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Article

A novel approach to prevent and restrict early stages of cancer cell growth using a combination of moringa and sesame in a *Drosophila* model

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SUMMARY

Cancer is a highly prevalent disease with many causes but no cure; here, we examined the efficacy of the naturally anti-inflammatory moringa and sesame plants to prevent or restrict cancer cell growth in *Drosophila melanogaster*. In our previous work, we showed that sesame and moringa individually repressed eye tumor formation in *D. melanogaster.* In this work, we studied combinations of moringa and sesame at different proportions to compare their effects in preventing cancer cell growth. We studied *D. melanogaster* eye tumors which allowed easy detection of the phenotypes with simple microscopes. As we increased the concentration of sesame and moringa in the mixture, the cancer symptoms improved. We showed that a diet consisting of a combination of 10% sesame and 15% moringa can be used to effectively prevent visible tumor growth in the *D. melanogaster* eye, and the endpoints were close to those of healthy, wild-type flies. This data showed improved tumor growth compared to the treatments used individually. We extended the study to restrict early stages of cancer using these treatments. Progeny of Gal4-Ret cross at four different stages of the lifecycle were treated with experimental food and compared to control sample flies. Our study showed this combination of Moringa and Sesame treatments during the second instar or third instar larvae could restrict the cancer cells growth. Therefore, we concluded that a combination of 15% moringa and 10% sesame could prevent cancer and restrict early stages of cancer growth in this specific model of cancer using *D. melanogaster.*

INTRODUCTION

Cancer is a group of diseases that still has no complete cure. It is estimated that the number of cancer cases will reach 24 million worldwide by 2035 (1). Cancer can affect any tissue or organ of the human body; breast, lung, prostate, liver, and stomach cancers are some of the primary cancers leading to death (2). Chemotherapy helps treat cancer for many patients, but not without significant adverse side effects such as hair loss, anorexia, malabsorption, and anemia, among others (3). As our body processes food, waste substances are produced which are known as free radicals also called reactive oxygen species. If the body cannot remove these free radicals effectively it can result in an oxidative stress that could harm cells and body function. Antioxidants are compounds that prevent the formation of these free radicals by inhibiting oxidation (4). Since chemotherapy can often be very taxing, we examined if a diet enriched with antioxidants could help prevent the onset of cancer or even lessen its progression in animals already affected by it.

Sesame (*Sesamum indicum*) and moringa (*Moringa oleifera*) have natural antioxidants that could be a way to prevent cancer or potentially even cure the early stages (5, 6). Sesame is an oilseed that has several anti-inflammatory and antioxidant properties and is very effective for the prevention of cardiovascular diseases (7, 8). Sesame consists of nutrients like proteins, carbohydrates, antioxidants, lignans, and tocopherols, among others (9). Sesame compounds inhibit the inflammatory mediator NF-κB and ERK/p38 MAPK signaling pathways, thereby suppressing inflammatory responses (10). Moringa is called the "miracle vegetable" because it is one of the most nutrient-dense plants on the planet (11). Studies have demonstrated that moringa has antiviral and anti-inflammatory properties and can treat viral infections and heart diseases as well as boost immunity (12-18). Moringa extract decreases NF-κB and increases the anti-inflammatory cytokines IL-10 and IκB-α(19).

Several animal species are routinely studied as cancer models in a laboratory setting. *Drosophila melanogaster* is one such model that has been extensively studied (20-21). 75% of disease-causing genetic variants in humans are also found in *D. melanogaster* where they have been shown to cause the same disease (22). *D. melanogaster* is a model that is very amenable to experimentation and study, particularly when working with the eye, as it allows for easy detection of phenotypes using simple microscopes. The RetMEN2B allele contains mutations observed in the multiple endocrine neoplasia (MEN) domain of *Ret*. *Ret* is an oncogene that causes retinoblastoma in *D. melanogaster* and causes lung and thyroid cancer in humans. Using the Gal4/UAS system, oncogenes such as *Ret* can be used to induce cancer in the *D. melanogaster* eye called a "rough eye" (an irregular and uneven, usually smaller eye). Gal4 is a tissue-specific transcriptional activator, derived from yeast, that can turn on activity for any gene with an upstream activator sequence (UAS) present. Male flies containing the UAS-RetMEN2B transgene can be crossed with a female GMR-Gal4 fly to produce offspring (Gal4-RET) that are susceptible to cancer (23-24). Expression of the RetMEN2B allele leads to phenotypes in the *D. melanogaster* eye that are similar to those observed in vertebrates, specifically increased proliferation and incorrect differentiation to neuronal cell types.

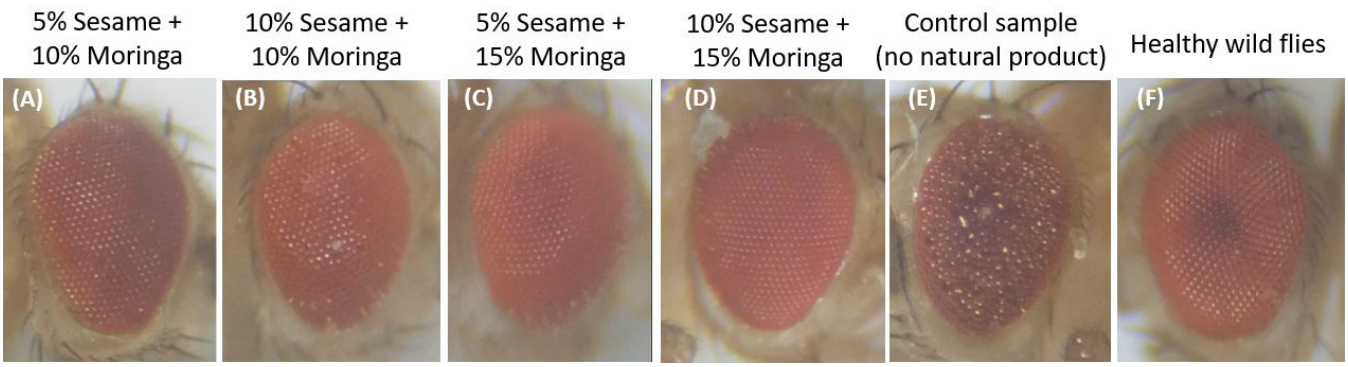

Figure 1. Flies treated with a 10% sesame and 15% moringa diet exhibited the most uniform ommatidia. A-F) Representative images of fly eyes from vial #1 (A; 5% sesame + 10% moringa), showing some nonuniform ommatidia and tumor symptoms (rough eye); vial #2 (B; 10% sesame + 10% moringa), also showing some nonuniform ommatidia and tumor symptoms; vial #3 (C; 5% sesame + 15% moringa), showing some nonuniform ommatidia but no tumor symptoms; vial #4 (D; 10% sesame + 15% moringa), showing no tumor symptoms and uniform ommatidia; vial #5 (E; water control), showing tumor symptoms and nonuniform ommatidia; and vial #6 (F; wildtype flies with water control), showing no tumors and uniform ommatidia. All images of progeny were taken five weeks after the parents mated. All experiments were performed with one trial.

In our previous study (25), we showed that individually, a 10% sesame diet showed some repressed tumor formation (showing smooth eye symptoms) and a 15% moringa diet showed good results in preventing cancer (showing both smooth eye and uniform ommatidia). In this work, we hypothesized that a combination of sesame and moringa in the right proportion would be even more effective as anti-cancer agent because of the great absorbance of the sesame (26) and presence of the anti-tumor compound niazimicin in moringa (27). We also hypothesized that we could find a combination therapy to restrict early stages of cancer.

RESULTS

Treatment of flies with combination of sesame and moringa for cancer prevention

To determine whether a dietary combination of sesame (Se) and moringa (Mo) could prevent *D. melanogaster* model of eye cancer better than either plant alone, we first wanted to determine the optimal concentration of each. In our earlier study, we tested doses in the range of 5–75% for sesame and 5–90% for moringa individually. We found that the optimal dose of sesame was 5–10% and of moringa was 10–15% when treated individually, so we used these doses in this current study (25). Gal4-RET flies were placed in four test vials, containing different combinations of sesame (5% or 10%) or moringa (10% or 15%). A 6th vial, containing healthy wild-type flies, was also taken without expression of any transgene (did not add any experimental food to the vial) for reference (**Table 1**). We also studied combinations of higher concentrations of sesame and moringa, including 15% sesame and 20% moringa, in 7th and 8th vials but flies did not survive (25). Higher concentrations killed the flies as it may be too high of a dose for the flies. The flies in all the vials were fed with Formula 4-24® Instant *Drosophila* Medium food (15ml), water (15ml) and 6-8 grains of yeast were added to each of the vials.

Fly eyes from vials 1 (5% Se + 10% Mo) and 2 (10% Se + 10% Mo) showed some nonuniform ommatidia and few tumor symptoms such as a rough eye phenotype, and fly eyes from vial 3 (5% Se + 15% Mo) showed a dark patch in the center with some nonuniform ommatidia (**Table 1, Figure 1**). Fly eyes from vial 4 (10% Se + 15% Mo) did not show any signs of tumors or nonuniform ommatidia. Fly eyes from control vial 5 (water only) showed clear, nonuniform ommatidia as expected. Fly eyes from the healthy wildtype flies in vial 6, which did not express any transgene, did not show any nonuniform ommatidia, as expected. Twenty flies were randomly chosen from each vial to score for tumors and uniformity of the ommatidia. We analyzed the percentage of flies that showed smooth eyes and uniform ommatidia in each vial (**Figure 2**). The percentage of smooth-eyed flies were 60%, 75%, 90%, 95%, 10% and 100% for vials 1–6, respectively. All of the experimental vials showed good improvement compared to the control vials. The combination of 10% sesame + 15%

Vial	Treatment	Type of flies	# of flies with smooth eyes	# of flies with uniform ommatidia	# of flies that died	Total # of flies tested
Vial #1	5% Se + 10% Mo	Gal4-RET cross	12	9	0	20
Vial #2	10% Se + 10% Mo	Gal4-RET cross	15	13	0	20
Vial #3	5% Se + 15% Mo	Gal4-RET cross	18	16	0	20
Vial #4	10% Se + 15% Mo	Gal4-RET cross	19	17	0	20
Vial #5	Water (Control)	Gal4-RET cross	2	3	0	20
Vial #6	Water	Healthy wildtype flies	20	20	0	20
Vial #7	15% Se + 15% Mo	Gal4-RET cross	N/A	N/A	20	20
Vial #8	15% Se + 20% Mo	Gal4-RET cross	N/A	N/A	20	20

Table 1. Description of the flies and type of food used in each of the 8 vials. Six vials, containing flies were given food with different combinations of sesame and moringa. 1 vial containing flies is given water and standard food and vial #6 had the healthy wild flies for reference. "N/A" indicates results not obtained due to fly death, while "Se" stands for sesame and "Mo" stands for moringa.

Figure 2. The combination of 10% sesame and 15% moringa prevented cancer symptoms in *D. melanogaster* eyes. A) The percentage of flies that showed smooth eye phenotypes were measured following treatment with the indicated combinations of sesame and moringa. The control sample consisted of mutant flies treated with a water control, whereas healthy wildtype flies were also treated with a water control. B) The percentage of flies that showed uniform ommatidia were measured following the same conditions as above. Twenty flies were randomly chosen from each vial to score for the tumors and uniformity of the ommatidia.

moringa showed the greatest effect, with 95% flies displaying smooth eye phenotypes. The percentage of flies with uniform ommatidia were 45%, 65%, 80%, 85%, 15% and 100% for each of the vials 1–6, respectively which showed that all the experimental vials have improved tumor symptoms compared to the control vials.

The lifespan of the flies, measured as the number of days flies were alive, with all the combinations of sesame and moringa was greater than the control flies (**Figure 3**). Survival percentage is the number of flies survived after certain days. As the number of days increased after a certain value, the survival percentage decreased and reached close to 0%. The median survival time for the control sample of Gal4-RET cross flies was 30 days and that of healthy wildtype flies was around 98 days. The median survival time for the test samples were between 55 days and 90 days. The median survival time for vial 1 (5% Se + 10% Mo), vial 2 (10% Se + 10% Mo), vial 3 (5% Se + 15% Mo), and vial 4 (10% Se + 15% Mo) were 55 days, 71 days, 71 days, and 90 days, respectively. This study also showed that as we increased the concentration of sesame and moringa in the mixture, the lifespan of the flies increased, with the best survival rate for the 10% sesame and 15% moringa combination. The slope of each of the curves appeared similar to one another as the number of days increases. This means that the number of flies that died per day was similar for each of the vials.

Treatment of flies with combination of 10% sesame & 15% moringa to restrict early stages cancer cell growth

Next, we wanted to test whether a combination of 10% sesame and 15% moringa could restrict early stages of cancer by administering this combination to the flies during different stages of the fly life cycle, specifically the second instar larval stage, third instar larval stage, pupal stage, and newly eclosed adult stage (**Table 2**). We examined the eyes of each fly for tumors and ommatidia appearance, two weeks after the larvae turned into adult flies. Eyes of the flies from vial 1 (second instar), showed a small area of tumor and uniform ommatidia in the rest of the area (**Figure 4A**). Fly eyes for the control sample (vial 2) compared to flies from vial 1 (second instar) showed complete rough eye (complete tumors, tumors formed on the entire area of the eye) and nonuniform ommatidia (**Figure 4B**). Thus, the 10% sesame and 15% moringa combination showed better performance in restricting cancer growth compared to the control sample.

Eyes of flies from vial 3 (third instar) showed few areas of tumor and uniform ommatidia in the rest of the area (**Figure 4C**). Fly eyes for the control sample (vial 4) compared to flies from vial 3 (third instar) showed complete rough eye and nonuniform ommatidia (**Figure 4D**) which indicates that the 10% sesame and 15% moringa combination had an effect in restricting cancer growth compared to the control sample.

Fly eyes from vial 5 (pupa) showed complete tumors and nonuniform ommatidia (**Figure 4E**) similar to the fly eyes for the control sample in vial 6 (**Figure 4F**). Fly eyes from vial 7 (newly eclosed) showed complete tumors and nonuniform ommatidia (**Figure 4G**) similar to the fly eyes for the control sample in vial 6 (**Figure 4H**).

In order to monitor the progression of cancer symptoms, we examined several flies that were treated during second and third instar larval stages several days to weeks after the pupae turned into flies (**Figure 5A-H**). In the first fly, a small tumor was found on the left side of the eye on the first day after the fly eclosed from the pupae (**Figure 5A**). After four days, the tumor size increased until nine days (**Figure 5B-C**) and did not change after that. A similar pattern was observed in a second fly (**Figure 5E-H**) where the tumor size did not grow after nine days.

DISCUSSION

We hypothesized that a combination of sesame and moringa would be more effective as an anti-cancer agent than either plant alone and could help prevent and restrict early stages of tumor growth. We determined the optimal combination of moringa and sesame to prevent tumors in the *Drosophila* eye to be 10% sesame and 15% moringa. In our

Vial #	Stage at which flies were treated	Treatment
Vial #1	2nd instar larval stage	Experimental (10% Se + 15% Mo)
Vial #2	2nd instar larval stage	Control (Water)
Vial #3	3rd instar larval stage	Experimental (10% Se + 15% Mo)
Vial #4	3rd instar larval stage	Control (Water)
Vial #5	Pupal stage	Experimental (10% Se + 15% Mo)
Vial #6	Pupal stage	Control (Water)
Vial #7	Newly eclosed adult stage	Experimental (10% Se + 15% Mo)
Vial #8	Newly eclosed adult stage	Control (Water)

Table 2. Description of the flies, type of food used and the stage at which flies started treatment in each of the eight vials.

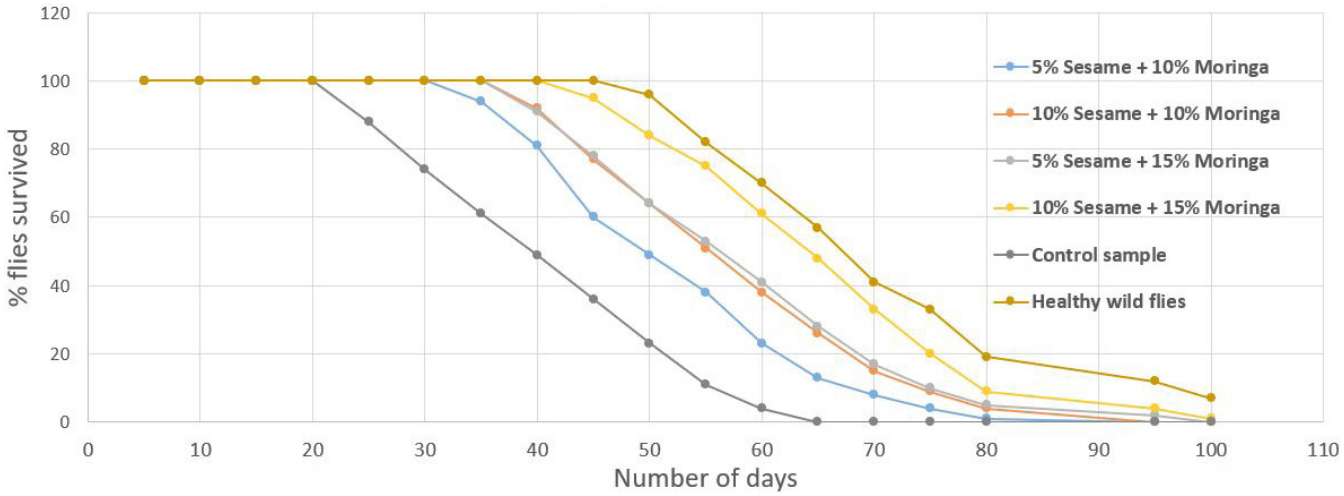

Figure 3. Treatment of flies with all tested combinations of sesame and moringa improved lifespan compared to controls. The lifespan of flies in each condition was calculated as the percent of flies surviving at each timepoint. Mutant flies were administered diets consisting of the indicated combinations of sesame and moringa or a water control. As an additional control, healthy wildtype flies were administered a water control. Twenty flies were analyzed for each vial.

earlier work (25), we showed that sesame at a concentration of 10% can be used to prevent rough eye phenotypes, while moringa at a concentration of 15% was found to be promising for preventing rough eye and uniform ommatidia in this fly model of cancer. In this work, we first wanted to determine the best concentration of each compound. We used different combinations of sesame and moringa. At higher concentrations, such as 15% sesame and 20% moringa, none of the flies survived in any of the samples, so we could not examine the eyes for rough versus smooth eye phenotypes. Past studies showed that high doses of these compounds may actually lessen their anti-oxidant effects (28) and that extremely high doses may cause buildup of iron which can cause gastrointestinal distress and hemochromatosis, a condition caused due to an overload of iron in the body (29).

The fly eyes treated with 10% moringa and any concentration of sesame showed few tumor symptoms and some nonuniform ommatidia. Fly eyes from vials with 15% moringa and 5% sesame showed no tumor symptoms but some nonuniform ommatidia. Fly eyes from vials with 15% moringa and 10% sesame prevented cancer in the *D. melanogaster* eye and the outcomes, that of smooth eye and uniform ommatidia, appeared similar to those of a healthy wildtype fly. The flies treated with 10% sesame and 15% moringa are gene muted flies and hence the eyes of the flies do not look similar to those of the healthy wildtype fly eyes. This work showed that the lifespan of the flies in all the combinations of sesame and moringa increased compared to that of the control flies (Gal4-RET + water). This study also showed that as we increased the concentration of sesame and moringa in the combination, the lifespan of the flies increased, with the best survival rate for 10% sesame and 15% moringa combination. The slope of all the curves are similar, which signifies that the death rate is similar for all the sample flies once the initial flies start to die.

Since we identified the best concentration of each compound to use in the combination, we then treated flies with this optimized combination. We started the treatment at the indicated stage (second instar larval stage, third instar larval stage, pupal stage, and newly eclosed adult stage)

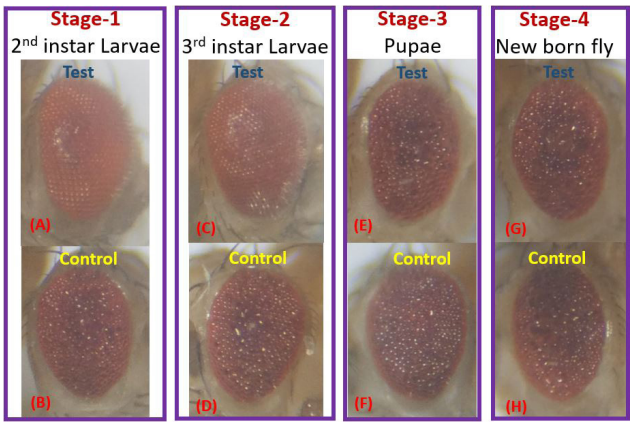

Figure 4. Flies treated during second and third instar larval stages restricted cancer growth compared to controls. A) Representative image of the eyes of the flies from vial #1 (second instar larvae with 10% sesame and 15% moringa combination) showing small area of tumor and uniform ommatidia everywhere else. B) Representative image of the eyes of the flies from vial #2 (second instar larvae with water control), showing cancer symptoms (tumor and nonuniform ommatidia). C) Representative image of the eyes of the flies from vial #3 (third instar larvae with 10% sesame and 15% moringa combination) showing few areas of tumor and uniform-ommatidia in the rest of the area. D) Representative image of the eyes of the flies from vial #4 (third instar larvae) showing cancer symptoms (tumor and nonuniform ommatidia). E) Representative image of the eyes of the flies from vial #5 (Pupa with 10% sesame and 15% moringa combination) showing cancer symptoms (tumor and nonuniform ommatidia). F) Representative image of the eyes of the flies from vial #6 (Pupa), showing cancer symptoms (tumor and nonuniform ommatidia). G) Representative image of the eyes of the flies from vial #7 (eclosed flies with 10% sesame and 15% moringa combination) showing cancer symptoms (tumor and nonuniform ommatidia). H) Representative image of the eyes of the flies from vial #8 (eclosed flies) showing cancer symptoms (tumor and nonuniform ommatidia).

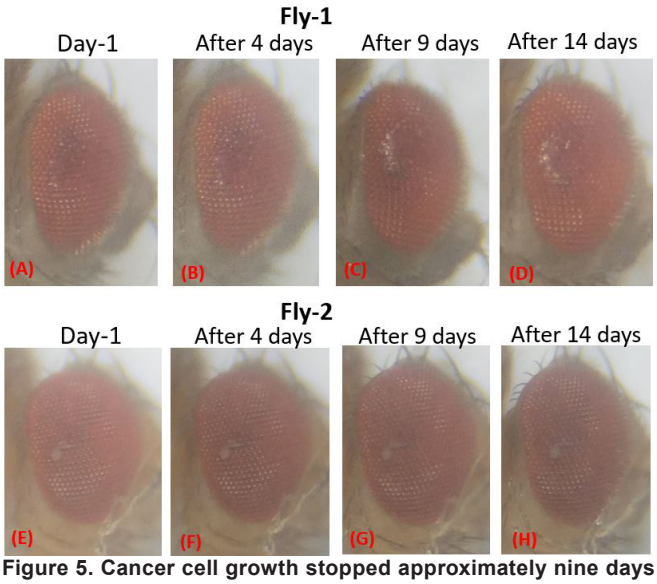

Figure 5. Cancer cell growth stopped approximately nine days after the fly eclosed from the pupal stage for the flies treated during second larvae stage. A-D) Images of the eye of fly #1 from vial #1 (mutant flies treated with 10% sesame and 15% moringa) (A) shortly after, (B) 4 days, (C) 9 days and (D) 14 days after pupae eclosion. E-H) Images of the eye of fly #2 from vial #1 (A) shortly after, (B) 4 days, (C) 9 days and (D) 14 days after pupae eclosion.

and continued through adulthood for the duration of the experiment. During the larval stages, imaginal discs of *D. melanogaster* eyes are formed, and during the pupal stage, eyes are developed (30). Since most of the food consumption in the flies occurs during the larval stages, we reasoned that if we treated the flies before the larval stage, we might prevent cancer occurrence using these natural products (25, 31). Our study revealed that the right combination of sesame and moringa could prevent cancer in *D. melanogaster.*

We were not able to restrict the tumor growth when we treated the flies in the pupal or early adult stages using the combination of sesame and moringa, likely because fly eyes were already developed by the pupal stage. We were also not able to prevent the cancer in the early stages (extract during second instar larvae and third instar larvae) when treated with the experimental food but were able to restrict the cancer cell growth to a certain extent. Cancer cell growth is restricted more severely when treated during second instar larval stage versus during the third instar larval stage. We tried treating the first instar larval flies using 10% sesame and 15% moringa combination but at this stage the larvae are so delicate, handling larvae at the first instar larval stage was lethal to the animals. The fact that we were not able to treat the cancer when treated in the initial stages may be because the natural products took some time to be absorbed into the fly or might require a longer amount of time to work.

The control samples for all the respective experimental stages showed tumors on the entire eye which indicated that the combination of 10% sesame and 15% moringa was indeed a potent combination to restrict cancer cell growth when administered during early stages of fly development. Moringa contains the anti-tumor compound niazimicin (27). Sesamin compound in sesame is known to have high absorbance into cells (25), which perhaps helps increase the absorbance of the compound Niazimicin when the combination of sesame and moringa was taken. The dosage concentrations recommended based on this work are 10% sesame and 15% moringa but in reality, these amounts may not be consumed by most people in their daily diets. Possible methods to increase the amount of moringa we consume is by adding moringa leaves to our soups and stews like lentil soup or chicken soup, adding them to drinks such as hot teas, or adding them to other foods such as scrambled eggs or egg rolls. Possible methods to increase the amount of sesame we consume could include using sesame to season meat or vegetables, to decorate baked goods with sesame seeds, to serve as salad dressing, or to make desserts with sesame oil (32).

Although we tried our best to minimize errors, there were some challenges and limitations in this study. The most challenging part was to collect the newly eclosed flies. Pupae were mostly stuck on the vials. We had to keep monitoring the pupae for eclosing and move them into fresh vials so that we did not mix them up with already present adult flies in the vial. The flies took several hours to eclose out of the pupae, so we had enough time to monitor them. The adult flies in the vial had to be flipped out into another vial on a frequent basis to transfer out the larvae, pupae, and eclosed flies. The microscope used in this study was not a high-resolution microscope, so some of the actual data may slightly vary from what was shown here, as we were unable to see smaller changes, but we do not think this would change the conclusions of the study. Although the flies were anesthetized during analysis, occasionally they moved slightly, making it difficult to capture high quality images. The procedure to pick the larvae could be improved in order to minimize the time the larvae spend exposed to the atmosphere. This might decrease the progression of the tumors size which would need further study. This study showed a combination of moringa and sesame treatments during the second instar or third instar larvae could prevent cancer and also restrict early stages of cancer growth in this specific model of cancer using *D. melanogaster*.

MATERIALS AND METHODS

Drosophila melanogaster care and crosses

Drosophila wildtype, the Oregon-R strain flies were purchased from Carolina Biological Supply Company. The RetMEN2B mutant and UAS-GAL4 driver lines were purchased from Bloomington *Drosophila* Stock Centre. The GMR-Gal4 line (STOCK# 1104) was crossed to the UAS-RetMEN2B line to drive expression of the RetMEN2B allele in the eye. The guidelines of the Carolina ® *Drosophila* manual was used to care the *Drosophila melanogaster* (32). The flies in all the vials were fed with Formula 4-24® Instant *Drosophila* Medium food (15 mL) and water (15 mL). Six to eight grains of yeast were added to each vial.

To treat flies with different combinations of sesame & moringa, at the indicated concentrations, for cancer prevention, four experimental vials along with a control vial without any natural products (water and regular Formula 4-24®) were used to compare with the experimental samples. A cross between UAS-RetMEN2B male flies and GMR-Gal4 female flies was set up by adding six to eight flies from each of the five vials. A sixth control vial was set up with just water and added healthy wildtype flies. After four to five days, the parental flies were separated, and the larvae were allowed to grow into adult flies. In about two weeks, most of the larvae turned into adult flies. Twenty flies were chosen randomly from each vial and scored for the tumors and uniformity on the ommatidia.

To treat flies with a combination of sesame & moringa, at the indicated concentrations, to restrict early stages of cancer cell growth, a cross was setup. The flies were allowed to mate for three to four days, and the parental flies were removed from the vial. Fifteen flies at each of the developmental stages were taken out and moved into eight vials for testing.

Experimental food preparation

Fifteen mL of Formula 4-24® Instant *Drosophila* Medium food was mixed with 15 mL of a liquid solution to make a total of 30 mL food. The liquid solution was a mixture of sesame, moringa, and water. Commercial liquid extracts of organic, Costa Rican *Moringa oleifera* (Pura Vida moringa) and Hawaiian *Sesame indicum* (Hawaii Pharm LLC) were used without further purification. For example, to prepare a sample containing 20% moringa, we added 3 mL of moringa extract to 12 mL of water and mixed well.

Handling and phenotypic analysis

All flies were examined under an AmScope SE306R-PZ-LED Stereo Microscope using 20X, 40X, and 80X magnification. The flies were put to sleep using a FlyNap® Anesthetic Kit (Item # 173010). We followed the procedures in the Carolina® *Drosophila* Manual (33). The difference between a rough eye and a smooth eye under the microscope is shown in **Figure 1E-F**. The *D. melanogaster* compound eye contains approximately 700-750 optical units known as 'ommatidia' (34). Ommatidia could be visibly seen under the microscope and we examined the uniformity qualitatively. The lifespan of the flies was initially measured by counting the number flies that died every five days, at the start of the experiment when deaths were relatively few. Once many flies were dying, we started counting the number of flies that survived.

To test whether the combination of sesame and moringa could restrict early stages of cancer growth, we transported most of the larvae carefully using a paint brush onto on a piece of paper. The second instar and third instar larval stages were separated based on the size of the larvae. Second instar larvae were 1–2 mm smaller than the third instar larvae. We note that it is important not to separate the larvae when they are moving since they elongate and stretch during this movement, making it possible to confuse a second instar for a third instar larva. We also picked some pupae from the vial, leaving some pupae to eclose into adult flies. We placed the vial horizontally and used the back of the paint brush to gently tap the pupae. Once detached from the surface, the pupae were placed on a clean surface.

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