁸ Protein quantification was measured using Pierce™ BCA Protein Assay Kit with bovine serum albumin as a standard.⁸ Sample absorbances were measured at 510nm (triglyceride assay) or 562nm (protein assay) using a SpectraMax i3 microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA) and SoftMax Pro 6 (version 6.5).

3.4 Pumping Rate, Locomotive Assay, and Body Size

Pumping rate was measured by counting pharyngeal contractions of randomly selected worms for 30 seconds. Locomotive behavior and body size were measured using WormLab system (model MSCOP-002, MBF Bioscience, Williston, VT, USA) and WormLab software (3.1.0 64-bit, MBF Bioscience, Williston, VT, USA). Worms were placed on low-peptone NGM plates with live *E. coli* OP50, then allowed to acclimate to the instrument's light for 20 minutes prior to measurement. Videos were recorded for 1

minute, where width, length, and speed were measured using WormLab software (3.1.0 64-bit, MBF Bioscience, Williston, VT, USA).⁸

3.5 Real-time PCR

Real-time PCR was performed using TRIzol® (Thermo Fisher Scientific, Inc., Middletown, VA) for total RNA extraction, followed by cDNA reverse transcription by high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Inc., Middletown, VA) and thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA).^{8,9} StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA) was used for measuring gene expression. The following were used for TaqMan gene expression assays (Thermo Fisher Scientific, Inc., Middletown, VA): *atgl-1* (Ce02406730_g1), *cebp-2* (Ce02421574_g1), *hosl-1* (Ce02494529_m1), *tub-1* (Ce02435686_m1), and *ama-1* (Ce02462726_m1) as an internal control. The delta-delta comparative threshold (Ct) method was used to analyze threshold values.^{8,9}

3.6 Statistical Analysis

Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA), followed by the Tukey test using SAS Software (version 9.4.1, Cary, NC). Significant difference will be considered as $p < 0.05$.

CHAPTER 4

RESULTS

4.1 Fisetin Reduced Fat Accumulation

Wild-type *C. elegans* were treated for 48 hours after reaching the adult stage.

Fisetin treated at 100 μM and 200 μM decreased triglyceride levels by 22% ($p = 0.0004$) and 39% ($p < 0.0001$) compared to the control, respectively (Fig. 4.1).

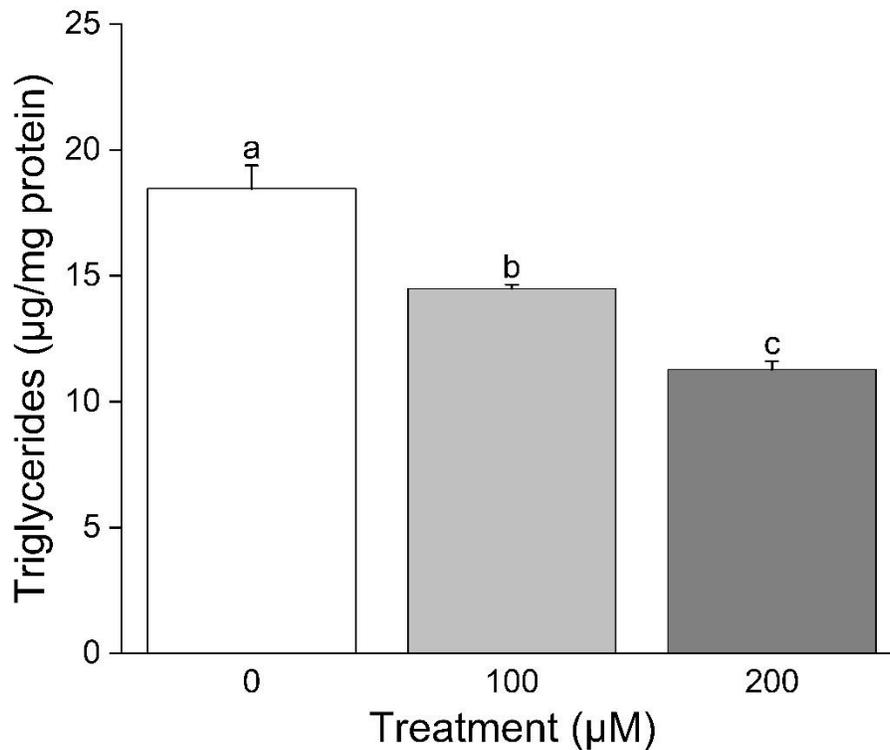


Figure 4.1 Effects of fisetin on fat accumulation in *C. elegans*. Adult worms (N2 Bristol, wild-type) were treated for 48 hours with 100 μM or 200 μM fisetin. Triglyceride levels were normalized by protein content. Data are means \pm S.E (n = 8, taken from two independent experiments, each sample consisted of ~1,200 worms). Means with different letters are significantly different at $p < 0.05$.

4.2 Fisetin's Effects on Body Size, Locomotion, and Pumping Rate

Wild-type *C. elegans* were treated for 24 hours after reaching the adult stage. Fisetin induced no significant changes on the worms' width compared to the control (Fig. 4.2A). Fisetin (100 μ M and 200 μ M) did significantly reduce the worms' length by 6% for both treatments ($p= 0.0074$ and $p = 0.0108$, respectively, Fig. 4.2B), which may be due to fisetin's fat reducing effects (Fig. 4.1).⁸ To determine if fisetin impacted the worms' energy expenditure, locomotive activity as movement speed was determined. Results showed no significant effect of fisetin on the worms' speed compared to the control (Fig. 4.2C). Lastly, pharyngeal contractions were counted to determine if fisetin impacted the worms' feeding behavior. No significant change by fisetin was seen in the worms' pumping rate compared to the control, suggesting fisetin had no effect on food intake (Fig. 4.2D).

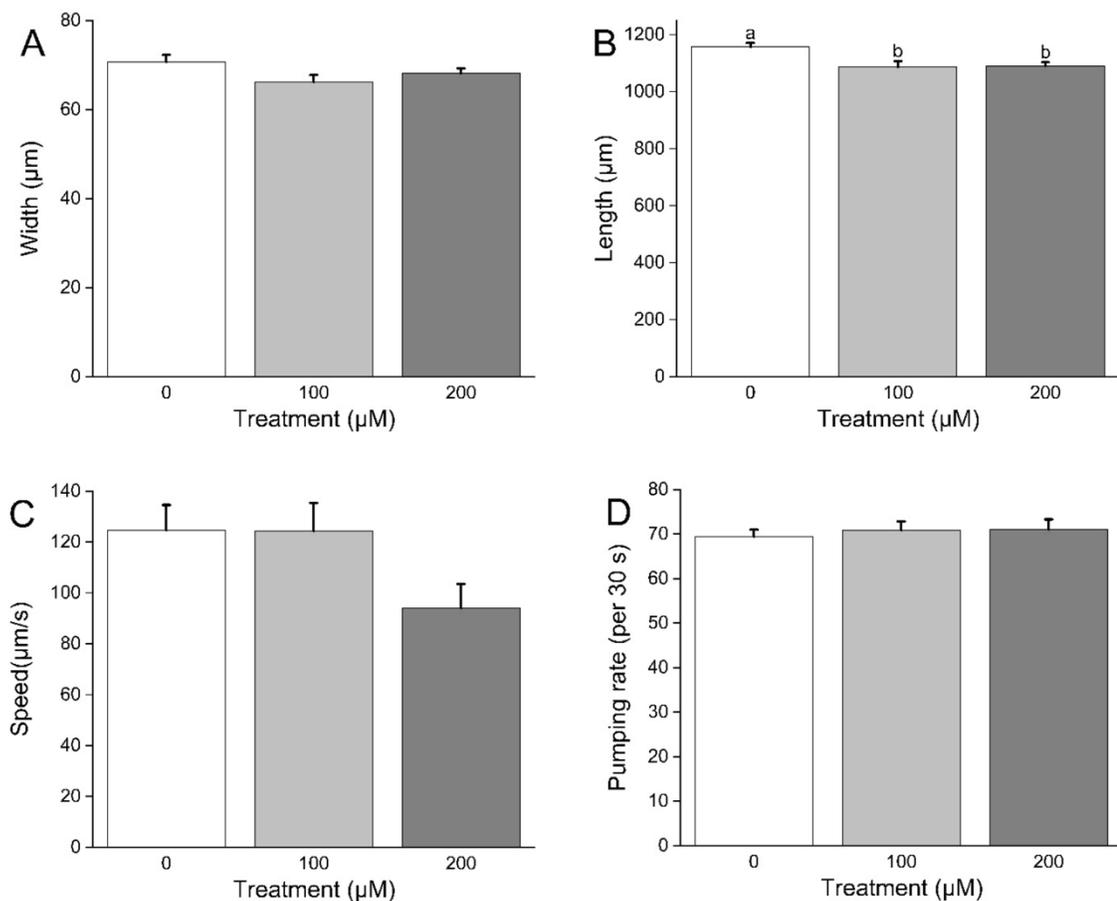


Figure 4.2 Effects of fisetin *C. elegans* body size, locomotion, and pumping rate.

Adult worms (N2 Bristol, wild-type) were treated for 24 hours with 100 μM or 200 μM fisetin. (A) worm width, (B) worm length, (C) worm speed, (D) pharyngeal contractions counted for 30 seconds of randomly selected worms on low-peptone NGM plates. Data are means ± S.E (A-C: n = 67 – 70, D: n = 12; taken from two independent experiments). Means with different letters are significantly different at $p < 0.05$.

4.3 Fisetin’s Effects on Fat Accumulation in Mutants

TUB-1, a homolog of tubby proteins in humans, is involved in energy balance, particularly in fatty acid β-oxidation via *kat-1*.^{77,78} Thus, *tub-1* mutants exhibit greater fat accumulation than wild-type worms along with deficiencies in chemotaxis and an extended lifespan.⁷⁸ Results showed no significance on fat accumulation in *tub-1* mutants

between treatment groups compared to the control, suggesting the *tub-1* gene plays a significant role on fisetin's fat lowering effects (Fig. 4.3).

NHR-49, a homolog of peroxisome proliferator-activated receptor alpha, is involved in energy homeostasis and plays key a role in fatty acid β -oxidation.^{79,80} Although there was no significant reduction in triglycerides with 100 μ M fisetin, 200 μ M fisetin significantly reduced fat accumulation by 31% ($p = 0.0057$) compared to the control (Fig. 4.3). This suggests that *nhr-49* may not play key roles in the effects of fisetin's fat reduction.

SBP-1, a homolog of mammalian sterol regulatory element-binding protein, which is involved in lipogenesis.⁸¹ The *sbp-1* mutants exhibit lower levels of fat accumulation.⁸¹ Fisetin treatment significantly reduced triglycerides by 17% with 100 μ M fisetin ($p = 0.0232$) and by 34% with 200 μ M fisetin ($p < 0.0001$) compared to the control, suggesting *sbp-1* does not play a role in fisetin's fat lowering effects (Fig. 4.3).

SIR-2.1, an ortholog to mammalian sirtuin, is involved in fat homeostasis.⁸²⁻⁸⁴ In addition, *sir-2.1* is a key player in aging and longevity and interacts with *daf-16* in the insulin/insulin-like growth factor-1 (IIS) pathway.⁸² Treatment with fisetin significantly reduced triglyceride contents by 21% at 100 μ M fisetin ($p = 0.0484$) and by 35% with 200 μ M ($p = 0.0024$) compared to the control, suggesting *sir-2.1* does not play a role in fisetin's fat lowering effects (Fig. 4.3).

DAF-2, a homolog to insulin/insulin-like growth factor receptor, is involved in energy homeostasis.^{8,85} DAF-16, an ortholog of mammalian Forkhead box O transcription factor, serves as a downstream target to *daf-2* in the IIS signaling pathway.^{8,86} In *daf-2* mutants, there was a significant reduction in triglycerides with 100

μM fisetin by 19% ($p = 0.0069$) and by 26% with 200 μM fisetin ($p = 0.0004$) compared to the control (Fig. 4.3). For *daf-16*, triglycerides were not significantly reduced by 100 μM fisetin, but did show a significant reduction by 31% with 200 μM fisetin ($P = 0.0012$) compared to the control (Fig. 4.3). These results suggest both *daf-2* and *daf-16* do not play significant roles in fisetin's fat lowering effects.

DAF-12, a homolog to farnesoid X receptor, plays a role in fat metabolism and dauer development.⁸⁷ Fisetin significantly reduced triglycerides by 28% with 100 μM ($p = 0.0118$) and by 44% with 200 μM ($p = 0.0007$) compared to the control, suggesting *daf-12* does not play a role in fisetin's fat lowering effects (Fig. 4.3).

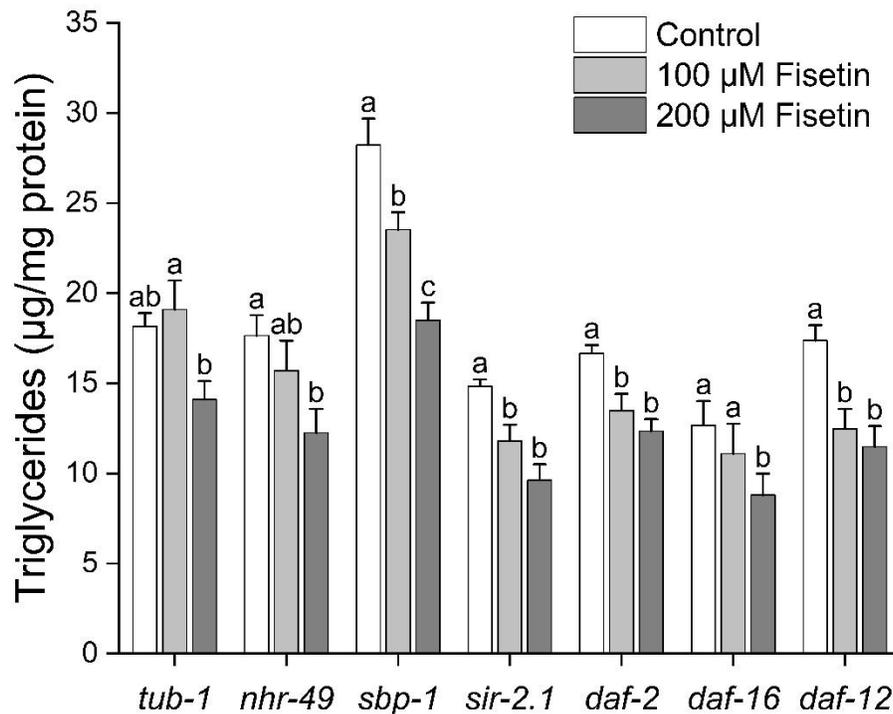


Figure 4.3 Effects of fisetin on fat accumulation in *C. elegans* mutants. Adult worms were treated with 100 µM or 200 µM fisetin for 48 hours. Triglyceride levels were normalized by protein content. Data are means ± S.E (n = 4-8, taken from one to two independent experiments, each sample consisted of ~1,200 worms). Means with different letters at each variable are significantly different at $p < 0.05$.

4.4 Fisetin's Effects on Gene Expression

The *tub-1* gene was chosen for real-time PCR based on the mutant study in Fig. 4.3. Interestingly, there was no significant difference on gene expression of *tub-1* between treatment groups (Fig. 4.4).

In addition to *tub-1*, other key genes involved in lipid metabolism were tested since these have no available mutants. CEBP-2, a homolog of CCAAT/enhancer-binding proteins, influences β -oxidation and fatty acid desaturation in *C. elegans*.⁸⁸ No significant

difference between groups were observed for *cebp-2*, suggesting *cebp-2* does not play a role in fisetin's fat lowering effects (Fig. 4.4).

ATGL-1, a homolog of mammalian adipose triglyceride lipase, and the HOSL-1, an ortholog of hormone-sensitive lipase homolog, are lipases in *C. elegans*, which influence lipolysis.^{37,76} Relative gene expressions were not significantly different between all groups for both *atgl-1* and *hosl-1*, suggesting neither genes play a role in fisetin's fat lowering effects (Fig. 4.4).

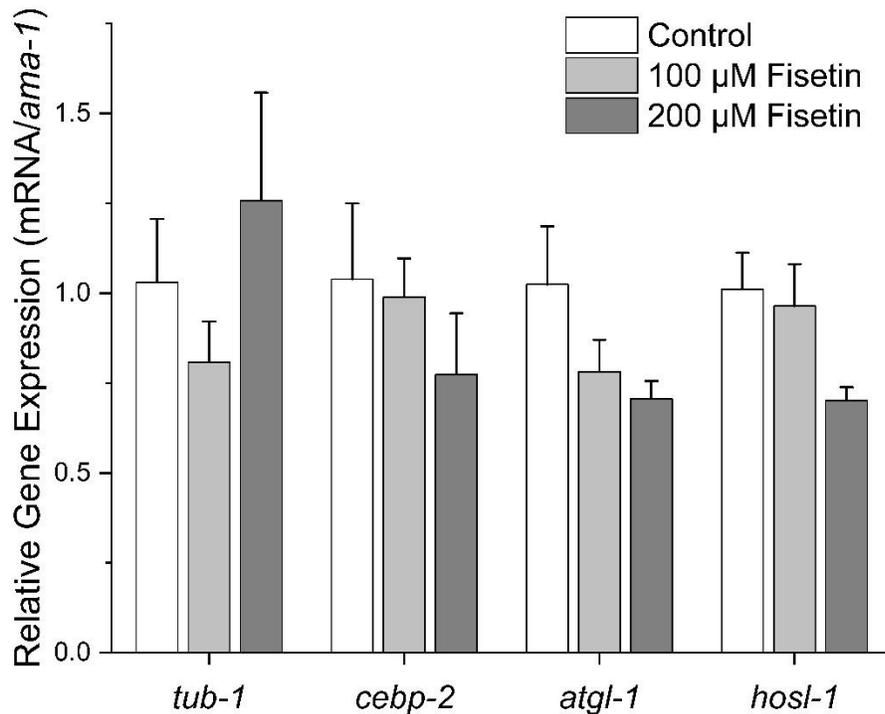


Figure 4.4 Effects of fisetin on fatty acid metabolism-related gene expression in *C. elegans*. Adult worms (N2 Bristol, wild-type) treated with 100 μM or 200 μM fisetin for 48 hours. Gene expression was measured by real-time PCR. Data are means ± S.E (n = 3, each sample consisted of ~6,000 worms). Means with different letters at each variable are significantly different at $p < 0.05$.

CHAPTER 5

DISCUSSION

5.1 Discussion

Fisetin, sourced from several plant sources^{11,67-69}, has shown several health benefits *in vitro* and *in vivo*, yet studies on lipid metabolism are currently limited.¹²⁻³⁰ Thus, this study evaluated fisetin's effects on lipid accumulation using *C. elegans* as a model system. Fisetin significantly reduced fat accumulation without affecting worm speed (representative of energy expenditure) or pumping rate (representative of food intake) in wild-type *C. elegans*. This effect of fisetin is mediated in part via *tub-1*, potentially by post-transcriptional regulation.

Previously, it was reported that fisetin decreases lipogenic gene expressions (PPAR γ , SREBP1, FAS, and SCD1) and increases fatty acid oxidation related genes (CPT1, CPT2, and COX8 β) in high fat-diet fed mice.³³ This is inconsistent with the current study showing no involvement of *nhr-49*, *sbp-1*, *daf-2*, nor *daf-16* by fisetin on fat accumulation. The current study only suggests the involvement of *tub-1*, a gene related to β -oxidation,^{77,78} however, without increased gene expression of *tub-1*. Thus, it is possible that fisetin regulates *tub-1* post-translationally. This can be determined by expressions of its downstream targets; *ech-1.1*, *rbg-3* or *rab-7*. *ech-1.1* is involved in fatty acid β -oxidation and increased expression reduces fat accumulation.^{8,79} Alternatively, *rbg-3*, a RabGTPase-activating protein regulating fat storage homeostasis, is another downstream target of *tub-1*, and this may contribute to fisetin's effect. This is supported by the report of *rbg-3* RNAi decreased fat storage.⁸⁹ Lastly, *rab-7*, both downstream of *tub-1* and *rbg-3*, functions in chemotaxis and fat storage homeostasis.⁹⁰

Both RBG-3 and its preferential target RAB-7 alter sensory molecules and neurons, which then play a role in regulating fat storage in *C. elegans* intestine.⁹⁰

There was a significant difference on fat accumulation between two fisetin treatments in *tub-1* mutants without significance from the controls. It is possible that there are additional factors involved, such as *kat-1*, which encodes for 3-ketoacyl-coA thiolase and functions in β -oxidation synergistically with *tub-1*.⁹¹ It was suggested that *kat-1* would compensate for defects in *tub-1*, even though both genes can be found in separate tissues, the intestines and neurons, respectively.⁷⁸ We speculate that the dose required for fisetin to act on *kat-1* may be greater, thus, higher a dose of fisetin (200 μ M) were effective, but not lower a dose tested (100 μ M) in the *tub-1* mutant. Thus, the role *kat-1* plays in fisetin's fat lowering effects warrants further investigation.

There is currently no clear method to compare dosages in *C. elegans* to other mammals, such as humans. Thus, it is not clear how to interpolate the significance of concentrations used in current studies for humans. Fisetin consumption in humans has not been widely studied, with only one study reporting an average daily intake of 390 μ g fisetin in 50 Japanese families.⁹² In the current study, 100 and 200 μ M fisetin, roughly 28.6 and 57.2 μ g/mL, respectively, induced fat reduction in *C. elegans*. Other studies using phytochemicals with fat reducing properties have used higher concentrations than the following study in *C. elegans*.^{9,79} Similarly, other studies have used 100 μ M fisetin on stress response in *C. elegans*, while another used 200 μ M fisetin in human pancreatic duct cells with no impact on cell viability.^{25,72} Additionally, as much as 300 mg fisetin/kg body-weight was fed to mice resulting in a reduction in tumor growth.²⁵ Future studies

would be needed to determine proper dose conversion from *C. elegans* to other animals and/or humans to properly interpret any observations from the current study.

5.2 Conclusion

The current study investigated fisetin's effects on fat metabolism in *C. elegans*. Fisetin reduced fat accumulation without affecting worm activity and pumping rate. The *C. elegans tub-1* knockout mutant abolished the effects of fisetin on reduced fat accumulation, although no effects on *tub-1* transcription levels were observed. These results suggest that fisetin reduces fat via the TUB-1 pathway, post-transcriptionally, leading to increased fatty acid β -oxidation. Additional studies are warranted to elucidate the mechanisms of fisetin on lipid metabolism in near future.

CHAPTER 6

FUTURE RESEARCH

The current study provides support for fisetin's fat reducing properties in *C. elegans*; fisetin reduces fat accumulation without altering locomotive activity or food intake rates. Although *tub-1* is a potential target of fisetin's fat lowering effect, gene expression did not support its regulation at the transcriptional level. To determine if fisetin's effects are on post-transcriptional levels, known targets of *tub-1* as well as mutants with TUB-1 bound to green fluorescence protein can be used to determine further how fisetin elicited its effects. Additionally, the role of *kat-1* in the *tub-1* mutant can clearly determine if *kat-1* is also involved in fisetin's fat lowering effects, especially at higher dosages.

Lastly, several genes were tested in this study, yet *C. elegans* still has many pathways which need to be explored. A previous study has shown fisetin to attenuate fat accumulation via multiple pathways; among them, those that were not tested in the current study should be investigated in this model, such as the mTROC1 pathway, FAS, and SCD1.^{31,33} The mechanistic target of rapamycin (mTOR) encodes *let-363* in *C. elegans*, which is involved energy homeostasis, while the *fasn-1*, homolog to FAS, and *fat-6* and *fat-7*, orthologs to stearoyl-CoA desaturases, are involved in lipogenesis.^{8,78} Additional targets of fisetin can be screened using the RNAseq method.

This research can offer a starting point for understanding fisetin's mechanism of lowering fat in *C. elegans*. Fisetin shows potential as an anti-obesity nutraceutical yet will still require human studies.

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