Identification of longevity, fertility and growth-promoting properties of pomegranate in *Caenorhabditis elegans*

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ABSTRACT

Background: Pomegranate (*Punica granatum* L.) is commonly consumed as fresh fruit and fruit juice. It is also used in the production of jam, wine, food coloring agent, and flavor enhancer. **Objective:** The aim of this study was to identify the possible longevity, fertility and growth promoting properties of different ethanolic extract concentrations of pomegranate in *Caenorhabditis elegans,* which is increasingly popular and has proven to be a very useful experimental model organism for aging studies as well as for testing antioxidants and other compounds for effects on longevity. **Materials and Methods:** In this study, five experimental groups (20, 10, 5, 2.5 and 1.25 mg pomegranate extract/mL and one control group) were used to determine the most effective dose of pomegranate in terms of longevity, fertility and growth parameters. **Results:** It was seen that, pomegranate extracts up to the concentration of 5 mg/mL, had the potential to promote for the longevity, formation of new generations, fertility of new generations and growth properties of *C. elegans* although higher concentrations significantly reduced these parameters. **Conclusion:** these findings indicated that pomegranate could be used as a supplement to enhance longevity, fertility and growth rate for the other living organisms and human beings, but the dose should be carefully adjusted to avoid adverse effects.

Key words: *Caenorhabditis elegans*, fertility, growth, longevity, pomegranate

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INTRODUCTION

Accumulation of oxidative damage upon the macromolecules increases progressively by the aging process. Various fruits and plants may provide protection differently against oxidative stress because they differ in many aspects, including the contents of vitamins, minerals, and fibers as well as their antioxidant capacities. Overexpression of antioxidant enzymes or supplementation of some antioxidants appears to be effective in extending the longevity in some nematodes and *Drosophila* strains and even in mouse models.[1-8] The pomegranate (*Punica granatum* L.) is one of the oldest edible fruits, widely grown in many tropical, subtropical countries and commonly consumed both as fresh fruit and fruit juice. Since pomegranate is a rich source of anthocyanins, ellagic tannins and other phenolic compounds, which are already proved to have antioxidant and antitumoral activity; it is also used in the production of jam, wine, liqueur, food coloring agent, and flavor enhancer.[9-11] In this study, the nematode *Caenorhabditis elegans* was used as a model organism. The key attributes of *C. elegans* as an experimental system for biological studies are its simplicity, easy cultivation in the laboratory, short life cycle, transparency, suitability for genetic analysis, and small genome size as well as its utilization to test antioxidants and other compounds for their effects on longevity when compared with other animals including mammals. Because the nematode *C. elegans* has proven to be a very useful experimental model for the studies on longevity, *C. elegans* has been included in various natural substances and commercial health food supplements studies to evaluate possible longevity effects of natural substances.[12-15] The aim of this dose-dependent study was to identify the possible longevity, fertility and growth promoting properties of different concentrations of pomegranate ethanolic extract in *C. elegans*, and to evaluate its potential to be used as an adjuvant in aging and reproduction.

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MATERIALS AND METHODS

The *C. elegans* wild-type (N2) strain and its food source *Escherichia coli* OP50 strain were obtained from Caenorhabditis Genetic Center at the University of Minesota, (USA). *C. elegans* cultivation media supplements were purchased from MERCK (Germany). The fruits of pomegranate (*P. granatum* L.) were purchased from local markets from its wild habitat in Irlıganlı Denizli province and unpeeled fruits were washed, cut into small pieces, lyophilized and ground to obtain fine powder. The powder was used for the preparation of the extract. The 10 g fine powder was soaked in 500 ml of ethanol for 24 h and stirred continuously. The mixture was filtered through a Whatman No. 1 filter paper. The filtrate was vacuum-dried in rotary vacuum evaporator at 40°C. The extract was lyophilized and stored at 4°C for further use. Pomegranate extract (2 g) was mixed with 100 mL nematode growth medium (NGM) and diluted to the proper concentrations before it was used for the experiments.

Quantization of constitutive egg-laying was performed according to the standard protocol described by Michael Koelle.[16] Briefly, 25 late (L4) nematodes were picked from an unstarved plate to a fresh plate. 36 h later, 20 animals were picked to a fresh plate. Especially in that period, it was ensured that no eggs were transferred. The animals were set at 20°C for exactly 30 min. Nematodes spawn average of 25–100 eggs in petri dishes in this period. The number of eggs was determined by ×20 objective microscopy at the end of 30 min. To help scanning across the plate systematically, parallel black lines drawn helped to place the plate inside a plate lid with it. The study was repeated daily intervals. The standard deviation between days was about 20%. The life span analysis experiments were performed according to the standard protocol described by Sutphin and Kaeberlein,[17] except for the concentrated OP50 bacteria, which were killed by incubating at 65°C for 30 min. Different concentrations of pomegranate were added to both NGM and lawn of bacteria to allow complete exposure of animals. In this study, one control and five experimental groups, containing 20, 10, 5, 2.5 and 1.25 mg pomegranate extract per milliliter of NGM were used. The worms were grown at 20°C, observed and counted daily. To prevent any statistical mistake, the escaping animals from the petri dishes were excluded from the replacement three petri dishes, which were under the same conditions with experimental groups. Furthermore, to determine the most effective dose of pomegranate on longevity, fertility and growth rate of worms, the whole process was carried out in three times for each experiment. There were not any differences between three times experiment results. On the other hand, One-way Analysis of variance, followed by Scheffe's test were performed to determine statistical differences between the groups with the aid of Statistical Package for the Social Sciences (SPSS) software version 11.0 (SPSS, Chicago, IL, USA). Statistical significance was defined as $P < 0.05$ for all tests and error bars were placed for all figures.

RESULTS AND DISCUSSION

Antioxidant substances are often considered as a promising strategy for modulating aging and extending longevity. Since, antioxidant studies in mammals are expensive and give ambiguous results, the interest for the determination of longevity-extending efficacy of antioxidants in *C. elegans* is becoming increasingly popular. The main advantages of the use of this model organism are the normal adult longevity, which is 14–20 days and if growth conditions are favorable, the nematode develops rapidly from fertilized eggs through four larval stages (L1–L4) to become an adult hermaphrodite within 3 days.[18,19] When the rates of the formation of the new generation between control and experimental groups were compared, it was observed that 2.5 and 5 mg/mL pomegranate extract in the growth medium induced the formation of the new generation. However, the formation of new generation potential of pomegranate significantly reduced at higher (10 and 20 mg/mL) concentrations [Figure 1]. There was no significant difference between the control group and the concentration of 1.25 mg/mL in terms of the forming new generation. These results suggested that the same extract, depending on its dose might also act as an inhibitor or as an activator on the same parameter. When the literature was examined in detail, we could not see detailed studies concerning dose properties of pomegranate about the forming new generation in *C. elegans*. This could be the first study providing information on this topic. On the other hand, there were two studies done in recent years with pomegranate in male rats. These studies showed that pomegranate had beneficial effects on male

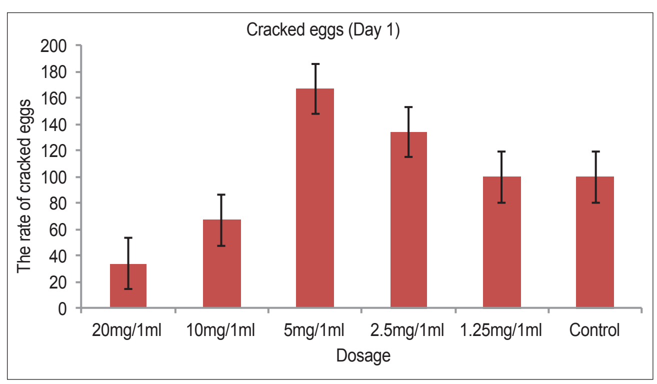

Figure 1: Comparing the rate of formation of new generation (from cracked eggs of *Caenorhabditis elegans*) of the control group and different concentrations of pomegranate extract

fertility, concentrations of spermatids, spermatocytes and spermatogonia.[20,21]

The effects of different concentrations of pomegranate on growth of the nematodes from the new generation were shown in Figure 2. The growth rates differed according to the different concentrations of pomegranate. Worms in a medium containing 2.5 or 5 mg/mL pomegranate extract grew more rapidly than the control. In contrast, higher concentrations of pomegranate (10 and 20 mg/mL) decreased growth rates of worms. These negative effects of the high concentrations of pomegranate on the growth of worms can be explained with high polyphenol content of pomegranate.[22] Because it is known that polyphenol may have prooxidant activity depending on the dose as well.[23] There were no differences between the control group and the concentration of 1.25 mg/mL in terms of growth rate of worms.

The hatching promoting effect of pomegranate on new nematodes generation increased significantly until concentrations of 5 mg/mL and also that promoting effects were observed both 3 and 4 days. But, pomegranate did not show an increasing effect on hatching promoting properties at higher concentrations (10 and 20 mg/mL). Furthermore, a significant decrease in the hatching and growth of worms was observed at higher concentrations [Figures 3 and 4]. These results may be explained with internal hatching, which is the retention of the eggs in the body. This rarely occurs in adverse environmental conditions, such as starvation and exposure to toxic compounds and bacteria. Therefore, internal hatching provides physical protection and transport for small larvae.[24-26] From these studies; we could conclude that hatching of worms could be inhibited by the high concentrations of pomegranate.

The longevity effects of different concentrations of pomegranate were observed day by day. After 23 days experimental period, it was seen that the longevity promoting effect of pomegranate increased with 1.25, 2.5 and especially 5 mg/mL concentrations. However, the same longevity promoting effect was not observed at 10, 20 mg/mL concentrations of pomegranate [Figure 5]. This effect might be attributed to dose-dependent toxicity of pomegranate. As a matter of fact, some studies reported that at higher doses, antioxidant substances could behave as inhibitor for longevity. In one of these studies, *Psoralea corylifolia* showed some *in vitro* antioxidant capacity and at low doses, caused significant longevity extension. However, *P. corylifolia* was also an inhibitor of the proteasome, cell division and mitochondrial function and at higher doses shows clear dose-dependent toxicity and reduction in

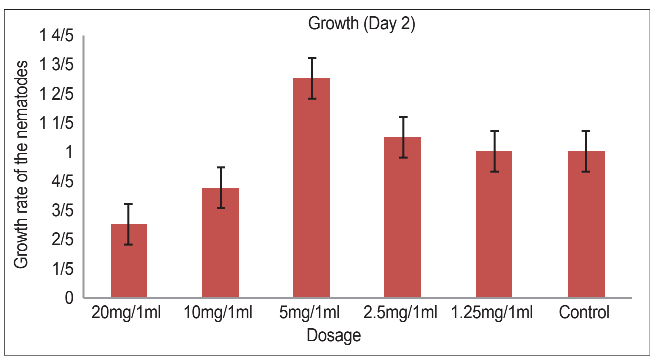

Figure 2: Comparing the growth rate of the nematodes from new genaration in control group and concentrations of pomegranate

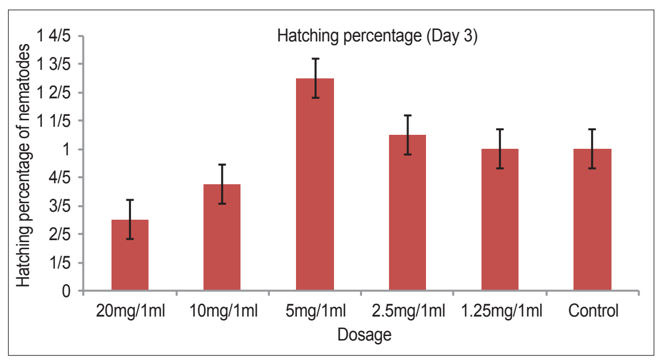

Figure 3: Comparing the fertility (hatching) promoting properties of new nematodes generation of control group and different concentrations of pomegranate extract

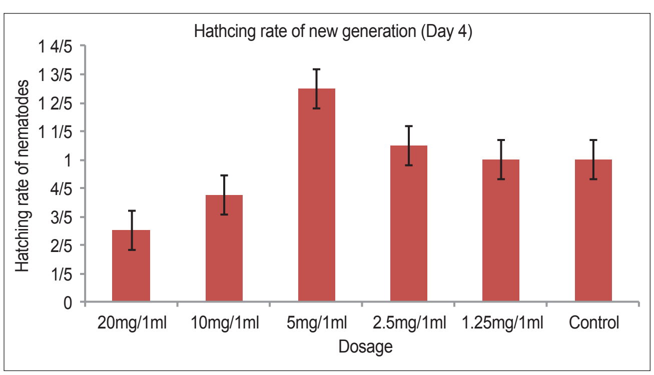

Figure 4: Comparing the fertility (hatching) promoting properties of new nematodes generation of control group and different concentrations of pomegranate

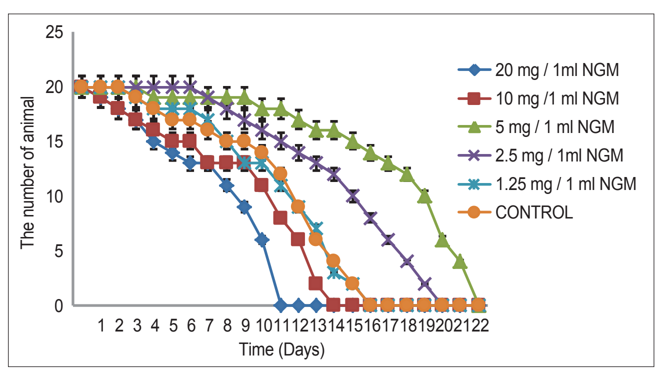

Figure 5: The longevity promoting properties of different concentrations of pomegranate extract in *Caenorhabditis elegans*

lifespan of *C. elegans*.[18,27,28] These studies were compatible with our study in that the same plant extract could have both longevity promoting effect and reducing effect, depending on its concentration. However, as far as we knew, there was no detailed study concerning dose properties and the longevity effects of pomegranate. This may be the first study providing information on this topic. Thus, it might be suitable to use this pomegranate concentration (of 5.0 mg/mL) at tissue level when used as a supplement for therapeutic regimens and longevity.

CONCLUSION

As a result, it was expected that this study could lead to a better understanding of the importance antioxidant substances for the dose-response studies and these findings also showed that the study of the edible antioxidant foods remains as a significant field to explore in terms of fertility, growth and longevity researches.

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