

Evaluation of the Pumpkin Seed Membrane as a Nutrient in the Fruit Fly Model, Fed With High Fat Diet

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Abstract: The pumpkin seed membrane (PSM), which is high in water retention in the study, was recycled as waste, intended to be used in terms of target-non-target creatures and to determine its effect on resistance formation. Nowadays, when fat intake is steadily increasing through nutrition, increased use of fat makes an individual vulnerable to stress. The study determined the effect of additional taken waste/nutrients on oxidative stress in obese individuals. The fatty diet and pumpkin seed effect were trialed in the model organism in the study. The amount of Malondialdehyde (MDA) and total oxidation (TOS) and total antioxidant effect (TAS) and glutathione-S-transferase (GST) activity in larvae, pupae, and adult of *Drosophila melanogaster* were calculated by adding different proportions of PSM (0.05-2 g) and fat (20%) to the diet. According to the data obtained: It was determined that the period with the highest oxidative stress index (OSI) is the pupae (pupae> larvae> female> male), the amount of lipid peroxidation (MDA) is higher in females, moreover, the third larvae is the most resistant stage. The results have shown that waste of seed membrane can't be useful in vivo as a nutrient due to usage limitation.

Keywords: *Drosophila*, glutathione-S-transferase, lipid peroxidation, nuts-pumpkin, oxidative stress index

Yüksek Yağlı Diyetle Beslenen Meyve Sineği Modelinde Besin Olarak Kabak Çekirdeği Zarının Değerlendirilmesi

Öz: Çalışma atık olarak geri dönüştürülebilir ve su tutma oranı yüksek olan kabak çekirdeği zarı (PSM), hedef dışı canlılar açısından kullanılması ve direnç oluşumuna etkisinin belirlenmesi amacıyla yapılmıştır. Beslenme yoluyla yağ alımının giderek arttığı günümüzde, artan yağ kullanımı bireyi strese karşı savunmasız hale getirmektedir. Obez bireylerde ek alınan atık/besin maddelerinin oksidatif stres üzerindeki etkisi de çalışma ile belirlenmiştir. Çalışmada model organizmada yağlı diyet ve kabak çekirdeği etkisi denenmiştir. *Drosophila melanogaster* diyetine %20 yağ ve farklı oranlarda PSM (0.05-2 g) eklenerek larva, pupa ve yetişkin bireylerde Malondialdehit (MDA) ve toplam oksidasyon (TOS) miktarı, toplam antioksidan etki (TAS) ve glutatyon-S-transferaz (GST) aktivitesi hesaplanmıştır. Elde edilen verilere göre: Oksidatif stres indeksinin (OSI) en yüksek olduğu dönemin pupa (pupa>larva>dişi>erkek) olduğu, kadınlarda lipid peroksidasyon (MDA) miktarının daha yüksek olduğu, ayrıca üçüncü larva en dirençli evre olduğu belirlenmiştir. Sonuçlar, kullanım sınırlaması nedeniyle tohum zarı atıklarının in vivo besin maddesi olarak yararlı olamayacağını göstermiştir.

Anahtar Kelimeler: *Drosophila*, glutatyon-S-transferaz, lipid peroksidasyonu, fındık-kabak, oksidatif stres indeksi

1. Introduction

Food products go through many stages until they arrive at the table from the field, where various waste can occur during

the production-consumption process.

Recently, studies on recycling food waste have become important (Töke and Ergülen, 2020; Güneysu, 2020; Filimonau et al.,

2021). Making food waste available by recycling alongside being of alarming size has set the stage for biological and biomedical research (Qiu et al., 2019; Güneş, 2020). Determining the availability of sustainable waste with little physiological impact on living things in a laboratory environment is important to solve or reduce this problem. Pumpkin seeds (*Cucurbita pepo* L.) oil and bark are a product used for herbal treatment in various diseases and as a feed additive (Syed et al., 2019; Valdez-Arjona and Ramírez-Mella, 2019; Alshehry, 2020; Majid et al., 2020). While the use of the pumpkin seed membrane (PSM) is available in the paper and clothing industry, there are few studies on the possibility of a nutritional environment (Güneş and Biçer Bayram, 2019).

However, nutrition affects the quality of life of living as an important factor in the occurrence of chronic diseases (Blüher, 2020). Compounds added to standard nutrients are increasing in proportion to increased nutrient intake. Excessive fat intake, especially with diet, has become a global problem (Genç and Köse, 2019; Bocharova and Teplyakova, 2020; Sağlam and Soylu, 2019; Blüher, 2020; Bendor et al., 2020). Chronic diseases, particularly obesity, are on the rise because of an imbalance in the number of calories taken and calories consumed, with increased work time and inactivity at home, especially

during the last one-year COVID-19 pandemic period. A high-fat diet (HFD) has been associated with obesity, diabetes, and heart diseases, it has been stated, which can increase aging and oxidative stress by researchers (Alzoubi et al., 2018a; Lorizola et al., 2018; Cormier et al., 2021). Phytochemical herbal products and compounds that have an antioxidant effect, such as polyphenol, are used to reduce stress and body weight in living things or to increase the lifespan (Chen et al., 2017; Chung et al., 2018; Güneş, 2020; Mohamad et al., 2020; Zhang et al., 2020; Salama et al., 2020). Therefore, the oxidative stress of plant products and the nutritional relationship with the fatty diet is a current issue studied by researchers (Koh et al., 2013; Llana-Ruiz-Cabello et al., 2015; Maduagwuna et al., 2020; Uysal ve Çelik, 2021). Therefore, the availability of the supplemental product needs to be evaluated well.

Model organisms are organisms from which biological events and processes can be easily studied and inferred, without ethical need (Matthews et al., 2020; Nainu et al., 2020). In studies of obesity-induced by oxidative stress in nutrition, in determining the effectiveness of phytochemicals, they are used fruit flies as models of human disease (*Drosophila melanogaster* Meigen) (Siddique et al., 2005; Hazır et al., 2020). Experimental use of *D. melanogaster*, which

contains tissues corresponding to human tissues, is also increasing in the study of some diseases (obesity, diabetes) that occur because of today's dietary habits (Lüersen et al., 2019; Poças et al., 2020; Cormier et al., 2021; Güneş, 2021). They are also considered to be used in COVID-19 treatment and drug production in recent studies (Nainu et al., 2020).

When evaluating the availability of pumpkin seed membrane as a nutrient environment due to its strong water retention capacity for in vitro studies (Çankaya and Özcan, 2017; Syed et al., 2019; Alshehry, 2020), tried determining the effect on oxidative stress (malondialdehyde-MDA, glutathione-S-transferase-GST, total antioxidant level-TAS, total antioxidant level-TOS, oxidative stress index-OSI) in the obese model. Thus, suggestions have been developed for usage possibilities according to the resistance of the insect fed with PSM waste.

2. Material and Method

2.1. Material

In the study, commercial PSM was received by Pinar nuts, Nevşehir type in 2018–2019. After PSM was sterilized (30–40 °C), and added to foods without freezing. *D. melanogaster*'s strain (W^{1118}) was incubated in 50 ml falcon tubes with $25 \pm 2^\circ\text{C}$ and 60%-70% relative humidity, 12/12 hours light/dark photoperiod. The standard

nutrient medium (SNM: mashed potatoes, sucrose, dry yeast, agar, nipagin, and ascorbic acid) prepared for culture food was used as described by Güneş and Büyükgüzel (2017). SNM was also used as an experimental diet and as a negative control group (1).

In the study, palmitic acid (20%, HFD, positive control group 2) was dissolved within Tween-80 and added to the hot SNM (Sun et al., 2010). Trial design groups; PSM 0.05 g/L (3), PSM 2 g/L (4), PSM 0.05 g/L + HFD (5), PSM 2 g/L + HFD (6) consist of these ingredients. The amount of PSM and HFN use were determined similar to the previous study (Güneş and Biçer Bayram, 2021). The females and males (5/1: about 100 individuals per bottle under carbon dioxide anesthesia) were taken for the culture, and they were mated for 48 h and the eggs were collected after six hours. Flies 1st stage larvae (100 individuals per bottle) were inoculated with experimental nutrient design and grown to the adult stage. 3rd stage larvae, pupae, and 3-day old virgin adult (20 female/20 male) stages were used for each trial in the biochemical analysis (lipid peroxidation product: MDA amount, antioxidant enzyme: GST activity, oxidative stress index: OSI, total antioxidant level and total oxidation level: TAS / TOS).

Samples were extracted with homogenization buffer (1.15% potassium

chloride, 25 mM dipotassium hydrogen phosphate, 5 mM ethylenediamine tetraacetic acid, 2 mM phenethylsulphonyl, 2 mM dithiollotitol, pH: 7.4) in an ultrasonic homogenizer at + 4 ° C. MDA amount (Jain and Levine method, 1995, 532 nm), GST (EC 2.5.1.18) activities (Habig et al., 1974, 340 nm), and total protein (Folin-Lowry, 1951, 600 nm) of samples were measured using a spectrophotometer. TOS ($\mu\text{mol H}_2\text{O}_2 \text{ Eq/L}$) and TAS (mmol Trolox Eq/L) of samples were measured, and oxidative stress index ($\text{OSI} = \text{TOS} / \text{TAS}$) was determined to the standard formula (Chen et al., 2017; Erel, 2005; Özgün et al., 2013; Güneş, 2021).

The statistical analysis of triplicate experiments included one-way analysis of variance (ANOVA) using SPSS software, followed by the least significant difference (LSD) test to identify significant differences between means. "Kruskal-Wallis" test was used to determine the changes in the stages of MDA and GST analysis. The significance of the mean between groups and stages was evaluated at the level of 0.05.

3. Results

About 0.05 g of PSM has no negative effects on the development of life, while the negatively affecting amount (LC_{50}) is 2 g (Güneş and Biçer Bayram, 2021) on insect.

3.1. Experimental Design and Analyzes

Looking at Table 1; the TOS levels of insects in the larval stage nutrition on PSM increased by $0.50 \mu\text{mol/L}$ in those fed with 2 g (S.E ± 0.1) compared to the control group. The oil addition (HFD + 2 g PSM) allowed this increase to increase to $2.00 \mu\text{mol/L}$. When the larval TAS levels were compared to control, the addition of PSM to the nutrient saw a severe decrease in total antioxidant activity from 8 to 0.005 mmol/L . According to the oxidative stress index, it is seen that stress starts with one gram of PSM and is highest at 2 g.

During the pupae stage, it was determined that oxidation with oil use increased to $32 \mu\text{mol/L}$. The insect was found to be feeding on PSM, causing it to drop to $1 \mu\text{mol/L}$. High concentration PSM and nutrition showed that oxidation continued at the same rate. Although the level of antioxidants increased in groups with no oil used during the same period, the use of PSM with oil was observed to decrease the level of TAS (1.00 mmol/L). Although the oxidative stress index showed fluctuations during the pupal period, the combination of 2 g of PSM and oil allowed us to rate it as the group with the highest stress (Table 1).

Table 1. TOS, TAS, and OSI values during fly developmental periods (standard errors are between 0.01–0.1 and are not displayed in the table; df: 35; P:Significance level)

Groups		TOS ($\mu\text{m/L}$)	TAS (mm/L)	OSI	P
Larvae (n:20)	1	1.5	8.0	0.2	*4 and 6 are similar, and different from others.
	2	1.8	9.0	0.2	
	3	0.5	4.0	0.1	
	4	2.0	0.1	20.0*	
	5	1.0	4.0	0.3	
	6	2.0	0.1	20.0*	
Pupae (n:20)	1	24.5	2.0	12.3	1,3,4,5 are similar, while others are different.
	2	32.0	5.0	6.4*	
	3	1.0	0.1	10.0	
	4	32.0	3.0	10.7	
	5	1.0	0.1	10.0	
	6	32.0	1.0	32.0**	
Female (n:20)	1	0.5	0.1	5.0	All groups are different from each other.
	2	1.5	0.1	18.8	
	3	3.0	0.1	27.3	
	4	5.5	0.2	32.4	
	5	1.5	0.1	12.5	
	6	8.5	0.2	53.1	
Male (n:20)	1	3.5	1.1	3.2	All groups are different from each other.
	2	4.5	3.6	1.3	
	3	7.0	0.1	70.0	
	4	3.0	0.4	7.5	
	5	9.5	0.1	95.0	
	6	8.5	0.1	106.0	

TOS activity in females increased by 3–3.5 $\mu\text{mol/L}$ with the use of PSM, has dropped by as much as 1.5 $\mu\text{mol/L}$ with the addition of oil. Oxidation at the highest concentration was determined to increase 8-fold to 8.5 $\mu\text{mol/L}$. However, TAS activity was found to be similar in female individuals with different concentrations of PSM intake. Oxidative stress index occurs at normal levels in female individuals in control groups, whereas feeding with PSM causes high stress and is even likely to produce lethal concentration (high oxidative stress) of HFD + 2 g of PSM (Table 1).

Although PSM increases oxidation in nutrition relative to TOS activities of the male individuals seen in Table 1, it has been stated that oxidation along with fat is approximately 3 times greater. Because total antioxidants are not sufficient in male individuals, OSI values were observed to be high. Although the oxidative stress index varied inversely in concentrations in PSM-fed men, the intake of PSM combined with high fat increased stress, resulting in reduced vitality and high oxidative stress.

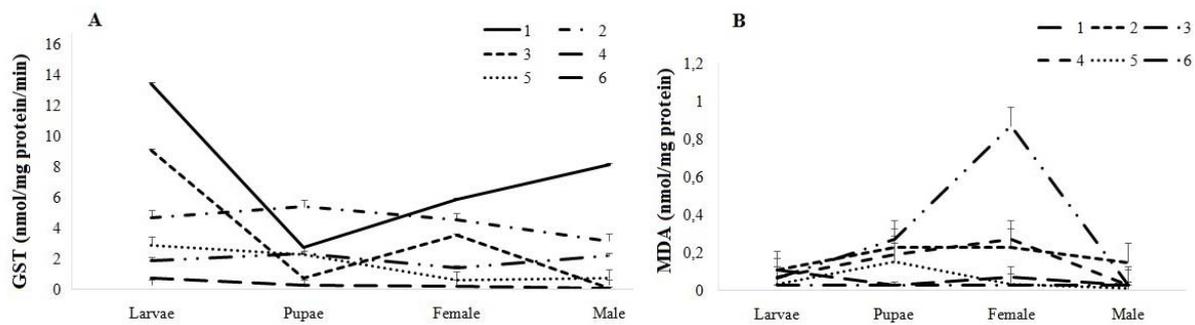


Figure 1. The adults antioxidant-resistance enzyme (A) and lipid peroxidation (B) during development stages, $P > 0.05$ (Kruskal-Wallis test)

According to Figure 1B, the amount of lipid peroxidation formed by larvae in the high-fat diet group was found to be the same as in the control group. Because of this result, PSM intake along with HFD for insects in the larval stage reduces the rate of lipid peroxidation. During the pupal stage, there are fluctuations in the amount of peroxidation, but it appears to increase the amount of MDA 5–9 times because of nutrition additional PSM to the fatty diet. While oxidation in the body fat of female individuals increased because of the amount of PSM, it was determined that with the use of fat this rising increased even higher, increasing of 0.87 ± 0.1 nmol/mg protein in the highest concentration of 2 g PSM + HFD. In male individuals, the use of PSM + fat was determined not to affect peroxidation, although statistically similar results were achieved with control.

If MDA analysis is interpreted between stages (Figure 1B); while the peroxidation of the pupae and female

individuals in the use of fat is excessive, the use of 0.05 g PSM does not appear to be effective between stages. There appears to be no change in female and male individuals despite reducing the amount of high fat intake MDA during the larval and pupal stage along with. The high amount of MDA in pupae and female individuals in high PSM groups indicates that females are more susceptible to oxidation. According to Figure 1A; Using PSM in the larval stage has decreased the resistance enzyme. With the addition of the high-fat diet, GST activity was further reduced, even dropping from 13.40 to 0.75 ± 0.1 (nmol/mg protein/min). During the pupal stage, pups in the HFD group were found to be about 2 times more resistant than control when comparing the resistance enzymes of insects to the control group. The resistance of pupae normalizes with increasing concentrations of PSM; GST activity decreased to 0.27 ± 0.1 (nmol / mg protein / min) in pupae fed with increasing concentrations of HFD + PSM.

GST activity has increased up to the use of 2 g PSM in females. However, individuals without nonresistant have formed. In men, resistance was not seen except for those who were fed only 2 g PSM.

If GST activity is interpreted between stages (Figure 1A); under normal conditions, it was found to be the most resistant stage (pupae < female < male < larvae) larval stage. With high fat intake, the resistance of the pupae phase appears to be increasing. However, in other stages (larval, female, male) resistance was reduced. The most resistant larvae in feeding at 0.05 g PSM were seen in females in the second. Besides fat intake, those fed 0.05 g PSM were determined to have greater GST activity in the larval and pupae stages. At 2 g, pupae and male individuals had the most GST activity. However, it was determined that the high-fat diet reduces resistance in stages or even reduces to amount to be called absent in male individuals (0.05 nmol/mg protein/min).

4. Discussion

Reactive oxygen species (ROS) cause oxidative stress by transporting molecular oxygen from the attack of unshared pairs of electrons; thus, fats, macromolecules such as proteins increase total oxidation in peroxide, and to *Drosophila* the toxic effect. (Guan et al.,

2017; Çolak and Uysal, 2018). Cellular oxidative damage is tried to be balanced with antioxidants taken from outside with cellular or nutrition. However, where the TAS mechanism is insufficient, a different response to stress (TAS <TOS, TAS = TOS, TAS > TOS) can be generated (Güneş, 2016 b). The amount of fat, protein, carbohydrates, vitamins found in the diet is as important in terms of removing tissue damage as the life parameters of insects (Adler and Bonduriansky, 2014; Unckless et al., 2015; Güneş, 2016a). The number of amino acids and proteins in flies fed rich nutrients by fatty diets can be reduced by being affected (Heinrichsen et al., 2014). So, experiments are planned considering the creation of obese model individuals. Our study shows that oxidative stress in the larval stage is highest at 2 g. In the pupae stage, 2 g of PSM taken with a high-fat diet was identified as the group with the highest stress. Holometabolic insects feed in the larval and adult stages, the oxidative stress index is in the form of larvae < pupae < adult (Güneş, 2016b). Female individuals are normally known to be oxidated individuals due to mating and egg production (Le Goff et al., 2006). Although virgin females were used in the study; according to OSI, it is determined that it causes high stress by feeding females with PSM and is even likely to produce fatal effects (high oxidative

stress) of HFD + 2 g PSM (Table 1). Male individuals appear to have high OSI values because total antioxidants are not sufficient. According to OSI, the nutrition of males with PSM varies inversely depending on concentration, but the intake of fat and PSM caused high oxidative stress, resulting in reduced vitality in individuals.

Antioxidant enzymes in *Drosophila*; (SOD, E.C. 1.15.1.1), (CAT, E.C. 1.11.1.6), (GSH, E.C. 2.5.1.18), Glutathione reductase (E.C. 1.8.1.10), GST, Disulfite reductase (E.C. 1.8.1.14), Methionine sulfoxide reductase (E.C. 1.8.4.12), Thioredoxin peroxidase (E.C. 1.11.1.15) (Missirlis et al., 2003; Güneş, 2016a and b). For example, in some studies, it was reported that CAT and GR amounts decreased and GST activity changed in *Drosophila* due to xenobiotics and aging (Durusoy et al., 1995; Fışkın and Asma, 1996; Yeşilada and Gelegen, 2000). It has been reported that the larvae are prevented from pupation because of not only chemicals or aging but feeding and the amount of lipid may increase in adult individuals in case of starvation, or dietary carbohydrate change may cause oxidative stress in the insect. Low unsaturated dietary fats may increase the amount of MDA (De Moed et al., 1999; Jordens et al., 1999; Sisodia and Sing, 2012; Güneş, 2016b). The study found that the uptake of HFD and PSM in the larval stage decreased the amount of MDA while increasing the pupal

stage by 5–9 times; while the amount of MDA due to the amount of PSM in females increased, the increase with the use of fat was seen to be even greater. In males, however, the use of PSM + oil was determined not to affect peroxidation. (Figure 1B). Adult individuals of 3–7 days are preferred for biochemical analyses. (Qiu et al., 2019). The three-day study of adults specifically determined that females were more open to lipid peroxidation than males (Figure 1B).

Pumpkin is a plant known as antioxidant effects (Nkosi et al., 2006; Perez Gutierrez, 2016). *Chenopodium ambrosioides* L. plant extracts can negatively affect *Drosophila* development and resistance (Wohlenberg and Lopes-da Silva, 2009). Which is more common in older and resistant individuals GST is a detoxifying enzyme and resistance enzyme (Piccoli et al., 2019). Using 10 mg of plants such as *Mangifera indica* causes GST activity to decline in *Drosophila* (Etuh et al., 2019). It can cause an increase or decrease in GST activity depending on the experimental model and the texture analyzed (Piccoli et al., 2019). In this study was found that while resistance decreased with the use of oil and PSM in the larval stage, GST activity decreased because of nutrition with HFD + PSM despite the formation of resistant individuals at the pupal stage. In female individuals, nutrition with PSM in

addition to the standard diet increased resistance, and the use of fat led to the formation of nonresistant individuals. With nutrition in males saw a decrease in the amount of GST. While GST activity is in the form of pupae < female < male < larvae among stages, it appears to reduce resistance in the pupae in another stage with high fat intake. High PSM nutrition, on the other hand, was found to be the most GST activity in pupae and male individuals but reduced resistance in the HFD stages (Figure 1A). GST activity is considered a biomarker of toxicity for environmental pollutants (Liu et al., 2015). While GST activity is excessive in the larvae, it supports the study (Vorojeikina et al., 2017).

There has been growing interest in clarifying the role of nutritional habits in the nervous system in studies of inferences with model organisms. It appears that increased HFD and refined sugar-rich diet can reduce cognitive function because of changing dietary habits. Also, there is an increasing interest in antioxidant functional foods and treatments (Alzoubi et al., 2018b). The addition of apple pulp to nutrients was found to be beneficial against oxidative stress and liver damage in mice whose obesity model was created over 30 days with HFD (Chen et al., 2017). Continuing to feed mice with

additional pineapple vinegar (produced from pineapple waste) to the HFD diet is known to lower cholesterol levels, lower blood glucose levels, increase antioxidant levels (Mohamad et al., 2020). *Nephelium lappaceum* L. shell waste added to the diet of obese individuals fed HFD is used to treat the damage caused by oxidative stress (Chung et al., 2018). While studies have shown that food waste can be useful in nutrition or in vivo as a nutrient environment, our study has not seen the same effect.

Conclusion

With high water retention capacity, PSM has been determined that high amounts cannot be tolerated by the insect, with small amounts of mixing of HFD-fed flies into the dietary environment increasing the insect's oxidative stress. Thus, although it was considered inappropriate to use PSM in the creation of a laboratory-fattening environment, it was believed that the resilient larval universe could be used in low quantities of PSM in designs as an experimental obesity model.

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