

# The Effects of Erzincan Grape (*Vitis vinifera* spp., Cimin) and Benzothiazol on a *Caenorhabditis elegans* Organism Model

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## ABSTRACT

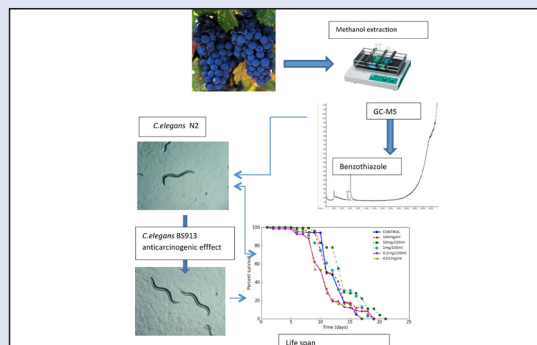
**Background:** Grapes and their products are known to have been used for the treatment of diseases throughout history. **Objective:** It was aimed to investigate the effects of Erzincan Cimin grapes on an organism model of *Caenorhabditis elegans* N2 wild type and *C. elegans* BS913 strains with gonad cancer. **Materials and Methods:** The effects of methanol extracts of the skin and seeds of Erzincan Cimin grapes were examined separately on *C. elegans* N2 wild type and an effect was determined on lifespan. By applying GS-MS analysis, a potential agent substance was determined in the skin and seed methanol extracts. This substance was purchased and the effects of this substance were investigated on lifespan and fertility in *C. elegans* BS913 strains with gonad cancer. In addition, the effects on young subjects exposed to this agent substance in L1 form were investigated. **Results:** Grape seed and skin methanol extract was observed to prolong the lifespan most at a dose of 10 mg/100 mL. Lifespan was determined to be at a maximum in a gonad cancer organism model with benzothiazol at a dose of 50 ppm. At the same dose, positive effects were determined on the fertility of strains with cancer. When the effects of benzothiazol were examined on young L1 forms, an evident retardation of growth was determined at doses of 10, 50, and 100 ppm. **Conclusion:** Owing to anti-carcinogenic effects of benzothiazol and benzothiazol-derived substances, they can be considered as agent substances in academic studies related to cancer.

**Key words:** *Vitis vinifera*, Cimin, Benzothiazol, *Caenorhabditis elegans*, Lifespan, Anti-carcinogenic effects.

## SUMMARY

- The effects of methanol extracts of the skin and seeds of Erzincan Cimin grapes were examined on *C. elegans* N2 wild type and an effect was determined on lifespan
- Through GS-MS analysis, benzothiazol was determined in the skin methanol extracts

- Benzothiazol was purchased and the effects of this substance were investigated on lifespan and fertility in *C. elegans* BS913 strains with gonad cancer
- The effects on young subjects exposed to benzothiazol in L1 form
- Grape seed, skin methanol extract, and benzothiazol was observed to prolong the lifespan
- Positive effects were determined on the fertility of gonad cancer strains.



**Abbreviations used:** GC-MS: gas chromatography and mass spectrometry; *C. elegans*: *Caenorhabditis elegans*; NGM: Nematode growth medium; *E. coli*: *Escherichia coli*; FUDR: Fluorodeoxyuridine; LDL: Low-density lipoprotein.

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## INTRODUCTION

Grapes, as the *Vitis* species of the Vitaceae family, are one of the oldest fruit species with cultural references from 5000 BC. They are a frequently consumed fruit due to both the taste and nutritional value and the health benefits. The regions of origin are Anatolia, Asia, and Caucasia. Compared with other fruits, grapes have the most varieties. It has been estimated that there are more than 15,000 varieties.

*Vitis vinifera* is an extremely important plant economically and medically. For many years, both the leaves and the fruit have been used as a folk medicine for treatment purposes. In various regions, the leaves have been used in astringent, hemostatic, analgesic, and hemorrhoid treatments and the fruit in diarrhea, hemorrhagia, and varicocele treatments and as an eye antiseptic.<sup>[1]</sup> The leaves are also used in Anatolia to lower the blood sugar level in diabetic patients.<sup>[2]</sup>

In addition to the leaves and fruit of *V. vinifera*, the fermentation products of grapes have been used for treatment purposes for many years. Viniculture and wine production is described in Egyptian hieroglyphics

dating from 2400 BC. The therapeutic value of wine has been known since ancient times, with reports that Hippocrates, Pliny, and Galen used wine in the treatment of several diseases.<sup>[1]</sup>

The therapeutic effects of *V. vinifera* on various diseases result from the fact that it contains a significant amount of flavonoids of the polyphenolic structure. The main phenolic compounds abundant in black grapes

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are phenolic acid flavonoids, anthocyanins, and proanthocyanidins. The phenolic compounds are related to the functions of reducing and eliminating the potential cardiac disease risk factors such as antioxidant activity, removal of free radicals, lipoprotein oxidation inhibition, and low-density lipoprotein (LDL) oxidation reduction. Flavonoids gain importance in that they are scavengers of free radicals, regulate enzyme activities, inhibit cell proliferation, and act as an antibiotic, antiallergic, antidiarrhea, antiulcer, and anti-inflammatory drug.<sup>[3,4]</sup> It was proved in the studies carried out that the proanthocyanidins in black grape have therapeutic effects in vascular disorders. Special pharmaceutical products containing the bioactive antioxidant proanthocyanidins in grape seed are used in the treatment of microcirculation disorders such as venous-lymphatic failure and peripheral capillary permeability increase.<sup>[5]</sup>

Erzincan Cimin grapes (*V. vinifera*, Cimin) are grown in the region of Erzincan in Turkey. They are a grape species that is black, each fruit weighing a mean 4g, with a mean of one to four seeds and a sweet mildly acidic taste. There is also a particular bloom on each grape.

It is known that grapes and grape products have been used in the past for the treatment of diseases. Although the health benefits of Erzincan Cimin grapes are known, there have not been sufficient academic studies in literature related to the effects on lifespan, reproduction, and cancer.

The aim of this study was to research the effects of Erzincan Cimin grape on the lifespan of *Caenorhabditis elegans* (*C. elegans*). In addition, by applying gas chromatography and mass spectrometry (GS-MS) to the methanol extracts of the fruit skin and seeds, a potential agent substance was determined and the effects of this substance on lifespan and fertility were tested on *C. elegans* strains with cancer.

## MATERIAL AND METHODS

In this study, the methanol extracts of the grape skin and seeds were tested separately. *V. vinifera*, Cimin, which is used in my research, was collected from 10 different grape grower's vineyard at Uzumlu district in Erzincan province. Fruit skin and seeds were separated from collected grapes. After being smashed in homogenizer, both skin and seeds were exposed to methanol, followed by filtration through Whatman No. 1 evaporated from obtained extract with the evaporator. This process is repeated two times. In the investigation of the effects of grape extract on lifespan, *C. elegans* N2 wild type was used [Figure 4]. As a result of the GS-MS analysis, benzothiazol (Sigma 101338) was determined. This was purchased in its pure form and to test the potential anti-

carcinogenic effects of benzothiazol, the mutant strain *C. elegans* BS913 with gonad cancer cells was used (Genotype: unc-32(e189) glp-1(oz112)/unc-36(e251) glp-1(q175) III, Description: Heterozygotes are WT and segregate WT and Uncs (both Unc-32 and Unc-36)). This has tumorous germline phenotype in both hermaphrodites and males and is semidominant and temperature sensitive. Even at permitted temperatures (15-20°C) brood size is very small (10-20 viable progeny) because of the overproliferation of germ cells at the expense of oogenesis. To examine the effects of benzothiazol on fertility, a control group was formed of *C. elegans* N2 wild type without cancer. To test the effects on fertility of the subjects with gonad cancer, the *C. elegans* BS913 strain was used. In the investigation of the effects of benzothiazol on physical growth, the L1 young form of *C. elegans* N2 wild type was used.

## Synchronization of *C. elegans* strains

Approximately 20 mature *C. elegans* were transferred to a nematode growth medium (NGM) petri dish containing *Escherichia coli* (*E. coli*) OP50. After laying eggs in a period of 4-6 h, the mature *C. elegans* were removed from the petri dish. For the synchronized formation of the young, these eggs were nourished. At the end of the third day, when they had reached adult form, they were used for the study. To be able to provide sufficient *C. elegans* for the study, this procedure was applied to five petri dishes simultaneously.

## Preparation of the NGM

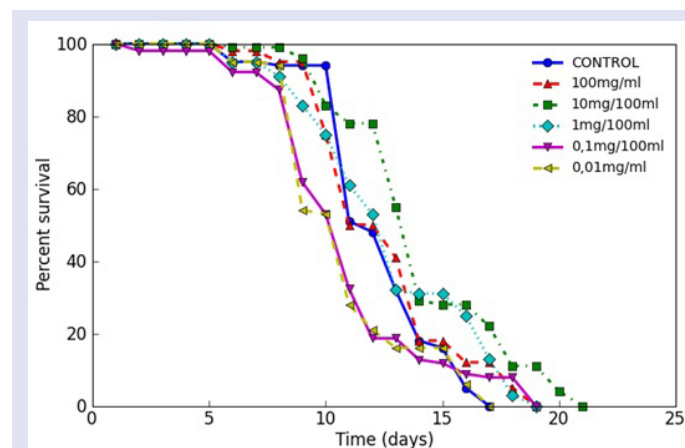
A 2.5 g peptone, 3 g NaCl, and 20 g Agar were dissolved in 1 L of distilled water. After autoclaving at 125°C for 15 min, the mixture was cooled to 55°C. Homogenization was obtained by adding 1 mL MgSO<sub>4</sub> (1M), 1 mL cholesterol (5 mg/mL), 1 mL CaCl<sub>2</sub> (1M), 25 mL KH<sub>2</sub>PO<sub>4</sub> buffer (pH 6), which had been previously prepared and filtered through a 0.2 µm mesh, to the medium. For the study, the grape seed extract, the skin extract, and the benzothiazol were added separately to the medium (for the grape seed extract and skin extract 100 mg/100 mL, 10 mg/100 mL, 1 mg/100 mL, 0.1 mg/100 mL, 0.01 mg/100 mL, for benzothiazol 10, 50, 100, 200, 400 ppm, respectively). Sufficient prepared NGM was transferred to the petri dishes, and after thickening, the *E. coli* OP50 strain was added to the NGM and the mixture was dried in a sterile cabinet. The control group medium was nourished without adding the grape extracts or benzothiazol.

## Lifespan analysis

In the lifespan analysis, the NGM to be used was prepared as described above. To prevent the development of eggs in the *C. elegans* during the lifespan analysis, fluorodeoxyuridine (FUDR) was added to the NGM at the preparation stage. The synchronized *C. elegans* prepared with the grape extracts and benzothiazol at the above-stated doses were transferred to the prepared petri dishes as 20 in each. Each day at the same time until all the organisms died, the live *C. elegans* were counted and compared with the control group.

## Fertility analysis

In the analysis of egg fertility, NGM not containing FUDR was used. Egg counting was applied according to the Koelle *et al.*<sup>[6]</sup> protocol. Twenty-five well-nourished L4 form *C. elegans* were transferred to each petri dish prepared with 10, 50, 100, 200, and 400 ppm benzothiazol. After 24 h, 20 were transferred to a new petri dish and kept at 20°C for 60 min. At the end of this period, eggs were counted at 20× magnification. One day after the number of eggs in the petri dish had been determined, the noncracked eggs were determined and counted; thus, the rate of fertility was calculated and compared with that of the control group. In this process, *C. elegans* BS913 mutant strain was used to investigate the effect of benzothiazol on the fertility of individual with gonad cancer. As the non-cancer control group, *C. elegans* N2 wild type was used.



**Figure 1:** The life span findings of grape seed methanol extract [Figure 1]. The results of grape seed extract on life span. It can be seen from the graph that the most effective dose was 10mg/100 mL and that doses of 1mg/100 mL and 0.1mg/ mL also prolonged the life span

## Control of physical growth

To examine the physical growth, equal numbers of *C. elegans* N2 wild type eggs were added to petri dishes prepared with NGM containing the same doses of benzothiazol with no FUDR added. The petri dishes with 90-100 eggs were checked each day in respect of hatching and physical growth and comparisons were made with the control group.

All the experiments were conducted at 18-20°C and for each dose, five petri dishes were used. Each experiment was conducted twice. The benzothiazol (Sigma 101338) determined in the skin extract as a result of the analyses was commercially purchased. The *C. elegans* strains used in the study were provided by Minnesota University Caenorhabditis Genetics Center, USA. The GS-MS analysis was applied in the Research Laboratory Application and Research Centre at Giresun University, Turkey.

## Statistical analysis

The statistical analyses were applied with SPSS v. 22 software (IBM). In the analysis of the percentage differences between the groups in respect of egg fertility, the chi-square test was used. The difference between the groups of the effect of the skin and seed extracts and benzothiazol on growth was calculated with the one-way ANOVA test. The cumulative survival rate of the *C. elegans* in each group was calculated according to the Kaplan-Mier estimator. A value of *P* less than 0.05 was accepted as statistically significant.

## RESULTS

When the effect was examined of the grape seed extract on the survival results, the most effective dose was determined to be 10 mg/100 mL. Doses of 1 mg/100 mL and 0.1 mg/100 mL were also seen to prolong the lifespan. In the comparisons of the statistical analysis of the grape seed extract and lifespan of the groups, a significant difference was found between the control group and the 10 mg/100 mL group and the control group and the 1 mg/100 mL group ( $P < 0.05$ ) and the differences between the other groups and the control group were not found to be significant ( $P > 0.05$ ) [Figure 1].

When the effect was examined of the grape skin extract on the survival results, the most effective dose was determined to be 10 mg/100 mL. Doses of 1 mg/100 mL and 0.1 mg/mL were also seen to prolong the lifespan. In the comparisons of the statistical analysis of the grape skin extract and lifespan of the groups, a significant difference was found

between the control group and the 10 mg/100 mL group and the control group and the 1 mg/100 mL group ( $P < 0.05$ ) and the differences between the other groups and the control group were not found to be significant ( $P > 0.05$ ) [Figure 2].

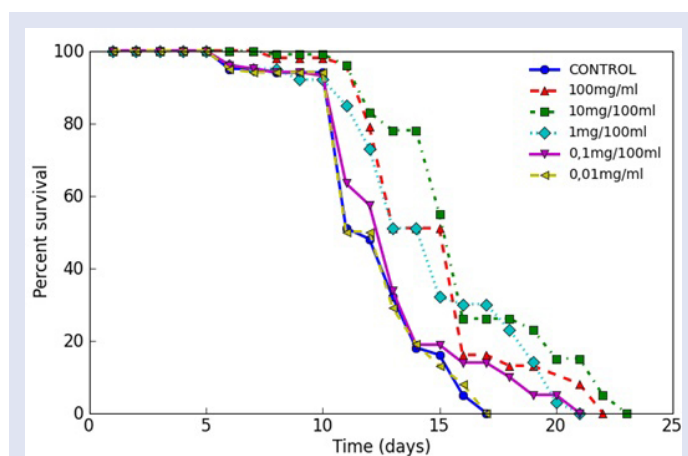
In the examination of the effect of grape seed extract and grape skin extract on lifespan, it was determined that the lifespan could be longer in the experiments made with the skin extract compared with the seed extract.

The potential agent substance, benzothiazol, which was determined in the skin extract as a result of the analyses applied, is not found in the seed extract. That lifespan was prolonged more by skin extract than seed extract was thought to be associated with the effect of benzothiazol. The amount of benzothiazol determined in the grape skin extract as a result of the GS-MS analysis was 21.2 ppm.

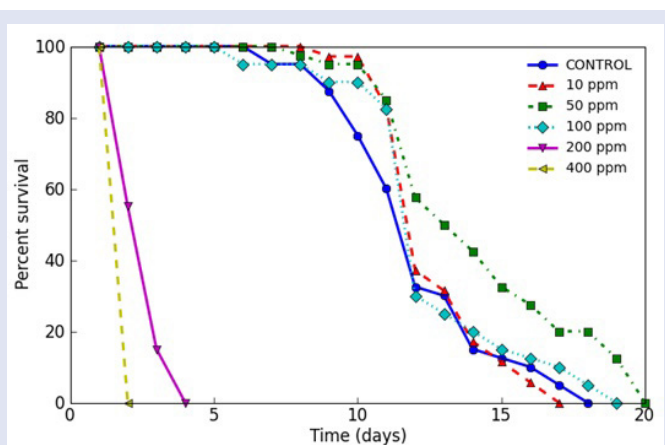
As a result of the experiments, it was determined that benzothiazol significantly prolonged the lifespan of *C. elegans* BS913 mutant strain with gonad cancer. This effect was observed in particular at 50 ppm dosage. Doses of 200 and 400 ppm were determined to be toxic, as all the subjects died at the end of the third day of 200 ppm dose and at the end of the first day of 400 ppm dose. A statistically significant difference was determined between the control group and the 50 ppm dose ( $P < 0.05$ ), and no statistically significant difference was determined between the control group and the 100 ppm dose ( $P > 0.05$ ). In the comparison of the data between the groups, statistically significant differences were determined between the 50 ppm dose and the 10 ppm dose and between the 50 ppm dose and the 100 ppm dose ( $P < 0.05$ ). The 200 and 400 ppm doses were not included in the evaluation [Figure 3].

When the effects of benzothiazol on fertility were examined, benzothiazol was found to have positive effects on the fertility of *C. elegans* BS913 with gonad cancer, and it was determined that there was a significant increase in the number of eggs at the 50 ppm dose in particular [Table 1].

To determine the effect of benzothiazol on young subjects, *C. elegans* N2 wild type L1 form was treated with 10, 50, and 100 ppm doses of benzothiazol and significant growth retardation was determined. As doses of 200 and 400 ppm were toxic, they were not included in the study. At the end of the third day, no statistically significant difference was found between the 10 ppm dose group and the control group ( $P > 0.05$ ), while the differences between the 100 and 50 ppm groups and the control group were determined to be statistically significant ( $P < 0.05$ ) [Figure 5].



**Figure 2:** The life span findings of grape skin methanol extract [Figure 2], The results of grape skin extract on life span. It can be seen from the graph that the most effective dose was 10mg/100 mL and that doses of 1mg/100 mL and 0.1mg/mL also prolonged the life span



**Figure 3:** The effect of Benzathiazol on the life span of *C. elegans* BS913 [Figure 3], The effect of Benzathiazol on the life span of *C. elegans* BS913. It can be seen from the graph that this effect occurred particularly at the dose of 50ppm. Doses of 200ppm and 400ppm were toxic as all the subjects treated with 200ppm died at the end of the 3rd day and those treated with 400ppm at the end of the 1st day





Figure 4: Microscopic images of *C. elegans* BS913 and N2 wild type

Table 1: The effect of Benzothiazol on the fertility of *C. elegans*

	Control		10ppm	50ppm	100ppm
	N2	BS913			
Fertility	102	28	30	39	26
Hatching from Eggs	99	27	28	37	23
%	97.0%	96.4%	93.3%	94.8%	88.4%

## DISCUSSION

The results of this study showed that grape seed methanol extract prolonged lifespan most at a dose of 10 mg/100 mL. The same dose of grape skin methanol extract was also determined to significantly prolong lifespan. In the comparison of skin and seed, the skin extract was seen to be more effective. The substance of benzothiazol, which was determined at an amount of 21.2 ppm in skin extract as a result of GS-MS analysis, was not found in seed extract. Maximum lifespan in a gonad cancer organism model was determined with benzothiazol at 50 ppm dose. At the same dose, positive effects were also determined on the fertility of strains with cancer (*C. elegans* BS913). The effects of benzothiazol were examined on young subjects in L1 form and significant growth retardation was observed at 10, 50, and 100 ppm doses.

Grapevines (*V. vinifera*) are cultivated throughout almost all the world, primarily in southern Europe and western Asia. The fruits, seeds, leaves, and fermentation products are used as both food and herbal medicine. Black grapes are a frequently used folk medicine for the treatment of diseases such as rheumoarthritis, ulcers, immunologic disorders, and inflammation. Grape extracts have been used for many years as herbal medicine due to the organic substances contained in grapes. Using various methods, different substances have been determined in previous research.<sup>[7-12]</sup>

Black grapes have long been known as an anti-carcinogenic food. *C. elegans* has been widely used in several recent scientific studies.<sup>[13-17]</sup> *C. elegans* is an apathogenic nematode, approximately 1 mm in size, which lives in soil. As it has a short life cycle (approximately 14-20 days at 20°C), it is very often used in studies of ageing.<sup>[17,18]</sup> It was the first organism to be determined with a genome series. It has approximately 20,000 genes and a total of six chromosomes. A significant proportion of these genes have been determined to show great similarity to human genes.<sup>[19]</sup>

In the formation of cancer and the ageing process, some of the reasons for the anatomic and functional degeneration observed in organs have been stated to be the damage caused by free radicals and reduced antioxidant capacity. As the known antioxidant property of grapes directly neutralizes free radicals with an oxygen source, it is thought that a protective effect is shown by the activation of antioxidant enzymes or the inhibition of pro-oxidative enzymes. In this context, by increasing the antioxidant capacity of the body,

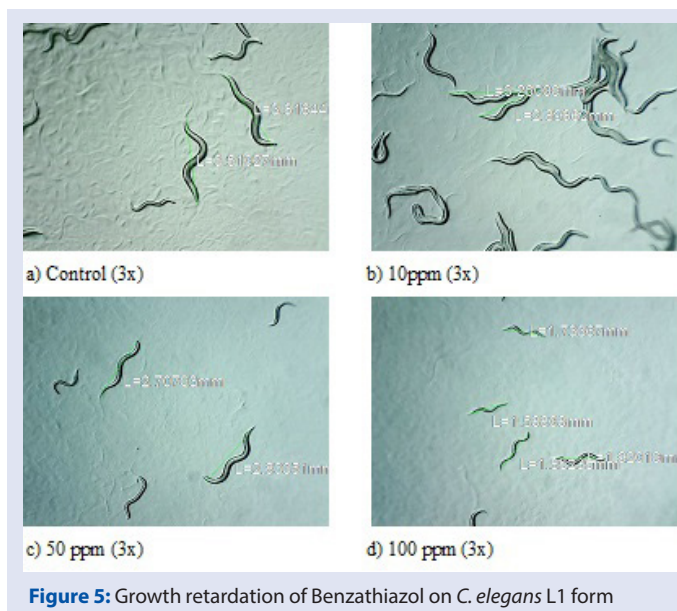


Figure 5: Growth retardation of Benzothiazol on *C. elegans* L1 form

grapes and grape extracts help apoptosis (programmed cell death) and, thus, it is thought that the formation of cancer could be prevented.

The ethanol extract of *V. vinifera* leaves has been tested on diabetic rats, and antidiabetic and antioxidant effects have been observed at a dose of 250 mg/kg. Condensed tannins and flavonoids are primarily thought to be responsible for the activity.<sup>[2]</sup> In another study, peroxidation events were observed to decrease in 13 individuals with mean age of 74.67 years with the daily consumption of 1 g/kg of dried black grapes.<sup>[20]</sup>

In another group, benzothiazol was determined in grape extract in the analyses applied by the researchers.<sup>[21,22]</sup> In the current study, benzothiazol was determined at an amount of 21.2 ppm as a result of GS-MS analysis. While this substance was determined in grape skin methanol extract, it was not present in the grape seed methanol extract.

Previous studies have researched the effects of benzothiazol on cancer tissues. In experimental tumor formations, suppressive effects have been seen.<sup>[23]</sup> In another study by Yoshida *et al.*,<sup>[24]</sup> benzothiazol and some derivative substances demonstrated high selective cytotoxicity against tumor cells, and there was shown to be a strong inhibitor effect on tumor cells *in vivo*. Kini *et al.*<sup>[25]</sup> applied synthesized benzothiazol and derivative substances to human cervical cancer cells and high cytotoxicity was observed to be demonstrated against the cancer cells.

In another study by Solomon *et al.*,<sup>[26]</sup> 30 benzothiazol and benzothiazol-derived substances were investigated, and it was emphasized that the effect of these substances on breast cancer cells was 10-15 times greater compared with non-cancerous breast epithelial cells. The most effective of these 30 substances was reported to be 6-methyl-benzothiazol-2-ylimino.

To the best of our knowledge, there is no study in literature that has investigated the effect of benzothiazol on young individuals. In the current study, *C. elegans* N2 wild type in L1 form was treated with 10, 50, and 100 ppm doses of benzothiazol, and a significant growth retardation was determined. As doses of 200 and 400 ppm were toxic, they were not included in the study. At the end of the third day, no difference was determined between the 10 ppm dose group and the control group ( $P > 0.05$ ) and a significant difference was determined in the 10 and 50 ppm dose groups ( $P < 0.05$ ).

In the results of the current study, similar to other reports in literature, development was seen to have been slowed by benzothiazol in a dose-

dependent manner. In a study by Solomon *et al.*,<sup>[26]</sup> it was reported that this effect was cellular based and was much greater in cancer cells. The treatment properties of benzothiazol found in cancer model organisms prolonged the lifespan of *C. elegans* BS913 subjects compared with the control group and were found to have positive effects on the fertility of strains with gonad cancer.

## CONCLUSION

In conclusion, the results of this study showed that the Erzincan Cimin grape significantly prolonged lifespan. Benzothiazol, which was determined in the grape skin extract, was found to have substantial positive effects on prolonging the lifespan of strains with cancer and also on the fertility of strains with gonad cancer. Fruits such as black grapes should be frequently consumed by humans and should be eaten whole with the skin. In addition, because of the anti-carcinogenic effects of benzothiazol and benzothiazol-derived substances, they can be considered as an agent substance in academic studies related to cancer.

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Nil

## Conflicts of interest

There are no conflicts of interest

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