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Investigation of Some Biological Activities of *Inula graveolens* (L.) Desf Species from Turkey

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Abstract

In this study, it was aimed to determine the phenolic and flavonoid content and different biological activities (antioxidant, enzyme inhibitor, anthelmintic) of the methanol extract of *Inula graveolens* (L.) Desf collected from Muğla (Turkey). As a result of the study, the total phenolic content was determined as 5.36±0.32 mg GAE/g, and the total flavonoid amount was determined as 3.49±0.05 mg QE/g extract equivalent. In the ß-carotene/linoleic acid method, the extract showed lower activity than the standard BHA used. The extract was determined to be equivalent to 4.28±0.24/0.47±0.03 mg TE/g extract in terms of copper and iron-reducing power capacity, respectively. Although the enzyme inhibitory activities of the extract increased with the increase in concentration, it was determined that it had lower activity than galantamine (89.41±0.05%) and kojic acid (73.93±0.10%) used as standard. Paralysis and death times of the extract at different concentrations (2.5,5,10,20 mg/mL) on *Tubifex tubifex* worms were determined. It was determined that the extract at high concentrations (20 mg/mL) exhibited an activity near that of andazole (10 mg/mL) used as a standard. According to these results, *I. graveolens* can be considered a good resource for the pharmaceutical industry due to its activities.

Keywords: Inula graveolens, antioxidant, enzyme inhibitor, Tubifex tubifex

Türkiye'den Toplanan *Inula graveolens* (L.) Desf. Türünün Bazı Biyolojik Aktivitelerinin Araştırılması

Öz

Bu çalışmada Muğla'dan toplanan *Inula graveolens* (L.) Desf türünün metanol ekstraktının fenolik ve flavonoid miktarı ve farklı biyolojik aktivitelerinin (antioksidan, enzim inhibitör, antihelmint) belirlenmesi amaçlanmıştır. Çalışma sonucunda total fenolik miktarı 5,36±0,32 mg GAE/g, total flavonoid miktarı ise 3,49±0,05 mg QE/g ekstrakt eşdeğeri olarak belirlenmiştir. ß-karoten/linoleik asit yönteminde ekstrakt standart olarak kullanılan BHA'ya göre daha düşük bir aktivite sergilemiştir. Ekstraktın bakır ve demir indirgeme gücü kapasitesi bakımından sırasıyla 4,28±0,24/0,47±0,03 mg TE/g ekstrakt eşdeğeri olduğu belirlenmiştir. Ekstraktın enzim inhibitör aktiviteleri konsantrasyon artışına bağlı artsa da standart olarak kullanılan galantamin (89.41±0.05%) ve kojik asite (73.93±0.10%) göre daha düşük bir aktiviteye sahip olduğu tespit edilmiştir. Farklı konsantrasyondaki ekstraktın (2.5,5,10,20 mg/mL) *Tubifex tubifex* solucanları üzerindeki paralize ve ölüm süreleri belirlenmiştir. Yüksek konsantrasyonlardaki ekstraktın (20 mg/mL) standart olarak kullanılan andazole (10 mg/mL) yakın bir aktivite sergilediği belirlenmiştir. Bu sonuçlara göre *I. graveolens* gösterdiği aktivitelerden dolayı ilaç endüstrisi için iyi bir kaynak olarak dikkate alınabilir.

Anahtar Kelimeler: Inula graveolens, antioksidan, enzim inhibitör, Tubifex tubifex

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Introduction

Due to the chemical diversity of metabolites in plants, they are widely used as food raw materials, treatment of diseases, and reducing the negative effects of conditions such as stress and aging (Ekor, 2014; Petrovska, 2012). Secondary metabolites are bioactive components that are important for human health. Plant secondary metabolites are raw materials in high demand in many industries, including health and food. The production of secondary metabolites by plants depends on meteorological conditions, geographical location, and growing conditions, and many plants can only grow and mature in certain seasons (Çölgeçen, 2015). Based on their biosynthetic origins, they are divided into 3 main groups as alkaloids, terpenes and phenolics. Phenolic compounds are one of the groups with the highest number of members in the plant kingdom (Tring et al., 2020).

Free radicals are molecules with one or more unpaired electrons. Due to their unstable structure, they can cause damage by binding to lipids, nucleic acids, proteins, and organelles in the cell (Karabulut & Gülay, 2016). In healthy individuals, there is a balance between antioxidants and free radicals (Gökbulut & Şarer, 2011). Antioxidants provide protection against various diseases (diabetes, cancer, ischemic and neurodegenerative disorders) that may occur by inhibiting free radicals. Tocopherols, ascorbic acid, flavonoids, and phenolic compounds are the most important natural antioxidant groups. Phenolics, which are important plant components, could remove radicals due to the presence of hydroxyl groups in their structures (Karabulut & Gülay, 2016). Antioxidants obtained from natural products in drug production have fewer side effects than synthetic antioxidants. Therefore, the interest in the search for antioxidant substances from natural products has gained importance over time. In addition, natural compounds with enzyme inhibitor potential have gained importance in the pharmaceutical industry because they are used in the treatment of many diseases such as cancer, and metabolic, cardiovascular, and neurological disorders (Abdioğlu, 2019).

Plants provide a rich medicinal resource against anthelmintics and insecticides. Ailments caused by helminths are caused by parasitic gastroenteritis, infections caused by different kinds of stomach and intestinal worms. As a result, discomforts such as fatigue, loss of appetite, and decreased productivity occur. Since chemotherapy or an effective vaccine against helminths has not been developed, metabolites obtained from natural products are the only effective treatment method to treat and control helminth infections. Random use of synthetic anthelmintic drugs can lead to parasite resistance. Herbal medicines have been used since ancient times for the treatment of parasitic diseases in humans and may be valuable in preventing the development of resistance (Hossain, 2015).

Inula graveolens (L) Desf, which grows widely in the Mediterranean region and is known as stinking grass among the people, is in the family of Asteraceae (Gökbulut & Şarer, 2011). *I. graveolens* is an herb with small yellow to yellow/white flowers that smell of camphor. *I. graveolens*, a perennial shrubby plant, can grow up to one meter. It is a multi-branched, heavily scented, linear-lanceolate plant covered with small glandular hairs (Karan et al., 2018). Sellem et al. (2020) investigated the antioxidant, antioxidant enzyme inhibitory activity, total substance amount as well as antimicrobial activity of extracts obtained from five different solvents (Cyclohexane - Dichloromethane - Ethyl acetate - Acetone - Acetonitrile) from *Inula graveolens* species. They demonstrated different biological activities. They determined that the highest amount of substance in its content belongs to phenolics.

The aim of this study was to determine the total phenolic and flavonoid amounts of the aboveground methanol extract of *I. graveolens* species and to evaluate different biological activities such as antioxidant (β-carotene, CUPRAC, FRAP), enzyme inhibition (Acetylcholinesterase, Tyrosinase), and anthelmintic (*Tubifex tubifex*).

In this study, besides the determination of antioxidant (β-carotene, CUPRAC, FRAP), enzyme inhibitor (Acetylcholinesterase, Tyrosinase), and anthelmintic activities of the aerial methanol extract of *Inula graveolens* species, the determination of the substance (flavonoid, phenolic) was performed.

Materials and Methods

Plant Material and Extract Preparation

Inula graveolens species was collected from the Kavaklıdere district of Muğla (Turkey) in 2021. The collected plant samples were dried and cut into small pieces with a blender. The fragmented plant samples (20 g) were transferred to the Erlenmeyer flask and 100 mL of methanol was added to it. Afterward, the Erlenmeyer flask was kept in a shaking incubator at 50 °C for 6 hours, and after this process, it was filtered into the balloon jug with the help of blotting paper. The incubation process was repeated a second time with the addition of solvent. After the filtration process was completed, the filtered samples were taken to the rotary evaporator (Heindolph LABOROTA 4011) to remove the solvent. In order to remove the water in the samples, they were kept in a lyophilizer (Thermo Savant) at -54 °C for 10 hours. After the lyophilization process, the samples were stored at -20 °C until they would be used (Turan & Mammadov, 2018).

Quantitative Analysis of Extracts and Determination of Antioxidant Activity

The total phenolic content of the extracts was calculated as gallic acid (mg GAE/g) equivalent using the Folin-Ciocalculate method (Singleton & Rossi, 1965). The total flavonoid amount was calculated as quercetin equivalent (mg QE/g) by modifying the method of Aryal et al. (2019). The ß-carotene/linoleic acid method for the determination of total antioxidant activity was performed according to the method of Amarowicz et al. (2004). The reducing power capacity of iron and copper was determined as Trolox equivalent (mg TE/g) according to the method of Benzie and Strain (1996), and Apak et al. (2004) respectively.

Enzyme Inhibitory Activity Methods

Acetylcholinesterase

125 µL of DTNB (0.5 mM), 25 µL of anticholinesterase (0.026 U/mL) was mixed onto 50 µL of water-dissolved solution extract solution (1 mg/mL) and incubated for 15 minutes at room temperature. After incubation, 25 µL of acetylthiocholine iodide substrate (1.5 mM) was added to initiate the reaction and incubated again for 10 minutes at room temperature. After incubation, absorbance measurement was performed at 405 nm. The same processes were performed in galantamine, which was used as a standard. By using absorbance values from all samples, % inhibitions were calculated with the help of the following formula (Ellman et al., 1961).

$$\% \text{ inhibitions} = [(A_c - A_s) / A_c] \times 100$$

A_c is the absorbance value of the control and A_s is the absorbance value of the extract.

Tyrosinase

After adding 40 µL of water-dissolved extract solution, 120 µL of phosphate buffer (20 mM pH: 6.8), and 20 µL of tyrosinase (480 U/mL) enzyme solution, it was incubated at room temperature for 15 minutes. After incubation, the reaction was started by adding 20 µL of L-DOPA (2.5 mM). Then, after incubation for 10 minutes at room temperature, absorbance values were measured at 492 nm. The same procedures were applied for kojic acid solutions as standard material. By using the absorbance values from all samples, the % inhibitions were calculated with the help of the following formula (Sharaf et al., 2014).

$$\% \text{ inhibitions} = [(A_c - A_s) / A_c] \times 100$$

A_c is the absorbance value of the control and A_s is the absorbance value of the extract.

Determination of Anthelmintic Activity

The anthelmintic activity of the plant extract was determined by Dash et al. (2002) method was modified. *Tubifex tubifex*, which is in the Annelida group, and is anatomically and physiologically similar to human intestinal worms, was used in the experiment. Aquarium worms such as *T. tubifex* are widely used for the initial evaluation of anthelmintic compounds in vitro due to their easy availability. *T. tubifex* was collected from the canals in the Köyceğiz district of Muğla. *T. tubifex* average size is around 1-2 cm. 20 ml solutions prepared at different concentrations (2.5, 5, 10, 20 mg/mL) by dissolving the plant extracts in distilled water were poured into Petri dishes. Later, 6 of these wolves were placed inside. Albendazole (10 mg/mL) was used as the reference standard. Distilled water was used as a negative control. The time taken for paralysis and death was then noted in minutes. The mean duration of paralysis at which movement was lost or no movement could be observed was noted, except when the worms were vigorously swayed. The time of death of each worm was recorded after it was determined that the worms did not move when shaken or externally stimulated.

Statistical analysis

All assays were performed in 3 replicates. The mean ± standard error was analyzed with Microsoft Excel. In studies conducted to determine free radical scavenging activity, the IC_{50} value was calculated using the Minitab 16 statistical program.

Results and Discussion

The potential antioxidant activity of methanol extract obtained from *I. graveolens* species was determined by ß-carotene/linoleic acid, FRAP, and CUPRAC methods (Table 1). It was determined that the extract (27.88±3.86%) exhibited a lower % inhibition than BHA (94.68±0.28%) used as a standard in the ß-carotene/linoleic acid method. The copper and iron reducing power capacities of the extract were calculated as Trolox equivalent. It was determined that it was 4.28±0.24/0.47±0.03 mg TE/g extract equivalent, respectively. There is a positive correlation between the total antioxidant activities of the extract and its phenolic content. Phenolic substances can inhibit the harmful structures of free radicals with hydroxyl groups in their structures. Within the scope of the study, the total phenolic and flavonoid substance amounts of the extract were determined (Table 1). The total phenolic amount was determined as gallic acid equivalent, and the total flavonoid amount was determined as quercetin equivalent. It was found to be 5.36±0.32 mg GAE/g and 3.49±0.05 mg QE/g extract equivalent, respectively.

Table 1. The Total Amount of Secondary Metabolites and Antioxidant Activity of the Extract

Sample/Assay	β-caratone/ Linoleic Acid (%)	FRAP (mg TE/g)	CUPRAC (mg TE/g)	Total Phenolic (mg GAE/g)	Total Flavonoid (mg QE/g)
I. graveolens	27,88±3,86	0,47±0,03	4,28±0,24	5,36±0,32	3,49±0,05
BHA	94,68±0,28	-	-	-	-

Ceyhan et al. (2021), antioxidant activities of 11 *Inula* L. species, DPPH (58.99-188.22 mg TE/g), ABTS (90.51-220.97 mg TE/g), CUPRAC (169.88-460.53) mg TE/g), FRAP (81.57-237.99 mg TE/g), metal chelation (8.31-25.39 mg EDTA/g), Phosphomolybdenum (1.55-2.49 mmol TE/g) methods were investigated. They found that the extracts exhibited significant antioxidant capacity. In a study, they determined the total phenolic content as well as the DPPH and ABTS scavenging activities of *I. viscosa* species collected from Algeria. They determined that the scavenging activity was 14.1±1.3-24.2±1.0 µg/mL in terms of IC_{50} value. They revealed that the total phenolic amount was 299.1 ± 34.5 mg GAE/g extract value (Brahmi-Chendouh et al., 2019). In another study on *I. viscosa*, DPPH free radical scavenging activity (157.72 ± 6.45 µM TE/g DW), oxygen radical absorbance capacity (4471.42 ± 113.16 µM TE/g DW), hydroxyl radical scavenging capacity (630.10 ± 17.81 µM) of ethanol extract TE/g DW) and total phenolic content (285.77 ± 3.68 mg GAE/g DW) were determined (Kheyar-Kraouche et al., 2018). Mohti et al. (2020) determined the antioxidant activity of the extracts obtained from the leaves and flower buds of *I. viscosa*, which they collected from Morocco, using different solvents and

methods. They determined that the highest scavenging activity was 54.24 ± 0.21 µg/mL (IC_{50}) flower bud (Sox-EtOH) extract. In another study, Imouzzer, Sefrou and Taounate investigated the antioxidant activities and total substance content of the ethanol and ethyl acetate extracts of *I. viscosa* collected. They revealed that *I. viscosa* extracts have significant antioxidant activities. They found that the highest amount of phenolic was in the ethanol extract, while the amounts of flavonoids were equal in the extracts (Chahmi et al., 2015). Gökbulut et al. (2013) determined the potential antioxidant activity of water, methanol, and ethyl acetate extracts obtained from leaves, flowers, and roots of 3 different *Inula* L. species collected from different regions of Anatolia by DPPH and ABTS methods. The highest scavenging activity was found in *I. helenium* flower methanol (0.14 ± 0.06 mg/mL, IC_{50}) extract and flower water extract (0.05 ± 0.02 mg/mL, IC_{50}), respectively. In a study, DPPH free radical scavenging antioxidant activity and total phenolic content of *I. crithmoides* hexane, methylene chloride, and methanol extracts were determined. They revealed that the methanol extract has both the highest activity and the highest amount of phenolic substances (Bucchini et al., 2015). Albayrak et al. (2015) investigated the antioxidant activities (DPPH, Phosphomolybdenum, ß-carotene/linoleic acid) and total phenolic contents of methanol, ethanol, water, and ethyl acetate extracts obtained from different taxa of *I. helenium*. Total phenolic amounts vary between 4.18±0.0-102.91±0.6 mg GAE/g. They determined that *I. helenium* ssp. methanol extract exhibited the highest activity. Tredafilova et al. (2020) examined the antioxidant activities (DPPH) and phenolic amounts of six different *Inula* L. species collected from Bulgaria in a study they conducted. They found the highest activity (69.41±0.55%) and phenolic substance content (119.92±0.95 mg GAE/g) in *I. ensifolia* flower methanol extract. Ozkan et al. (2019) investigated the antioxidant activities (DPPH) and total substance amounts (Phenolic, Flavonoid) of water and methanol extracts obtained from *I. viscosa* species they collected from Manisa. They determined that the highest amount of phenolic (107.0±0.0001 mg GAE/g) and flavonoid (158.35±0.0002 mg CE/g) was in the methanol extract. The methanol extract (93.78±0.0003) showed the highest activity in terms of DPPH free radical scavenging activity of the extracts at different concentrations. Asraoui et al. (2021) determined the antioxidant activity and total phenolic and flavonoid content of *I. viscosa* leaf extracts of methanol, ethyl acetate, and chloroform. The extracts showed gallic acid and catechin equivalence as high as 87.2 ± 0.50 mg GAE/g and 78.6 ± 0.55 mg CE/g, respectively. They found that ethyl acetate extract exhibited higher antioxidant activity in DPPH (0,6±0,03 µg/mL; IC_{50}), ABTS (8,6±0,08 µg/mL), and FRAP (634,8 mg ± 1,45 AAE/g) methods compared to methanol and chloroform.

The acetylcholinesterase and tyrosinase enzyme inhibitory activities of the extract were determined (Table 2, Table 3). In both studies, an increase in % inhibition is observed depending on the increase in concentration. It exhibited lower percent inhibition than galantamine (89.41±0.05%) and kojic acid (73.93±0.10%) used as standard.

Table 2. Acetylcholinesterase Enzyme Inhibitory Activity of the Extract (% inhibition)

Plant/Standard	0,025 mg/mL	0,05 mg/mL	0,01 mg/mL	0,2 mg/mL	IC_{50}(mg/ml)
I. graveolens	-	13.01±0.24	22.57±0.62	37.83±0.52	0.228±0.002
Galantamine	73.09±0.05	79.08±0.47	84.76±0.08	89.41±0.05	-

Table 3. Tyrosinase Enzyme Inhibitory Activity of the Extract (% inhibition)

Plant/Standard	0,025 mg/mL	0,05 mg/mL	0,01 mg/mL	0,2 mg/mL	IC_{50}(mg/ml)
I. graveolens	-	11.80±0.12	21.04±0.34	61.73±0.05	0.172±0.001
Kojic acid	45.52±0.14	54.81±0.17	61.73±0.05	73.93±0.10	0.037±0.003

In a study, acetylcholinesterase (3.56-5.13 mg GALAE/g, butyrylcholinesterase (1.49-7.34 mg GALAE/g), tyrosinase (112.31-122.13 mg KAE/g), α-glucosidase (0.77-2.08 mmol)) of 11 *Inula* L. species ACAE/g) and α-amylase (0.73-0.90 mmol ACAE/g) were found to be active enzyme inhibitors (Ceyhan et al., 2021). In a study, acetylcholinesterase and tyrosinase enzyme inhibitory activities of 6 different Inula species were investigated. The highest acetylcholinesterase enzyme inhibitory activity was observed in *I. ensifolia* flower methanol (17.0%) extract. *I. bifrons* flower methanol (0.123±0.000

mg/mL, IC_{50}) extract exhibited the highest tyrosinase enzyme inhibitory activity (Trendafilova et al., 2020). In a study, the enzyme inhibitory activities of α-glucosidase and α-amylase of leaf methanol, ethyl acetate, and chloroform extracts of *I. viscosa* species were investigated. Methanol extract showed the highest α-glucosidase (22.3 ± 2.82 mg/mL, IC_{50}) and α-amylase (27%) enzyme inhibitory activity (Asraoui et al., 2021). In a study, acetylcholinesterase (38.5 mg/mL, IC_{50}), butyrylcholinesterase (34.65 mg/mL, IC_{50}), glutathione S-transferase (77.0 mg/mL, IC_{50}) and α- glucosidase (40.76 mg/mL, IC_{50}) enzyme inhibitory activities were determined (Bursal et al., 2021). Güçlü et al. (2022) determined that acetylcholinesterase (75.94±0.09%), butyrylcholinesterase (78.63± 0.02%), α-glucosidase (53.26±0.12%), α-amylase (18.07±0.03%) and tyrosinase (59.21±0.08%) enzyme inhibitory activities of *I. auccheriana* ethanol (80%) ethanol extract. In a study, acetylcholinesterase, butyrylcholinesterase, and α-amylase enzyme inhibitory activities of *I. salicina* extracts prepared with different solvents were investigated. The highest α-amylase (0.290±0.001 mg/mL, IC_{50}) and acetylcholinesterase (0.577±0.012 mg/mL, IC_{50}) enzyme inhibitory activities were observed in ethyl acetate extract. They found that the highest butyrylcholinesterase enzyme inhibitory activity was in methanol (0.279±0.004 mg/mL, IC_{50}) extract (Yıldırım et al., 2022). Although the methanol extract exhibited lower inhibition than the standards in enzyme inhibitor assays, the results were moderate because it was tested at the same concentrations as the pure standard compounds.

The anthelmintic activity of the extract at different concentrations (2.5, 5, 10, 20 mg/mL) was determined by determining the duration of action (paralysis and death) on *Tubifex tubifex* helminths (Table 4). Depending on the increase in concentration, there was a decrease in the duration of paralysis and death. Concentrations of 10 mg/mL and 20 mg/mL exhibited anthelmintic activity near andazole used as a positive control.

Table 4. Anthelmintic Activity of *Inula Graveolens* Methanol Extract

	Concentration (mg/mL)	P (min)*	D (min)**
I. graveolens	2,5	18	32
	5	13	23
	10	7	12
	20	3	5
Pozitive Control***	10	4	10
Negative Control****	-	-	-

P: Paralysis time for worms, **D: Death time for worms, * Positive Control: Andazol®, ****Negative Control: Distilled water.*

In a study, they investigated the anthelmintic activity (*Panagrellus redivivus*/Tubifex worms) of different concentrations (2, 2.5, 3 mg/mL) of methanol extract obtained from *Blumea lacera* species in the Asteraceae family. They found that the extract exhibited an anthelmintic activity close to levamisole, which is used as a standard (Haque et al., 2014). Das et al. (2011) investigated the anthelmintic activity of Tamarindus indica leaf, bark ethanol, and water extracts (*Pheretima posthuma, Tubifex tubifex*). Extracts from the bark showed activity close to the standard (Piperazine) in both worms. It was determined that the bark methanol extract at a concentration of 15 mg/mL (death time: 20.66±1.33) exhibited a higher anthelmintic activity than Piperezine (45.33±1.20). In a study, the anthelmintic activity (*Tubifex tubifex*) of *Hopea odorata* leaf methanol, ethanol, and water extracts was investigated. The methanol extract (20 mg/mL) exhibited an anthelmintic activity close to the standard Levamisole (1 mg/mL) (Hossain et al., 2015). Dey and Ghosh (2010) investigated the anthelmintic activity of *Amorphophallus paeoniifolius* methanol extract at different concentrations (25,50,100 mg/mL) on *Pheretima posthuma* and *Tubifex tubifex* in their study. The extract at a concentration of 100 mg/mL (time to death: 38.66±2.906) showed higher anthelmintic activity than Piperazine at a concentration of 10 mg/mL (time to die: 64±0.881). In a study, the anthelmintic (*Pheretima posthuma, Tubifex tubifex*) activity of different concentrations (25, 50, 100 mg/mL) of *Tragia involucrata* leaf methanol extract was investigated. It was observed that the death and paralysis

times were shortened due to the increase in concentration. They found that the paralysis time at the highest concentration was 19.33 minutes, and the dead time was 40.00 minutes.

Conclusion

Plants have always been used for medicinal purposes as well as for ethnobotanical use. Species found in the genus *Inula* L. have been very valuable in this respect. This study on *I. graveolens* shows us that the extracts of the species are at moderate levels in terms of phenolic component, although they do not have high activity. Although methanol extracts did not exhibit as high activity as the standards, data close to them were obtained in the studies conducted to determine the enzyme inhibitory activity on acetylcholinesterase and tyrosinase. The results obtained in the anthelminthic studies revealed that the lethality of the species is high. This may be due to the effect of glycoside saponins on the structure of the plant. All these revealed that *I. graveolens* is a species that can be evaluated pharmacologically in future studies, as it has antioxidant, enzyme inhibitor, and anthelmintic effects.

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Author Contribution

Ramazan Mammadov, wrote the project and conducted and directed the studies. *Bayram Kaya*, contributed to all the works as the project coordinator. *İlayda Cansu Atıcı*, participated in the laboratory work. *Mehmet Özgür Atay*, made a direct contribution to the work and writing of the article. All authors have read and approved the article.

Ethics Statement

There are no ethical issues with the publication of this article.

Conflict of Interest

The authors state that there is no conflict of interest.

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