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The Effect of Nettle (*Urtica dioica* L.), Carob (*Ceratonia siliqua* L.), and Chaste (*Vitex agnus-castus* L.) Plants on Fertility in *Caenorhabditis elegans*

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ABSTRACT
This study was carried out to determine the effects of different concentrations of water extract of Nettle (*Urtica dioica* L.), Carob (*Ceratonia siliqua* L.), and Chaste (*Vitex agnus-castus* L.) plants, which are traditionally used in the treatment of infertility among the people, on fertility in *Caenorhabditis elegans* (*C. elegans*), which is a model organism. Scope of work; 0.1%, 0.05%, 0.02%, and 0.01% concentrations of the water extracts of the plants were applied to the *C. elegans* standard medium and egg counts were made for 3 days according to the Koelle protocol. In addition, one day after each egg count, uncracked eggs were determined, and egg productivity was calculated. The analyses were performed in triplicate, the averages were determined and the differences between the groups were statistically compared with the SPSS program. According to the results obtained from the study, it was determined that Nettle, Carob, and Chaste plants have positive effects on fertility. Among the plants, it was determined that the plant that affected fertility the most was Nettle, followed by Chaste and Carob plants, respectively. In addition, in terms of dosage applications, the best effect in all three plants has been observed at the highest dose of 0.1%.

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Keywords
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Fertility

Isırgan (*Urtica dioica* L.), Keçiboynuzu (*Ceratonia siliqua* L.) ve Hayıt (*Vitex agnus-castus* L.) Bitkilerinin *Caenorhabditis elegans*' ta Fertiliteye Etkisi

ÖZET
Bu çalışma, halk arasında geleneksel olarak infertilite tedavisinde kullanıldığı bilinen Isırgan (*Urtica dioica* L.), Keçiboynuzu (*Ceratonia siliqua* L.) ve Hayıt (*Vitex agnus-castus* L.) bitkilerinin su ekstraktının farklı konsantrasyonlarının, bir model organizma olan *Caenorhabditis elegans* (*C. elegans*)' ta fertilite üzerine olan etkilerini belirlemek amacıyla planlanmıştır. Çalışma kapsamında; bitkilerin su ekstraktlarının %0.1, %0.05, %0.02 ve %0.01'lik konsantrasyonları *C. elegans* standart besiyerine uygulanmış ve Koelle protokolüne göre 3 gün boyunca yumurta sayımı yapılmıştır. Ayrıca, her yumurta sayımı yapıldıktan bir gün sonra çatlamayan yumurtalar tespit edilerek, yumurta verimlilikleri de hesaplanmıştır. Analizler üç tekrarlı olarak yapılmıştır ve ortalamalar belirlenerek SPSS programı ile gruplar arasındaki farklar istatistiksel olarak karşılaştırılmıştır. Çalışmadan elde edilen sonuçlara göre, Isırgan, Keçiboynuzu ve Hayıt bitkilerinin fertilite üzerinde olumlu etkilere sahip olduğu belirlenmiştir. Bitkiler arasında fertiliteye en fazla etki eden bitkinin Isıgan olduğu bunu sırasıyla Hayıt ve Keçiboynuzu bitkilerinin takip ettiği belirlenmiştir. Ayrıca, doz uygulamalarında her üç bitkide de benzer şekilde en iyi etki en yüksek doz olan %0.1' lik uygulamada tespit edilmiştir.

Botanik

Araştırma Makalesi

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Anahtar Kelimeler
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INTRODUCTION

Humans' use of plants for medicinal purposes dates back to ancient times. Archaeological findings from the earliest civilizations indicate that people utilized plants to obtain food and address health issues (Özpınar & Yüksek, 2019). Additionally, it is known that many drugs used in modern medicine today are derived from plants (Saraç et al., 2018). Scientific research conducted on the effectiveness and safety of commonly used medicinal and aromatic plants in folk medicine plays a significant role in Turkish traditional medicine and its proper utilization (Fugh-Berman & Kronenberg, 2003). Because, even though herbal remedies have been known for a long time, the specific ways in which each plant affects human physiology are not well understood. Therefore, the scientific community has focused on plant extracts as therapeutic agents for 30-40 years (Bora & Sharma, 2010; Çetin, 2012). Contrary to popular belief, many plants have been discovered to have medicinal effects, and the discovery of such plants continues (Beşen & Beji, 2014). In our country, which has a rich potential in terms of medicinal and aromatic plants, herbal treatment is preferred by the public due to its low cost, easy accessibility, and concerns about the side effects of chemical drugs. It is particularly widely used in the treatment of infertility (Beşen and Beji, 2014; Daştan & Saraç, 2018). Studies conducted in countries where plants affecting fertility are extensively used have found that the medicinal plants used in these studies not only lead to a significant increase in pregnancy rates and ovulation but also result in a decrease in miscarriage rates (Tan et al., 2012; Yılmaz, 2019). In modern times, efforts are being made to document plants that affect fertility. Therefore, their effects are being investigated and extensively discussed through scientific publications and projects (Gaware et al., 2009; Beşen & Beji, 2014). For example, in a study conducted by Telefo et al. (2011) in Cameroon, 46 different plants used in female infertility were documented.

Urtica dioica L. is one of the most common species in the family Urticaceae. In studies with the species, which is a perennial herbaceous plant, the extracts used have been found to have many pharmacological effects such as antioxidant, anti-inflammatory, anti-diabetic, anticancer, antiulcer, etc. (Esposito et al., 2019). *Ceratonia siliqua* L. is a species of the Fabaceae family that is widespread in the Mediterranean region. Due to the many bioactive components contained in the fruit of this species and the products obtained from this fruit; positive effects have been observed on diabetes, inflammation, digestive system disorders, hyperlipidemia, oxidative stress, etc.. (Brassesco et al., 2021). *Vitex agnus-castus* L. species is referred to as the chaste tree that spreads in Central Asia, the Mediterranean Region, and Southern Europe. It is a species of the Verbenaceae family. Studies carried out in recent years show that this type can be effective in the treatment of PMS and menstrual disorders, menopausal problems, and also its antioxidant, antitumoral, antimicrobial, antiepileptic, anti-inflammatory, osteopenic, etc. effects can be observed (Niroumand et al., 2018).

In this study, Nettle *(Urtica dioica* L.), Carob (*Ceratonia siliqua* L.), and Chaste (*Vitex agnus-castus* L.), which have been determined as plant materials, are traditionally used for various human-specific disorders, primarily infertility treatment (Ezer & Avcı, 2004; Ugulu et al., 2009; Edirne et al., 2010; İpekoğlu & Oral, 2019).

Currently, various model organisms are used to demonstrate the effects of many substances, such as plant extracts, on human health. *C. elegans* is a microscopic, non-pathogenic soil nematode (roundworm) that is approximately 1 mm long and 65 µm thick. It freely lives in organic-rich environments such as the soil near tree roots (Hertweck et al., 2003; Ünlü & Erdem, 2010). Due to its fundamental characteristics such as simplicity, transparency, and short lifespan, *C. elegans* has been extensively used as a model organism in basic biological research for many years (Olsen et al., 2006; Porta de la Riva et al., 2012; Savaş et al., 2018; Özpınar, 2020). *C. elegans* can easily sustain its life in laboratory conditions by feeding on bacteria (*Escherichia coli* OP50 strain) on an agar substrate in a petri dish (Ünlü & Erdem, 2010). Additionally, it can be stored indefinitely as a stock at -80°C or in liquid nitrogen (Springer, 2005). Its lifespan under laboratory conditions is approximately 3 weeks, and its life cycle (from embryo to reproductive adult) takes about 3.5 days at 20°C (Olsen et al., 2006) (Figure 1). This study investigated the effects of water extracts obtained from Nettle, Carob, and Chaste plants at concentrations of 0.1%, 0.05%, 0.02%, and 0.01% on *C. elegans*' fertility.

MATERIAL and METHOD

Plant Materials

The Nettle leaves, Carob fruits, and Chaste seeds used as plant material in the study were obtained from an herbalist shop located in Sivas and later diagnosed (Figure 2).

Preparation of Plant Extracts

For extraction, the leaves of Nettle, the fruits of Carob, and the seeds of Chaste were used. The dried plant materials were first ground into a powder using a laboratory-type mill. Then, 100 g of the powdered plant samples were weighed and placed in beakers, to which 500 mL of distilled water (dH_2O) was added as the solvent. The plant samples were macerated at room temperature (25±2°C), 150 rpm, using an electronic

shaker for 24 hours. After maceration, the plant extracts were filtered twice through filter paper (Whatman No. 1). The solvent in the filtrate was removed using a rotary evaporator at 40°C. The resulting dry extracts were stored at +4°C until analysis.

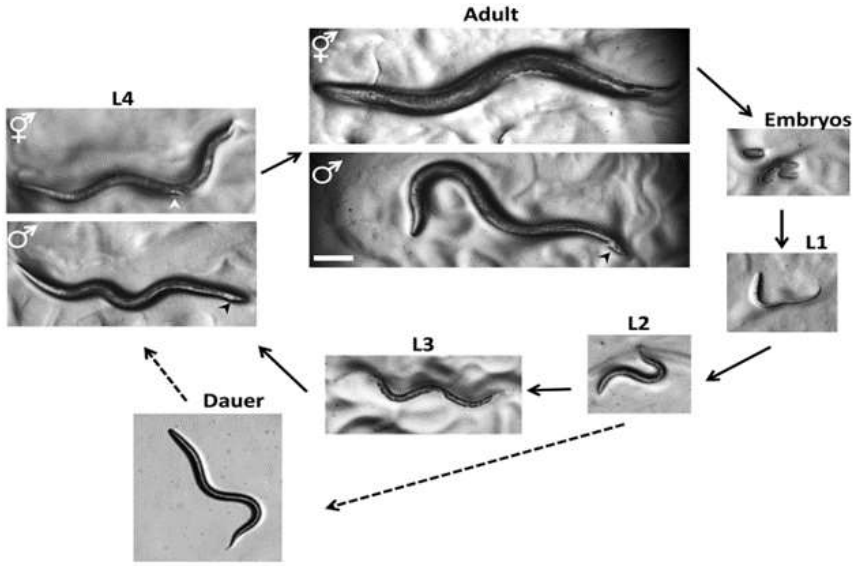

Figure 1. Life cycle of *C. elegans* (Corsi et al., 2015)
Şekil 1. C. elegans'ın yaşam döngüsü (Corsi ve ark., 2015)

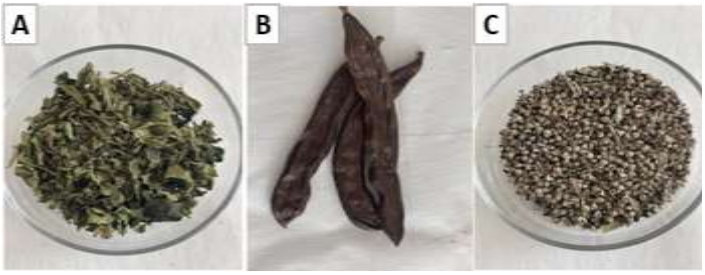

Figure 2. Plant materials used in the study A. Nettle leaf, B. Carob fruit, C. Chaste seed
Şekil 2. Çalışmada kullanılan bitki materyalleri A. Isırgan yaprağı, B. Keçiboynuzu meyvesi, C. Hayıt tohumu

Preparation of TBX Agar Medium for *E. coli* OP50 Strain

To prepare TBX Agar (Tryptone Bile Glucuronide Agar) medium for *E. coli* OP50 strain, 9.125 g of TBX Agar was weighed using a precision balance, and then 250 mL of dH_2O was added to it. The mixture was then placed on a magnetic stirrer to ensure the agar's dissolution, and the medium was autoclaved at 125°C for 15 minutes for sterilization. After autoclaving, the agar medium was allowed to cool down to 55°C.

Purification of *E. coli* OP50 Strain

After preparing the TBX Agar medium for the *E. coli* OP50 strain, approximately 10 mL of the medium was poured into each 60 mm petri dish. The medium was allowed to solidify. Once solidified, the TBX Agar plates were ready for use. Using a sterile loop, *E. coli* OP50 strain was streaked onto the agar surface, near the flame of a Bunsen burner. The plates were then incubated at 37°C for 24 hours. After colony formation was observed, specific blue colonies belonging to the *E. coli* OP50 strain were identified. This step was performed to purify the *E. coli* OP50 strain by identifying the colonies specific to it before transferring the *E. coli* to a liquid medium, to avoid the possibility of contamination.

Preparation of Liquid Culture Medium for *E. coli* OP50 Strain

A liquid culture medium has been prepared for the transfer and propagation of the purified *E. coli* OP50 strain. For this purpose, 9.125 g of Lauryl Sulfate Tryptose Broth (LST Broth) was weighed on a precision balance, and then 250 mL of dH_2O was added. The mixture was autoclaved at 120 °C for 15 minutes to ensure sterilization. Once sterilized in the autoclave, the medium was cooled to 37 °C. Subsequently, a single colony of *E. coli* OP50 strain was inoculated into the LST Broth using a loop taken from a TBX Agar culture. During this process, work was conducted inside a laminar flow (sterile cabinet) and near a Bunsen burner to minimize the risk of contamination. After transferring the liquid culture medium, it was incubated at 37°C in an incubator for 24 hours. Following successful growth, the medium was stored at +4°C for later use (the cloudiness of the liquid indicates the proliferation of *E. coli* OP50 strain).

Preparation of Nematode Growth Media (NGM)

2.5 g of Peptone, 3 g of NaCl, and 20 g of Agar were dissolved in 1 L of dH_2O using a magnetic stirrer until the boiling temperature was reached. The mixture was then autoclaved at 125 °C for 15 minutes and subsequently cooled down to 55 °C. After cooling, the NGM was homogenized by adding the previously prepared and filtered components, including 1 mL of $MgSO_4$ (1M), 1 mL of Cholesterol (5 mg/mL), 1 mL of $CaCl_2$ (1M), and 25 mL of KPO_4 buffer (pH: 7).

Culturing of *C. elegans* on NGM

After homogenization, NGM was poured into 60 mm Petri dishes, approximately 10 mL each, to allow it to solidify into an agar consistency. Once the NGM solidified, about 400 µL of *E. coli* OP50 strain was added to the centre of each dish. The petri dishes with added *E. coli* were then left to dry in a sterile environment for approximately 1-2 days. Care was taken to prevent the NGM from completely drying out to maintain its agar consistency. Once the bacterial solution dried, a small piece was cut from the stock culture and placed upside down onto the newly prepared NGM. This allowed the *C. elegans* to be cultured on the NGM medium, providing an environment suitable for their growth and development.

Preparation of NGM with Plant Extract

The dried plant extracts weighed for 0.01%, 0.02%, 0.05%, and 0.1% concentrations, were individually added to separate NGM. After adding the extracts, the mixture was rapidly stirred and poured into petri dishes to solidify. Control petri dishes were prepared without adding any concentration of plant extract (Figure 3). Petri dishes were prepared in triplicate for all doses of the plant extracts studied. Subsequently, all prepared Petri dishes were wrapped with aluminium foil and stored at +4 °C to be used after the synchronization process of *C. elegans*.

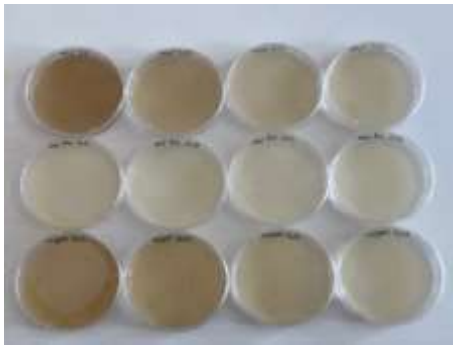

Figure 3. NGMs prepared with water extracts at concentrations of 0.01%, 0.02%, 0.05%, and 0.1%.

Şekil 3. Su ekstraklarının %0.01, %0.02, %0.05 ve %0.1 konsantrasyonları ile hazırlanmış NGM'ler

Synchronization of *C. elegans*

1 g of NaOH was weighed on a precision balance and then 5 mL of dH_2O was added to dissolve it. Subsequently, 1 mL of sodium hypochlorite and 0.5 mL of NaOH solution were transferred to a centrifuge tube. The previously prepared NGM, where *C. elegans* had been cultured, was used to wash the eggs by pipetting with dH_2O. After pipetting, the eggs were also transferred to the centrifuge tube, which was then centrifuged at 3000 rpm for 10 minutes, and the supernatant was discarded. The remaining pellet containing the eggs was transferred to new NGM plates. These eggs constitute synchronized larvae, and when they reach the adult form (L4) by the end of the third day, they are used for fertility analysis (Koelle, 2005).

Fertility Analysis

To determine the effects of plant extracts on fertility, evaluations were conducted on egg counts, the number of individuals hatched from the eggs, and egg productivity. The egg counting for fertility analysis followed the protocol by Koelle (2005). Accordingly, 15 well-fed L4 stage *C. elegans* were transferred to each petri dish prepared with different concentrations of plant extracts (0.01%, 0.02%, 0.05%, 0.1%). After 36 hours, 10 individuals from each dish were transferred to a new petri dish and left at 20°C for 30 minutes. After the specified time, egg counting was performed under a stereo microscope using a 20x objective. Additionally, the number of *C. elegans* individuals hatching from the eggs was counted in the same Petri dishes, and any unhatched eggs were identified.

Statistical Analysis

Fertility analysis was repeated three times for each plant extract and its doses. Statistical analysis of the fertility data was performed using SPSS 22.0 (IBM Corporation, Armonk, New York, United States) software. Differences between the means were determined using the Tukey test with a significance level of $P<0.05$.

RESULTS and DISCUSSION

The effects of water extracts of different concentrations of the Nettle, Carob, and Chaste plants on fertility were determined in the study, and the findings obtained are presented in Tables 1, 2, and 3.

According to the fertility results obtained from a 3-day count of 10 *C. elegans* individuals transferred at the L4 stage, Nettle water extract doses have caused significant increases in both parameters (egg counts and individual counts). This is because significant statistical differences exist between the applied dose groups and the control ($P<0.05$). All applied dose groups have positively influenced fertility. This effect

is proportional to the dose amount. As the dose amount increases, the egg and individual counts increase (Table 1).

All doses of Nettle water extract have increased egg productivity in *C. elegans* compared to the control. Egg productivity was determined as 99.1% at a dose of 0.01%, 99.4% at a dose of 0.02%, 99.2% at a dose of 0.05%, and 99.1% at a dose of 0.1%. The egg productivity of the control is 97.9% (Figure 4).

Table 1. The effect of Nettle water extract on fertility in *C. elegans*
Çizelge 1. Isırgan su ekstraktının C. elegans'ta fertiliteye etkisi

Doses (%)	Egg counts	Individual counts
Control	857.66±4.04^{d}	839.66±4.16^{d}
0.01	958±3^{c}	950.33±4.50^{c}
0.02	964.66±3.21^{bc}	959.66±2.51^{bc}
0.05	969.66±3.78^{b}	962.66±3.21^{b}
0.1	999.33±2.51^{a}	991.33±4.04^{a}

^{a,b,c} Values within a column with different superscripts differ significantly at P<0.05

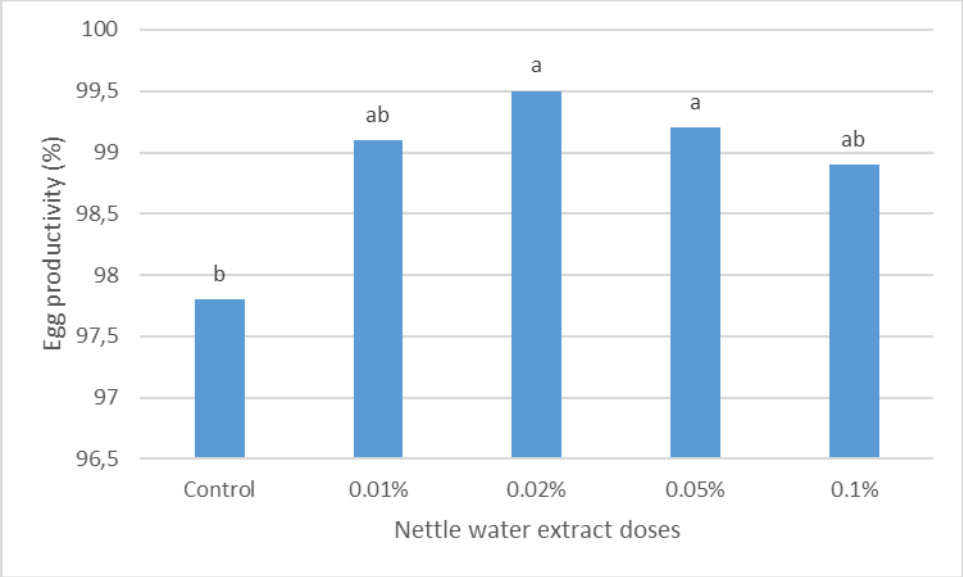

Figure 4. The effect of Nettle plant on egg productivity in *C. elegans*
Şekil 4. Isırgan bitkisinin C. elegans'ta yumurta verimine olan etkisi

Table 2. The effect of Carob water extract on fertility in *C. elegans*
Çizelge 2. Keçiboynuzu su ekstraktının C. elegans'ta fertiliteye etkisi

Doses (%)	Egg counts	Individual counts
Control	866.66±4.16^{b}	846.66±4.72^{c}
0.01	861.66±2.08^{b}	851.66±3.21^{bc}
0.02	864.66±1.52^{b}	856.66±4.16^{ab}
0.05	868.66±3.21^{ab}	860±2^{ab}
0.1	874.66±3.05^{a}	865±3.60^{a}

^{a,b,c} Values within a column with different superscripts differ significantly at P<0.05

When Table 2, which demonstrates the effects of different doses of Carob water extract on fertility, is examined, it is determined that the highest dose of the Carob plant, which is the 0.1% application, and partially the 0.05% application, have a positive effect on egg counts. Compared to the control, no significant difference is observed in other dose applications. When the number of individuals hatched from the eggs is evaluated, a significant increase is determined depending on the dose increase (Table 2). This indicates that the Carob plant has a positive effect on fertility.

The graph showing the egg productivity of different doses of Carob water extract and the control is presented in Figure 5.

When examining Figure 5, it can be observed that all doses of Carob water extract have increased egg productivity compared to the control. The egg productivity, which was 97.6% in the control, reached an average of 99% in Carob extract applied at different concentrations.

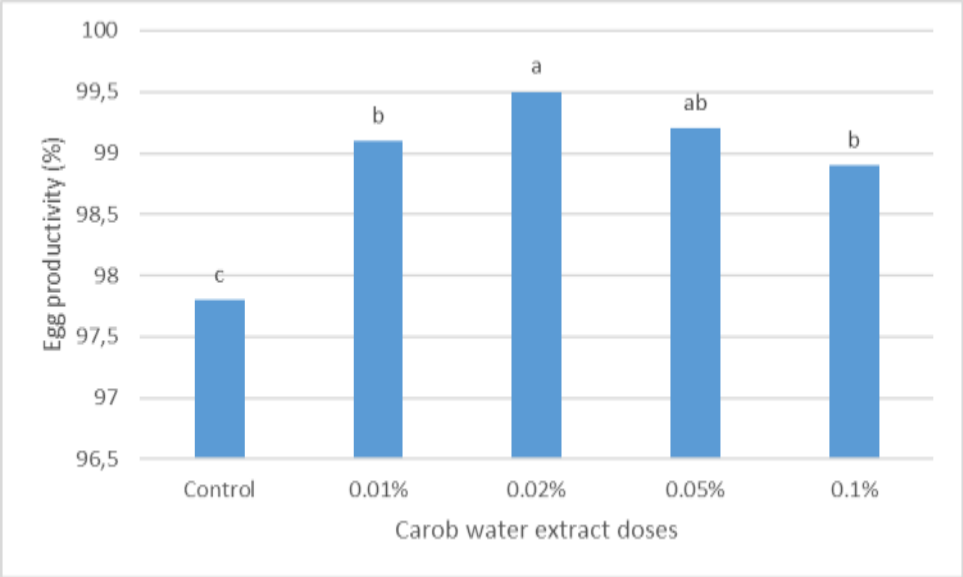

Figure 5. The effect of Carob plant on egg productivity in *C. elegans*
Şekil 5. Keçiboynuzu bitkisinin C. elegans'ta yumurta verimine olan etkisi

All doses of Chaste water extract (0.01%, 0.02%, 0.05%, 0.1%) have shown an increase in egg counts and individual counts in *C. elegans* compared to the control. This difference between the control and dose groups is statistically significant (P<0.05). Chaste water extract has had a positive effect on fertility, increasing egg counts and individual counts with higher doses. The highest numbers of eggs (998.66±3.21) and individuals (988.66±1.52) were observed in the 0.1% dose, while the lowest numbers of eggs (868.66±4.72) and individuals (849.66±4.50) were observed in the control (Table 3).

The graph showing the different doses of water extract of Chaste seeds and the control's egg productivity is given in Figure 6.

Table 3. The effect of Chaste water extract on fertility in *C. elegans*
Çizelge 3. Hayıt su ekstraktının C. elegans'ta fertiliteye etkisi

Doses (%)	Egg counts	Individual counts
Control	868.66±4.72^{e}	849.66±4.50^{e}
0.01	939±4.35^{d}	931.33±3.21^{d}
0.02	951.33±4.04^{c}	946.66±2.51^{c}
0.05	966±4.35^{b}	958.33±2.51^{b}
0.1	998.66±3.21^{a}	988.66±1.52^{a}

^{a,b,c} Values within a column with different superscripts differ significantly at P<0.05

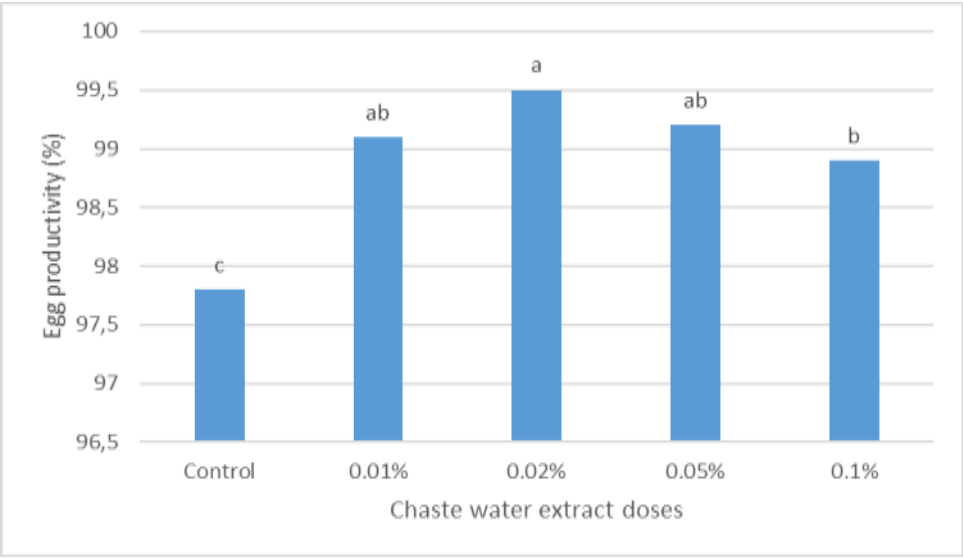

Figure 6. The effect of Chaste plant on egg productivity in *C. elegans*
Şekil 6. Hayıt bitkisinin C. elegans'ta yumurta verimine olan etkisi

The egg productivity of *C. elegans* for the application doses of the Chaste plant at 0.01%, 0.02%, 0.05%, and 0.1% were determined as 99.1%, 99.5%, 99.2%, and 98.9%, respectively. In the control, the percentage of hatched larvae from the eggs is 97.8% (Figure 6).

There are many different studies on the number of eggs and egg productivity of *C. elegans* individuals of different plant extracts and active ingredients (Ozpınar et al., 2017; Özpınar et al., 2017). However, not many studies were found that demonstrate the effects of Nettle, Carob, and Chaste plants on fertility in model organisms found in the conducted literature review.

Yılmaz (2019), in his study investigating the effects of commonly used plants in Turkey, namely Nettle, Rosemary, and Carob, on sperm function parameters under in vitro conditions. He treated sperm samples obtained from 40 patients with extracts of these plants at concentrations of 0.1%, 0.05%, and 0.01% for 30 minutes, 1 hour, and 24 hours, respectively. Sperm motility and vitality parameters were evaluated according to the World Health Organization (WHO) criteria. The study found that progressive sperm motility and vitality were improved with the in vitro application of Nettle, Rosemary, and Carob extracts in normozoospermic samples. Vafaei et al. (2018) reported a study in which they aimed to determine the effects of Carob (*Ceratonia siliqua*) extract on sperm quality, testicular structure, testosterone levels, and oxidative stress in Busulfan-induced infertile mice. In this study, adult male mice were treated with 10 mg/kg of busulfan along with Carob extract at doses of 800, 400, 200, 100, and 50 mg/kg for 35 days. According to the research findings, the administration of 800 mg/kg Carob extract for 35 days improved sperm quality, biochemical parameters, germinal epithelium thickness, and testosterone levels in Busulfan-induced infertile mice. In another study, the effect of orally administered Carob fruit in capsule form and vitamin E on sperm parameters in men with idiopathic infertility was investigated. In the study, it was determined that the daily use of 1500 mg oral Carob fruit capsule for 90 days had a significant effect on sperm motility, but compared to vitamin E, it did not have a significant effect on morphology and count (Sanagoo et al., 2021). Jalili et al. (2014) conducted a study to investigate whether the hydroalcoholic extract of *Urtica dioica* could inhibit the negative effects of nicotine on the viability, count, motility, testicular histology, and testosterone hormone levels of sperm cells. In their research, they determined that the hydroalcoholic extract of *Urtica dioica* could enhance spermatozoa quality and inhibit the negative effects of nicotine on sperm parameters. In a different study, the effects of *Vitex agnus-castus* extract on the reproductive potential of women with premature ovarian ageing (POA) were investigated. The study aimed to determine the impact of *Vitex agnus-castus* extract as a plant that promotes fertility. The results indicated that when used within established dosage guidelines, *Vitex agnus-castus* extract is considered a safe and effective botanical intervention in medical practices for women with POA, encouraging fertility. (Hossein-Rashidi & Nemati, 2017).

CONCLUSION

This study aimed to determine the effects of different concentrations of water extract from Nettle (*Urtica dioica* L.), Carob (*Ceratonia siliqua* L.), and Chaste (*Vitex agnus-castus* L.) plants on fertility in *C. elegans*. It was found that all dosage applications of Chaste and Nettle plants increased the number of eggs and the number of individuals hatched from eggs compared to the control. The Carob plant, on the other hand, showed a positive effect on the number of eggs with its 0.1% and 0.05% applications. The dosage application that yielded the best results in all three plants was 0.1%. Furthermore, according to the study results, it can be said that the water extract of the Nettle plant has a greater impact on fertility overall.

When the data obtained from the study are evaluated as a whole, it is believed that medicinal plants, as one of the traditional methods, can be used to treat infertility, which is widely seen today, instead of using chemically based drugs or lengthy and costly treatment methods. Many plants are being discovered to have more medicinal effects than previously known or can be used in different areas. Scientific research and studies help us better understand the effects of plants on health and lead to discoveries. As more information is obtained about the bioactive compounds found in plants and their effects on the human body, herbal medicines, and treatment methods can evolve. This process is a dynamic field where discoveries are constantly being made in the field of herbal medicine.

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Researchers' Contribution Rate Statement Summary

The authors declare that they have contributed equally to the article.

Conflict of Interest Statement

The article's authors declare that they do not have any conflict of interest.

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