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Use of Activated Sludge Process in Molasses Industry Wastewater Treatment

Pekmez Endüstrisi Atık Sularının Arıtılmasında Aktif Çamur Prosesinin Kullanımı

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Abstract

Water is an important component in production of molasses and it takes an important role in cleaning, cooling and heating process. Therefore, the process of molasess leads to release large amount of wastewater containing organic components. Due to the fact that this wastewater mainly consists of organic components, the wastewater needs high level of biological oxygen (BOD) and chemical oxygen (COD). Thus, the wastewater from the molasses process can causes of environmentally serious problems. To prevent these environmental problems, biological treatment systems are currently used in the molasses industry. Active sludge process is one of the most common processes used in the industry. Carbon, phosphate and nitrogen can be removed from the wastewater by active sludge treatment process. Also, active sludge treatment systems are very effective, cheaper, and eco-friendly. This study aims to show real-time analytical data from the wastewater from molasses industry by using active sludge treatment system (According to the governmental regulations on wastewater treatment). In the molasses industry, COD was 2500-5500 mg/L, BOD

Özet

Su, pekmez üretim prosesi için önemli bir kaynaktır ve üretim esnasında gerçekleştirilen temizlik, soğutma ve ısıtma gibi çok çeşitli işlemlerde önemli rol oynar. Bu nedenle, pekmez üretim aşamasında çok fazla su kullanır ve bunun sonucu olarak, yüksek miktarda organik madde içeren atık su açığa çıkar. Pekmez üretim atık sularının kimyasal içeriği genellikle organik maddelerden oluştuğundan yüksek biyolojik oksijen (BOİ) ve kimyasal oksijen (KOİ) ihtiyacına sahiptir. Bu nedenle, bu atık su ciddi çevresel sorunlara neden olmaktadır. Pekmez endüstrisi atık sularında yüksek organik madde içeriği nedeniyle biyolojik arıtma yöntemiyle etkili arıtma sağlanabilmektedir. Aktif çamur prosesi biyolojik arıtma yöntemleri içerisinde en fazla kullanılan yöntemdir. Aktif çamur prosesi ile atık suda bulunan karbon, azot ve fosfat biyolojik olarak uzaklaştırılmaktadır. Arıtma sürecinde farklı görevleri olan mikroorganizma topluluğu olarak bilinen aktif çamur prosesi çevre dostu, ekonomik ve etkili bir arıtma yöntemidir. Bu çalışmada pekmez endüstrisi atık sularının aktif çamur prosesi ile (atıksu arıtma tebliğine uygun olarak) arıtılması sağlanmıştır. Pekmez üretim

was 800-1800 mg/L, TSS was 1.10-1.40 g/L, conductivity was 1800-2600 µS/cm and pH was 3.5-5.5. After the active sludge treatment process, COD and BOD were 30-70 mg/L and 8-24 mg/L, respectively. Conductivity was 1100-1200 µS/cm. Also, total nitrogen, nitrite, nitrate and total phosphate were analysed to observe the low level of them. After the treatment process, environmental pollution was reduced to minimum levels.

endüstrisi atık suyunun giriş değerleri KOİ 2500-5500 mg/L, BOİ 800-1800 mg/L, AKM 1.10-1.40 g/L, iletkenlik 1800-2600 µS/cm ve pH 3.5-5.5 arasında değişmektedir. Aktif çamur prosesi sonrası KOİ ve BOİ değerleri sırasıyla 30-70 mg/L ve 8-24 mg/L arasına, pH 7.6-8.0 ve iletkenlik ise 1100-1200 µS/cm getirilerek tebliğe uygun halde getirilmiştir. Ayrıca arıtılmış suyun toplam azot, nitrit, nitrat, toplam fosfat gibi değerleri de analiz edilerek uygunluğu kontrol edilmiştir. Bu sayede yüksek kirliliğe sahip olan atık su arıtılarak çevre kirliliği önlenmiştir.

Keywords: Molasses, Active sludge, Wastewater

Anahtar kelimeler: Pekmez, Aktif çamur, Atık su

Abbreviations: COD, chemical oxygen demand; BOD, biological oxygen demand; TSS, total suspended solids; TDS, dissolved solids; MBR, Membrane bioreactor

1. INTRODUCTION

1.1. The Importance of Water

Although more than %70 of the earth is covered by water, only % 0.5 of all water can be used as drinkable water. Because of this limited water supply and increasing human population, water became more important issue all over the world (Aladag, 2011; Sarkar et al., 2006). Unfortunately, water scarcity and drought are now a very important problem not only in dry zone, also in wetland areas (Al-Isawi et al., 2016). Within last 50 years, the amount of water used in industry increased by 3 times, and we consume more water than the amount which is essential for sustainable environment (Katip, 2018).

Accelerating industrial development and urbanisation has negative effect on the seas and freshwater bodies. These industrial activities cause of serious environmental problems. Over time, more industrial activities, and cities force the environment negatively. Lakes, rivers, and coasts are exposed to pollutants due to bigger cities and industry. Sometimes, these pollutants can cause of destroying of the water supplies irreversibly (Chan et al., 2009; NG Wun, 2006).

1.2. Wastewater

Until 19th century, the relationship between water pollution and diseases could not be determined. In 1854, Dr. John Snow could detect that epidemic cholera was happened because well water which was contaminated with sewage was used as drinking water (Aladag, 2011). After this determination, treatment of water became more important issue.

Last century, demand to drinking and utility water increased by ten times. Correspondingly, pollution level of water increased. Therefore, the pollution criteria (BOD, TSS, oil, nitrogen etc.) of domestic and industrial wastewater should be investigated (Cırık & Eskikaya, 2018).

Industrial wastewaters can be classified based on the industry field and raw materials that were used in the process. Some industrial wastewaters can contain too-much organic components, high amount of inorganic or toxic components, or easily decompose to biological matters. Which means that suspended solid (TSS), biological oxygen (BOD) and chemical oxygen (COD) can be in a wide range from 1 mg/L to 1000 mg/L (NG Wun, 2006).

1.3. Content of Molasses

Molasses is a food containing high amount of carbohydrate, mineral and organic acids. It is made of fresh or dried fruits such as grape, mulberry, fig, locust bean, apple, watermelon, sugar beet etc. each kind of molasses was called as the fruit that was used in the process. Fresh or dried grape is treated with calcium carbonate and then some enzyme. The final product is highly viscose product (Çümen, 2021). Also, molasses contains large amount of carbohydrade like glucose and fructose.

1.4. Active Sludge Process

Physical, chemical and bacteriological features of water can be regained partly or completely with water treatment systems. Water treatment systems involve all processes of physical, chemical, and biological operations to cleanse wastewater before wastewater releases to receiving environment (Chan et al., 2009; Stainer, 1976). Water treatment stations are generally succession of activities involving tanks and pools where wastewater is purified (Anlı & Şanlı, 2019).

Activated sludge process is the most used method among biological treatment methods (Martins et al., 2004). Activated sludge process, which was developed in England as a result of the studies carried out by Arden and Lockett in 1913-1914, is a turning point in biological wastewater treatment (Metcalf & Eddy, 1991). Microorganisms in wastewater benefit from

organic materials in wastewater that have biological value for structure change and energy gain. Thus, the organic matter taken into the microorganism is removed from the environment as activated sludge. This removed sludge is considered as activated sludge since it shows biological activity (Akdemir, 2011). Activated sludge method, which aims to transform wastewater into activated biological flocs, is a process equivalent to naturally self-treatment of polluted water under aerobic conditions (Berkun, 2006).

In activated sludge systems, organic matter resides in the pool where the aerobic bacterial culture is suspended. In the pool, the stoichiometric transformation of the bacterial culture takes place as in reactions (1) and (2) below. In case of oxidation and synthesis;

$$COHNS + O_2 + \text{Nutrient} \xrightarrow{\text{Bacterium}} CO_2 + NH_3 + C_5H_7NO_2 + \text{other products} \quad (1)$$

In the reaction equation, COHNS represents the organic matter in the wastewater. When the amount of substrate is insufficient, some of the cell's storage materials are oxidized by its own mass. This internal respiration is expressed by equation (2).

$$C_5H_7NO_2 + 5O_2 \xrightarrow{\text{Bacterium}} 5CO_2 + 2H_2O + NH_3 + \text{Energy} \quad (2)$$

Equations (1) and (2) describe the complete oxidation of organic matter (Akdemir, 2011; Metcalf & Eddy, 1991).

In wastewater treatment with activated sludge process; decomposition, degradation or oxidation of carbonaceous wastes, degradation or oxidation of nitrogenous wastes, removal of fine solids and removal of heavy metals take place and these processes are given in Table 1 (Gerardi, 2003).

Table 1. Processes in the activated sludge system

Aim	Oxidation and removal equation
Biochemical Oxygen Demand (cBOD) oxidation involving carbon	$cBOD_{(protein)} + O_2 \longrightarrow C_5H_7O_2N + CO_2 + H_2O + NH_4^+ + SO_4^{2-} + HPO_4^{2-}$
Nitrogen-containing Biochemical Oxygen Demand (cBOD) oxidation	$nBOD_{(ammonium\ ions)} + O_2 \longrightarrow C_5H_7O_2N + NO_3^- + H_2O$
Fine solids removal	Colloids, split growth, insoluble substances
Removal of heavy metals	Al, Cr, Cu, Cd, Fe, Pb, Hg, Ni, Zn

Basic components of an activated sludge system; aeration tank and settling basin or clarifier (Figure 1). Activated sludge; mixed liquid containing the source of microorganisms such as bacteria, fungi, protozoa and high-form organisms includes biological flocs containing

biodegradable and non-degradable suspended, colloidal and dissolved organic and inorganic substances (Akdemir, 2011; Spellman, 2000; Wang et al., 2009).

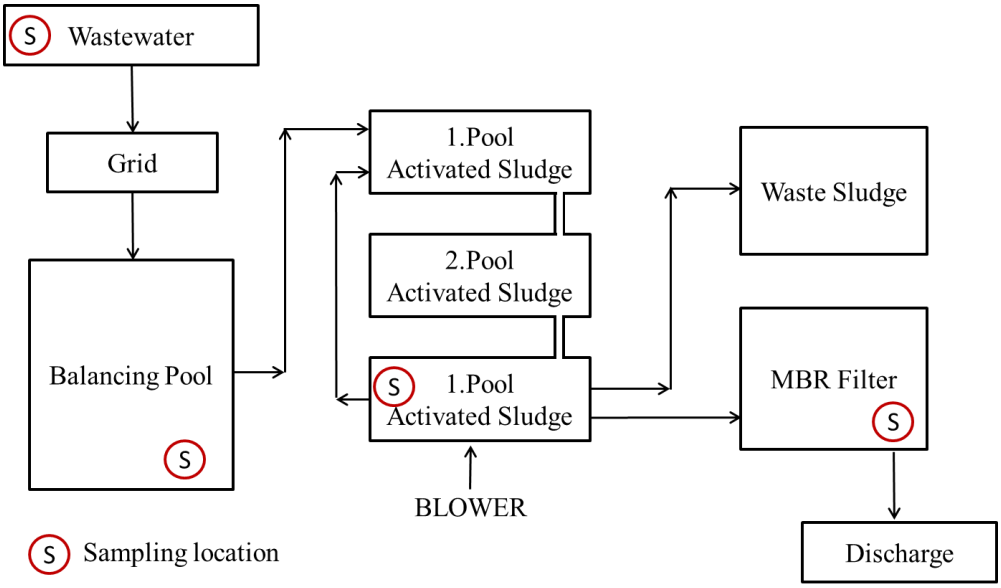


Figure 1. Semas Food basic activated sludge process diagram

In this study, it was aimed that the wastewater profile of Semas Gıda, which produces molasses, was formed and the wastewater of the molasses industry was treated in accordance with the activated sludge process and the wastewater treatment communiqué. For this purpose, 30 days pH, conductivity, dissolved oxygen, total solids (TS), suspended solids (TSS), dissolved solids (TDS), chemical oxygen demand (COD) of molasses production wastewater, activated sludge pool and MBR filter outlet. , biological oxygen demand (BOD), total kjendahl nitrogen, nitrate, nitrite and total phosphate values were tested for compliance with the communiqué. The effluent water features that should be in the wastewater treatment pool are as in Table 2.

Table 2. Purified water criteria

Analysis	Results
pH	6-9
SS (mg/L)	<70
COD mg/L	<150
BODmg/L	<50

2. MATERIALS and METHODS

2.1. Sampling

In this study, samples were taken from the points indicated in Figure 1 in the wastewater facility, at intervals of 4 hours, for 30 days, 5000 mL per day from each sample. Then, these samples

were mixed and homogenized and the following tests were carried out. Table 3 shows the daily sampling time and amount. Samples were taken according to Table 3 for 30 days.

Table 3. The times of taking daily water samples

	1stvsample	**2nd sample**	**3rd sample**	**4th sample**	**5th sample**
Time	08:00	12:00	16:00	20:00	24:00
Quantity	1000 mL	1000 mL	1000 mL	1000 mL	1000 mL

2.2. Microscope Analysis

Samples taken from the wastewater facility were examined daily with A. Krüss Optronic brand microscope with 10X, 40X and 100X magnifications. In microscope analysis; vitality in wastewater and MBR outlet, vitality in activated sludge and morphological structure analysis were performed.

2.3. pH Determination

The pH analyzes of the samples taken from the facility were made with Ohaus Starter2100 brand pH meter and Ohaus ST310 brand probe.

2.4. Conductivity Determination

Conductivity analyzes of the samples taken from the facility were made with Emaf EM77G brand conductivity meter and Mettler toledo 950-K19/120 brand probe.

2.5. Dissolved Oxygen Determination

Dissolved oxygen of the samples taken from the facility was made with Hanna HI2004 brand oxygen meter.

2.6. Determination of Total Solids (TS), Suspended Solids (TSS) and Dissolved Solids (TDS)

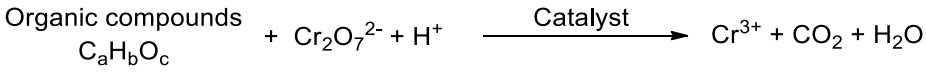
A well-mixed 250 mL sample is evaporated in a constant weight capsule and dried in an Ankyralab brand oven until a constant weight is obtained at 103-105°C, then cooled in a desiccator and weighed. The weight gain in the empty capsule represents the total solids content.

After keeping it in an Ankyralab oven at 105 °C for 1 hour and cooling it in a desiccator, the vacuum filtered sample from filter paper (0.45 µm membrane for clean water, glass fiber for

wastewater) is carefully taken on an aluminum or stainless steel tray by using forceps. It is dried in an oven at -105°C for 1 hour. It is cooled in a desiccator and weighed. The weight of the filtrate residue refers to the suspended solids. The filter residue obtained for the suspended solids (105°C) is in a capsule brought to a constant weight at 550±50°C in an Elimko 70 brand muffle furnace at 550±50°C and in a muffle furnace at 550±50°C. It is burned for 20 minutes. It is cooled in a desiccator and weighed. The solid remaining after the weight loss caused by the combustion of volatile solids; is a volatile suspended solid (Baird, 2017).

2.7. Chemical Oxygen Demand Determination (COD)

Chemical oxygen demand (COD) is one of the important parameters used to determine the organic pollution level of domestic and industrial wastewater. Chemical oxygen demand (COD) is the amount of oxygen required for the chemical oxidation of oxidizable substances in water (Dedkov et al., 2000).



5 mL of the sample is taken into the reaction flask and 2.5 mL of potassium dichromate solution (80 g of mercury sulfate is dissolved in 800 mL of water. 100 mL of 1.84 g/mL sulfuric acid is added and 11,77 gr $K_2Cr_2O_7$, dried at 105 °C, is added and cooled and made up to 1000 mL) is added and mixed. 7.5 mL of silver sulfate/sulfuric acid solution (10 g silver sulfate is dissolved in 35 mL of water, and 965 mL of 1.84 g/mL sulfuric acid is added) is added to the refrigeration system. The reaction is continued for 2 hours at 150 °C. The cooled samples are tested with a Shimadzu 1201V brand spectrophotometer at 600 nm. Potassium hydrogen phthalate was used as a standard (1000 mg/L COD sodium phthalate; 0.8 g sodium phthalate dissolved in 1000 mL water).

2.8. Determination of Biological Oxygen Demand (BOD)

The BOD is defined as that mass of oxygen that is consumed by chemicals in the course of n days to oxidatively degrade the organic substances present in 1 Lof water at 20 °C. The dissolved oxygen oxidizes manganese(II) to manganese(III). In acidic solution, this reacts with Titriplex® II to form a red complex that is determined photometrically (modified Winkler method). The BODn is calculated as the difference between the oxygen concentrations determined immediately after sampling and that after n days of incubation of a water sample to which allyl thiourea has been added to inhibit nitrification. The method is analogous to EPA 405.1 (BOD Cell Test 100687).

Check the pH of the sample, specified range: pH 6-8. If required, add dilute sodium hydroxide solution or sulfuric acid drop by drop to adjust the pH. Fill oxygen reaction boots each with pretreated sample and 2 glass bads to overflowing. Close bubble-free with the slanted ground-glass stoppers. Fill oxygen reaction boots each with inoculated nutrient-salt solution and 2 glass bads to overflowing. Close bubble-free with the slanted ground-glass stoppers. Use one bottle of pretreated sample on done of inoculated nutrient-salt solution fort the measurement of the initial oxygen concentration. Incubate one bottle of pretreated sample a done of inoculated nutrient-salt solution closed in a thermostatic incubation cabinet at 20°C for 5 days. After incubation, use one bottle of pretreated sample a done of inoculated nutrient-salt solution for the measurement of the final oxygen concentration. Add 5 drops of BOD-1K and then 10 drops of BOD-2K, close bubble-free, and mix for approx. Add in one minute 10 drops of BOD-3K, reclose and mix. Place the cell into the cell compartment. Align the mark on the cell with that on the spectrophotometer 500 nm.

Calculation:

BOD of measurement sample: Result 1-Result 2 (measurement sample) = A in mg/L

BOD of blank1 – Resut 2 (blank) = B in mg/L

BOD of original sample in mg/L = (A-B)*dilution factor

2.9. Determination of Total Kjeldahl Nitrogen

The 100 mL sample is taken into the Kjeldahl flask containing the boiling stone. 50 mL of digestion solution (67 g of K_2SO_4 is dissolved in 400 mL of distilled water and 67 mL of 1.84 g/mL H_2SO_4 is added to it and completed to 500 mL by adding 3.65 g of $CuSO_4$). It is boiled until 15-20 mL remains in the solution and cooled. 250 mL of distilled water is added. Distillation is carried out with 50 mL of neutralization solution (250 g NaOH and 12.5 g $Na_2S_2O_3.5H_2O$ are taken, completed to 500 mL and dissolved in distilled water) (200-250°C). 50 mL of H_3BO_3 solution (10 g of H_3BO_3 is dissolved in ammonia-free freshly distilled water and made up to 500 mL. 5 mL of mixed indicator is added) into a 500 mL flask. When pH is 3.4-3.5 mixing stopped. The distillate is collected until a minimum of 200 mL has accumulated (green color). Titrate with 0.02N H_2SO_4 until the initial pH value is determined. Glutamic acid, 1 g/L equivalent to 100 mg N, was used as a standard. (Mixed indicator; 1 g Methylene blue + 1 g Methylene Red + 100 mL ethyl alcohol)

Calculation:

$$TKN = \frac{(A-B) \times N \times 14000}{V} = \frac{(A-B) \times 280}{V}$$

N=0.02N

V= sample volume A=Titrant amount B= Witness titrant

2.10. Nitrate Determination (with 2,6 – dimethylphenol spectrophotometric method)

In the presence of sulfuric acid and phosphoric acid, nitrate reacts with 2,6-dimethylphenol to form 4-nitro-2,6-dimethylphenol. The reaction time is about 5 minutes. The absorbance of the reaction product is measured spectrometrically at a wavelength of 324 nm and the nitrate concentration in the analysis sample is read from the calibration curve. 35 mL of sulfuric acid-phosphoric acid mixture (250 mL of sulfuric acid + 500 mL of ortho-phosphoric acid is carefully mixed and 0.020 g of amidosulfonic acid is added to a 100 mL flask). 5 mL of wastewater sample and 5 mL of 2,6-dimethylphenol solution (0.6 g of 2,6-dimethylphenol is dissolved in 500 mL of glacial acetic acid) are added and left for 30 minutes. Samples are tested with a Shimadzu 1201V brand spectrophotometer at a wavelength of 324 nm. In this method, potassium nitrate solution in the range of 1-50 mg/L was used as a standard (Huifa et al., 2003).

2.11. Nitrite Determination

Nitrite (NO_2^-) is determined by the principle of reacting with sulfanilamide and N-(1 naphthyl)-ethylenediamine dihydrochloride under acidic conditions to form a reddish-purple azo dye. The resulting product is measured spectrophotometrically at a wavelength of 543 nm and the concentration of nitrite in the sample is determined. 50 mL of sample, passed through a 0.45 µm filter, is taken into a 100 mL flask and 2 mL of color developing reagent is added and mixed. After waiting for 20 minutes, the samples are tested with a Shimadzu 1201V brand spectrophotometer at 543 nm wavelength. In this method, $NaNO_2$ solutions containing 0.05-0.5 µg N were used as standard (Nagaraja et al., 2001).

2.12. Determination of Total Phosphorus with Stannous Chloride

One of the phosphorus determination methods is the colorimetric method in which Tin (II) Chloride is used as the reducing agent. In this method, phosphate ions form ammonium molybdate and ammonium phospho molybdate. The molybdenum blue complex is formed as a result of the reduction of this compound with stannous chloride. Since the color of this complex

is proportional to the phosphate concentration, the orthophosphate concentration is determined colorimetrically by measuring the color intensity (Arnold et al., 1992).

4 mL Molybdate reagent (25 g $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ is dissolved in 175 mL distilled water to 100 mL of wastewater or effluent sample, carefully added to 266 mL concentrated H_2SO_4 400 mL distilled water in a separate container, cooled, molybdate solution is added. and diluted to 1 L) and 0.5 mL (10 drops) of stannous chloride reagent (2.5 g of fresh $SnCl_2\cdot 2H_2O$ dissolved in 100 mL of glycerine. Heated in a water bath and dissolved by mixing with a glass baguette). The ammonium phosphomolybdate complex is reduced, revealing the molybdenum blue color depending on the phosphorus concentration. After the reagents are added, the following reaction takes place:

$$(NH_4)_3PO_4.12MoO_3 + Sn^{2+} \longrightarrow \text{molybdenum blue} + Sn^{4+}$$

After 10 minutes, the developed color is measured in the spectrophotometer at 690 nm and the result is compared with the calibration curve and the phosphorus content is found. As a standard, a solution of KH_2PO_4 in the range of 0.05-1.2 mg/L is used (219.5 mg of anhydrous potassium dihydrogen phosphate is dissolved in pure water and made up to 1 L. 1 mL of this solution is 50 µg PO_4-P).

Calculation:

$$P = \frac{mg\ P\ \times 1000}{mL\ sample}$$

3. RESULTS and DISCUSSION

As a result of the tests, the average of the 30-day chemical and biological results of the molasses industry wastewater and the average of the results of the MBR effluent after the activated sludge process are given in Table 4.

Table 4. Average results of all analysis over 30 days

Analysis	Unit	Wastewater	Sludge Pool	MBR output
pH	-	4.54±0.63	7.28±0,09	7.71±0,13
Conductivity	µS/cm	2263.01±221.54	1535.10±40.02	1213.03±21.71
Dissolved oxygen	mg/L O_2	0.4±0.27	5.23±0.21	5.88±0.10
TSS (Total suspended solids)	g/L	3.68±0.35	6.14±0.48	n.d.
SS (Suspended solids)	g/L	1.21±0.09	5.92±0.48	n.d.
TDS (Total dissolved solids)	g/L	2.44±0.34	0.21±005	n.d.
COD (Chemical oxygen demand)	mg/L O_2	3889.03±944.80	121.03±26.56	52.53±10.27
BOD (Biological oxygen demand)	mg/L O_2	1297.22±324.36	41.74±8.87	17.75±3.68
Total Kjeldahl Nitrogen	mg/L N	119.85±46.17	not tested	1.01±0.21
Nitrate	mg/L NO_3^-	82.66±27.21	not tested	0.33±0.07
Nitrite	mg/L NO_2^-	5.84±1.39	not tested	n.d.
Total Phosphate	mg/L PO_4-P	55.34±8.42	not tested	1.01±0.25

3.1. Microscope Analysis

Samples taken from the wastewater facility were examined daily with A.Krüss Optronic brand microscope at 10X, 40X, 100X and 200X magnifications. In microscope analysis; a high rate of bacterial and yeast viability was observed in the wastewater, but the species were not determined. No vitality was observed in the MBR output. In the activated sludge, the vitality and morphological structure analyzes were made daily and the species specified in Table 5 were determined.

Table 5. Types of microorganisms determined in activated sludge

Type	Genus	Type	Genus
Bacterium	*Pseudomonas* spp.	Protozoa	Ciliates
	Arthobacter spp.		Flagellates
	Bacillus spp.		Rhizopoda (Amoeba)
	Cytophaga spp.		Rotifers
	Zooglea spp.	Fungi	Geotrichum
	Acinetobacter spp.		Penicillium
	Nitrosomonas spp.		Cephalosporium
	Nitrobacter spp.		Cladosporium
	Sphaerotilus spp.		Alternaria

3.2. pH, Conductivity and Dissolved Oxygen Analysis

The results of the inlet, activated sludge pool and effluent samples taken from the wastewater pool, showing the 30-day graphs of pH, conductivity and dissolved oxygen, are given in Figure 2-4.

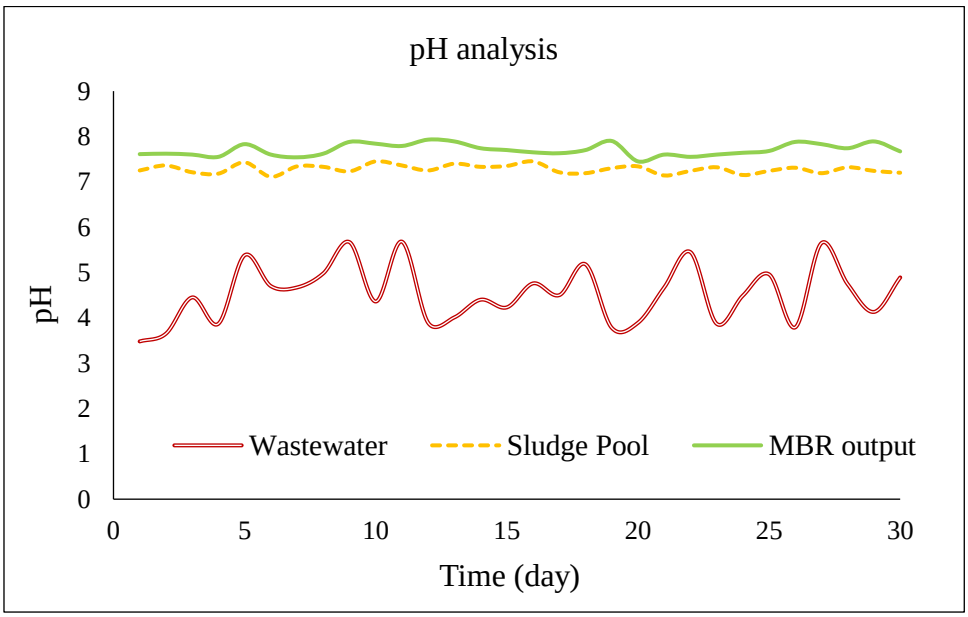


Figure 2. 30-day pH graph of wastewater, activated sludge pool and MBR effluent

The pH of the wastewater inlet for 30 days was 4.54±0.63, while the pH of the effluent was 7.71±0.13.

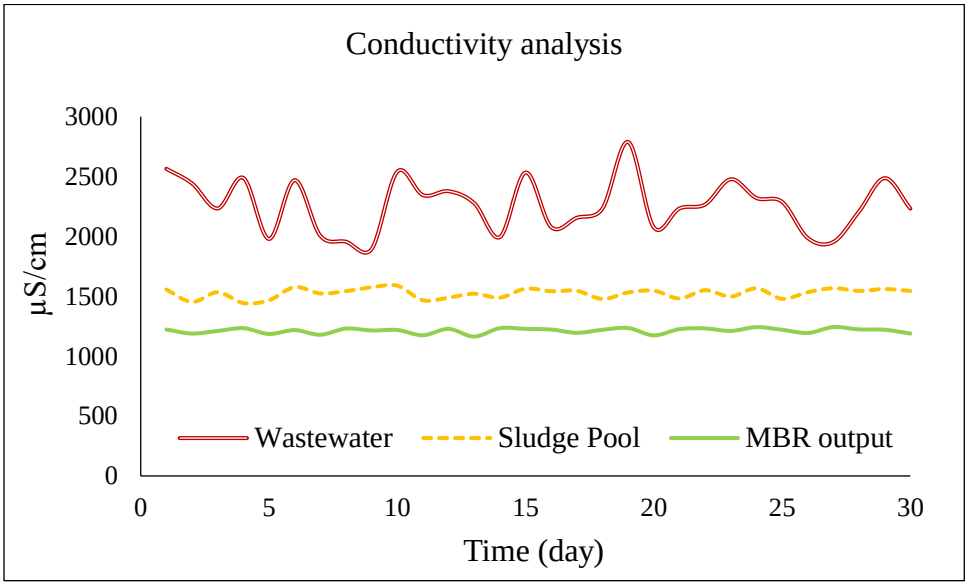


Figure 3. 30-day conductivity graph of wastewater, activated sludge pool and MBR effluent

In addition, the conductivity of the wastewater inlet was 2263±221.54 µS/cm, while the conductivity of the effluent was 1213±21.71 µS/cm.

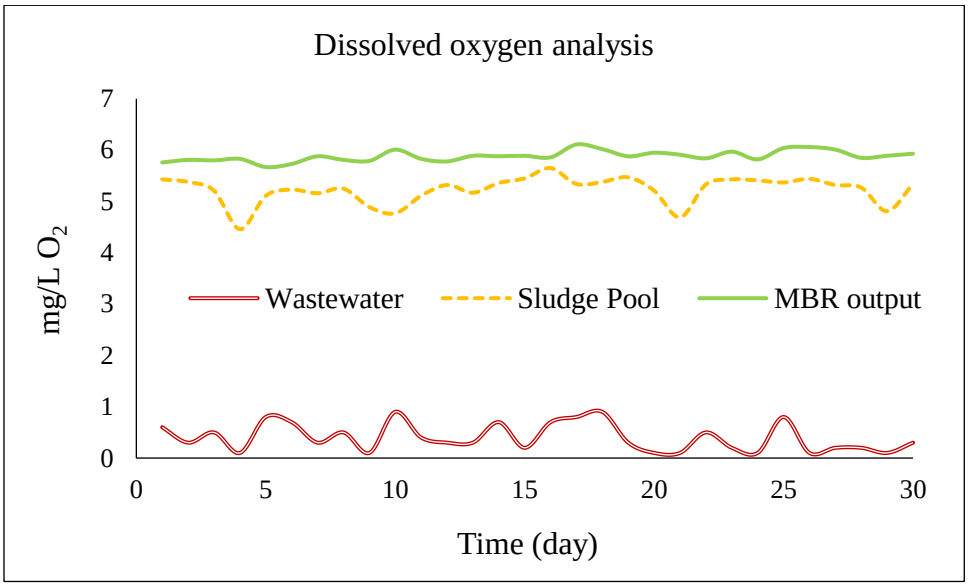


Figure 4. 30-day DO graph of wastewater, activated sludge pool and MBR effluent

Finally, the dissolved oxygen of the wastewater inlet was 0.4±0.27 mg/L O_2, while the dissolved oxygen of the effluent was 5.88±0.10 mg/L O_2.

3.3. Total solids (TS), Suspended Solids (TSS) and Dissolved Solids (TDS) Analysis

The results showing the 30-day graphs of the total solid, suspended solids and dissolved solids amounts of the inlet, activated sludge pool and effluent samples taken from the wastewater pool

are given in Figure 5. As a result of the analyzes, the TSS of the wastewater pool inlet samples was 1.21±0.09, while no solid matter was detected in the effluent.

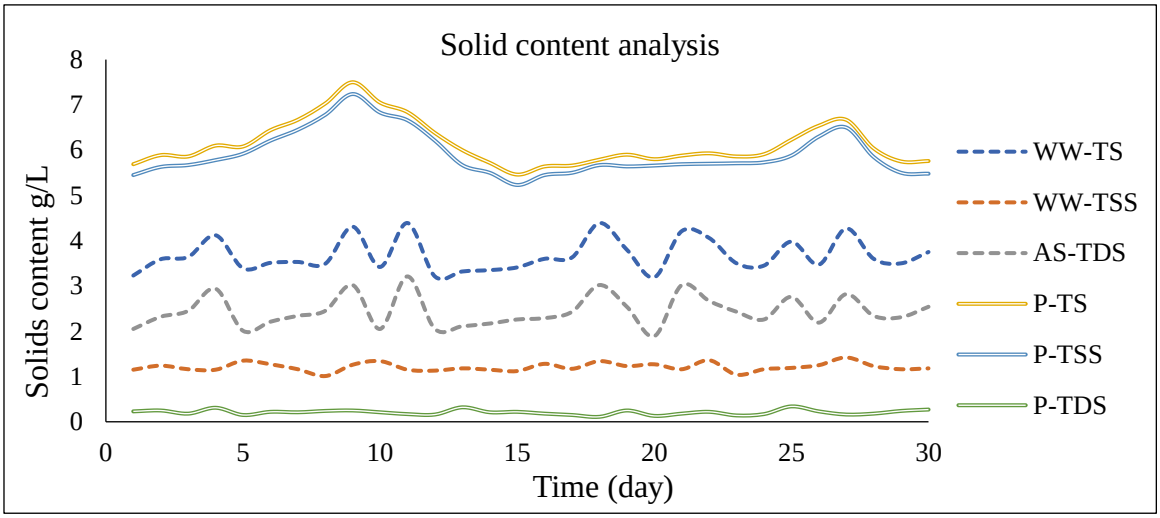


Figure 5. 30-day solids graph of wastewater and activated sludge pool

3.4. Chemical Oxygen Demand Determination (COD)

The results showing the 30-day graphs of the chemical oxygen demand analyzes of the inlet, activated sludge pool and effluent samples taken from the wastewater pool are given in figures 7 and 8. Results were calculated against the potassium hydrogen phthalate standard (Standard graph figure 6). The standard graphic equation gives the equivalent COD value of potassium hydrogen phthalate at different concentrations. It is seen that there is a linear change between these two variables. As a result of the analyzes made, the mean COD value of the wastewater inlet was 3889.03±944.80 mg/L O_2, while the MBR effluent was 52.53±10.27 mg/L O_2.

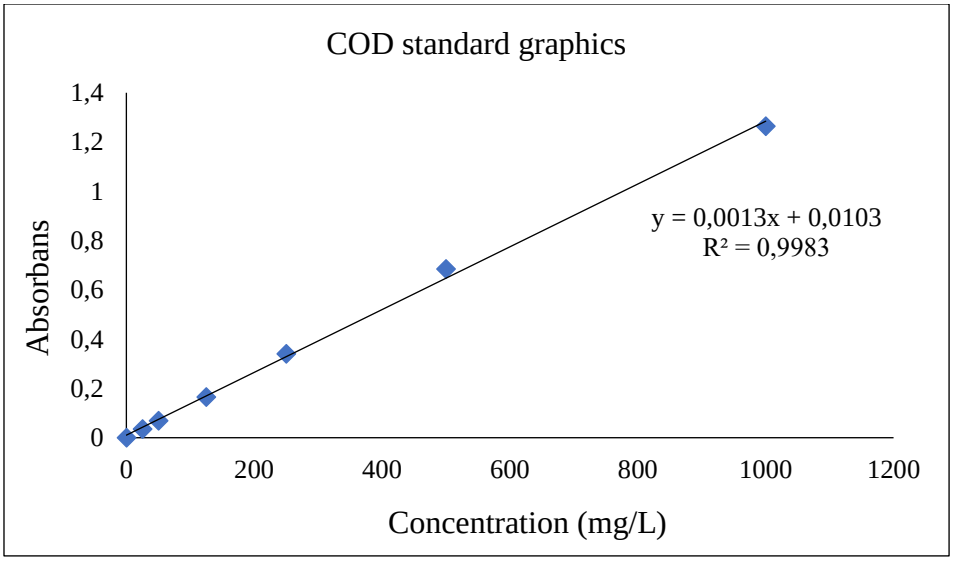


Figure 6. Potassium hydrogen phthalate standard graph

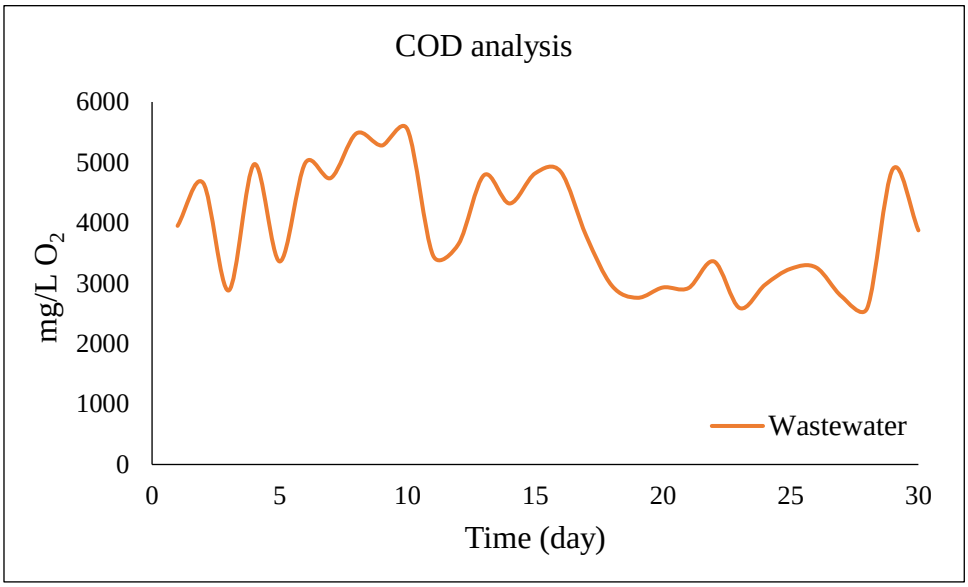


Figure 7. 30-day COD graph of wastewater

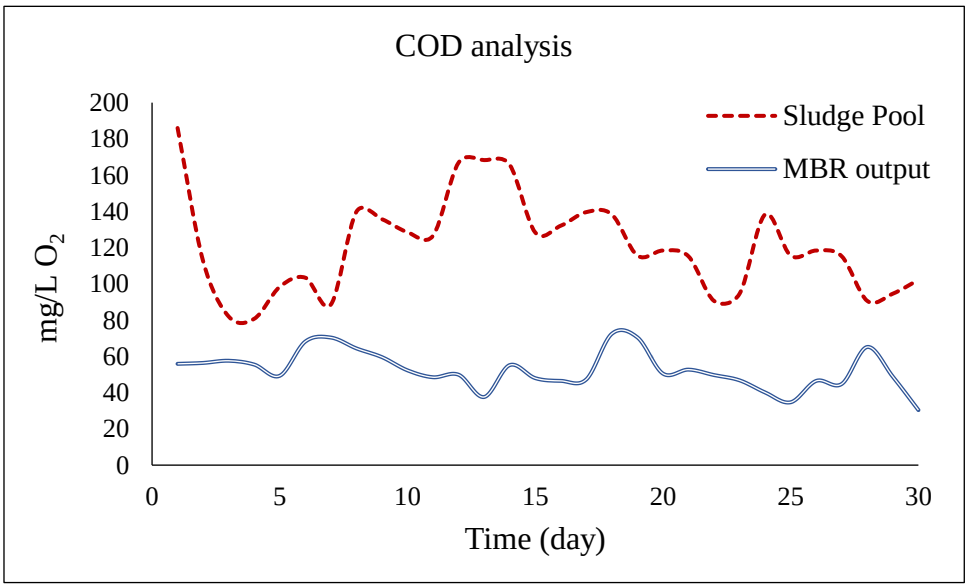


Figure 8. 30-day COD graph of activated sludge and MBR effluent

3.5. Determination of Biological Oxygen Demand (BOD)

The results showing the 30-day graphs of the biological oxygen demand analyzes of the inlet, activated sludge pool and effluent samples taken from the wastewater pool are given in figures 9 and 10. As a result of the analyzes, it was seen that the average BOD value of the wastewater inlet was 1297.22±324.36 mg/L O_2, while the MBR effluent was 17.75±3.68 mg/L O_2.

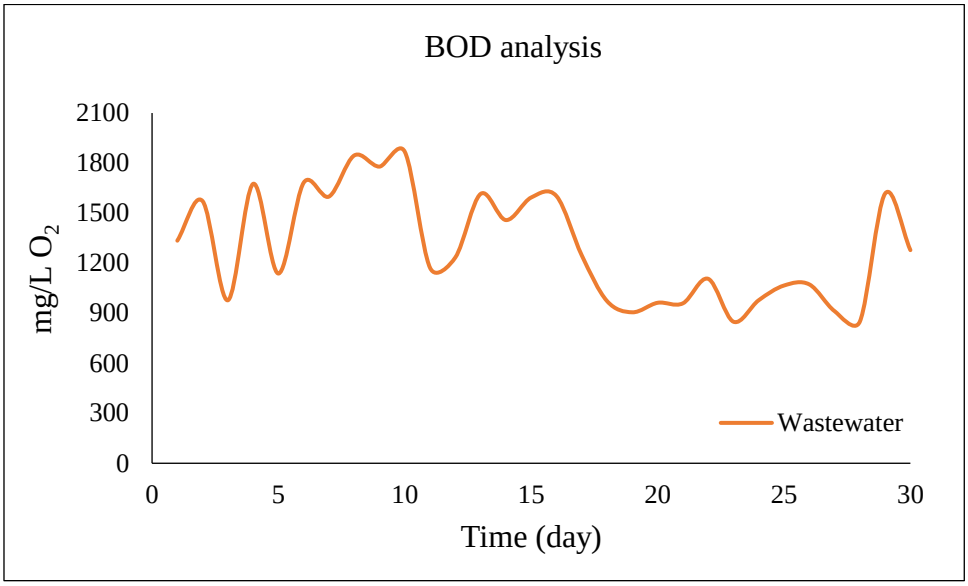


Figure 9. 30-day BOD graph of wastewater

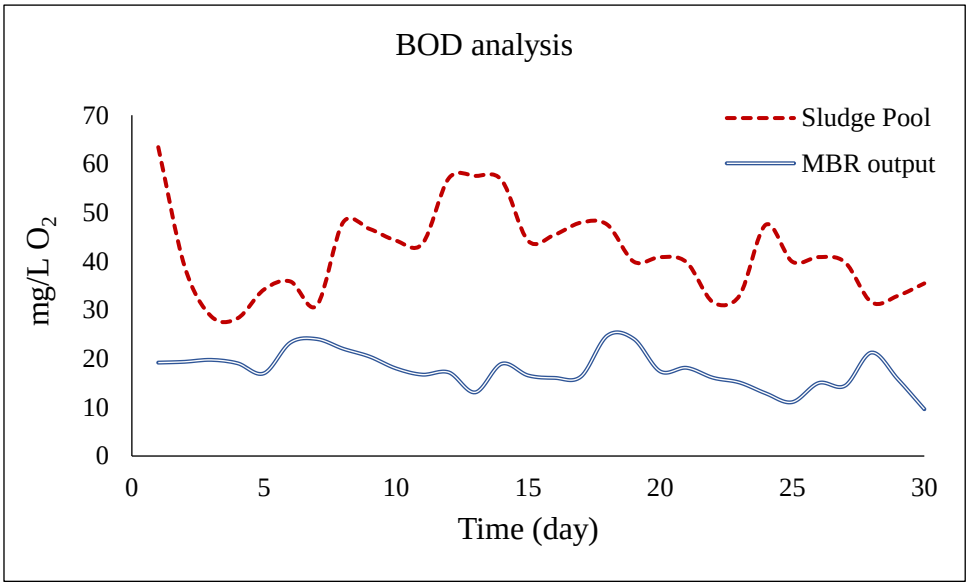


Figure 10. 30-day BOD graph of activated sludge and MBR effluent

3.6. Determination of Total Kjeldahl Nitrogen

The results showing the 30-day graphs of the total nitrogen analyzes of the inlet and outlet water samples taken from the wastewater pool are given in Figures 11 and 12. As a result of the analyzes, it was seen that the total nitrogen of the wastewater inlet was 119.85±46.17 mg/L N on average, while the MBR outlet water was 1.01±0.21 mg/L N.

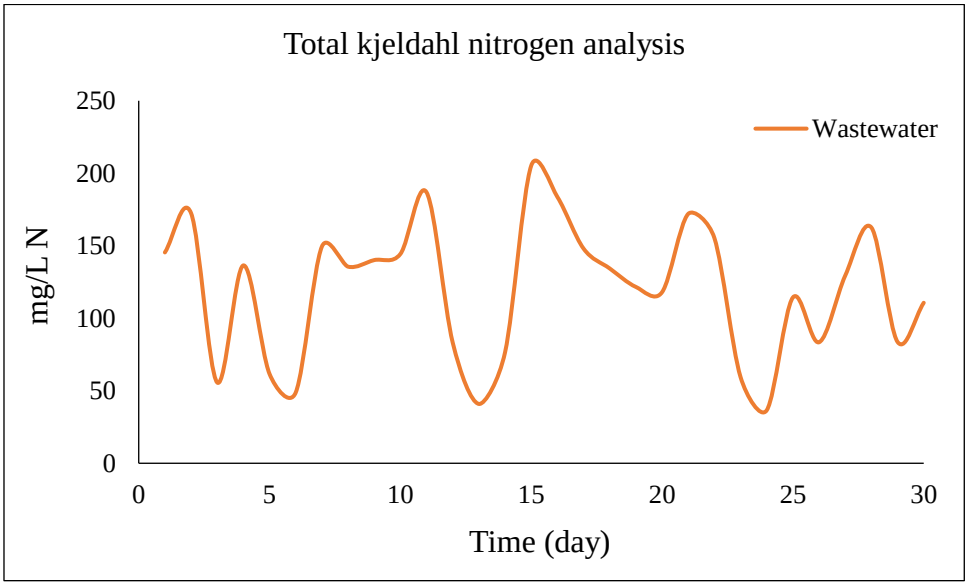


Figure 11. Graph of 30-day total Kjeldahl Nitrogen of wastewater

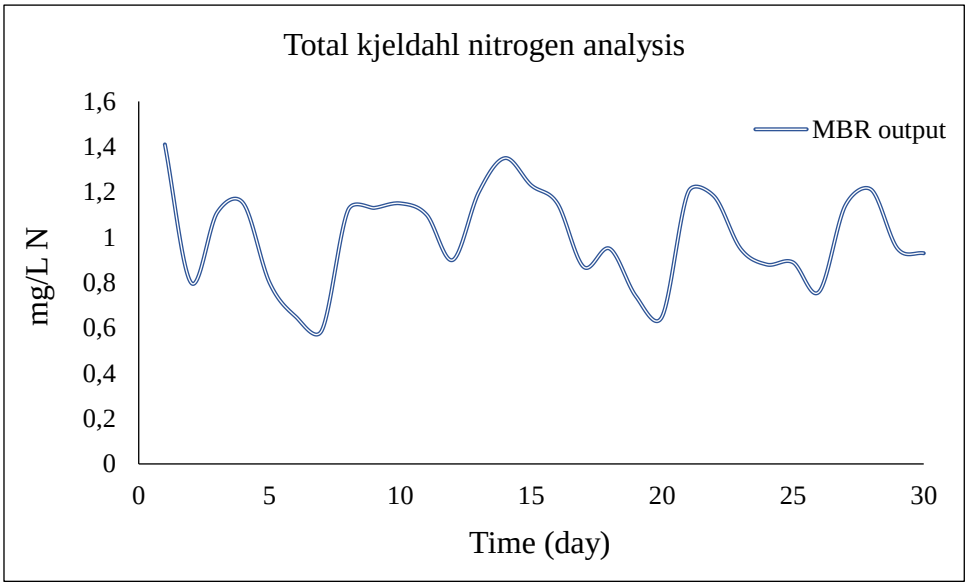


Figure 12. 30-day total Kjeldahl Nitrogen graph of MBR effluent

3.7. Determination of Nitrate

The results showing the 30-day graphs of nitrate analyzes of the inlet and outlet water samples taken from the wastewater pool are given in figures 14 and 15. In addition, the potassium nitrate standard graph used to calculate the results is given in Figure 13. The standard graphic equation gives the equivalent nitrate value of potassium nitrate at different concentrations. It is seen that there is a linear change between these two variables. As a result of the analysis, it was seen that the nitrate of the wastewater inlet was 82.66±27.21 mg/L NO_3^- on average, while the MBR outlet water was 0.33±0.07 mg/L NO_3^-.

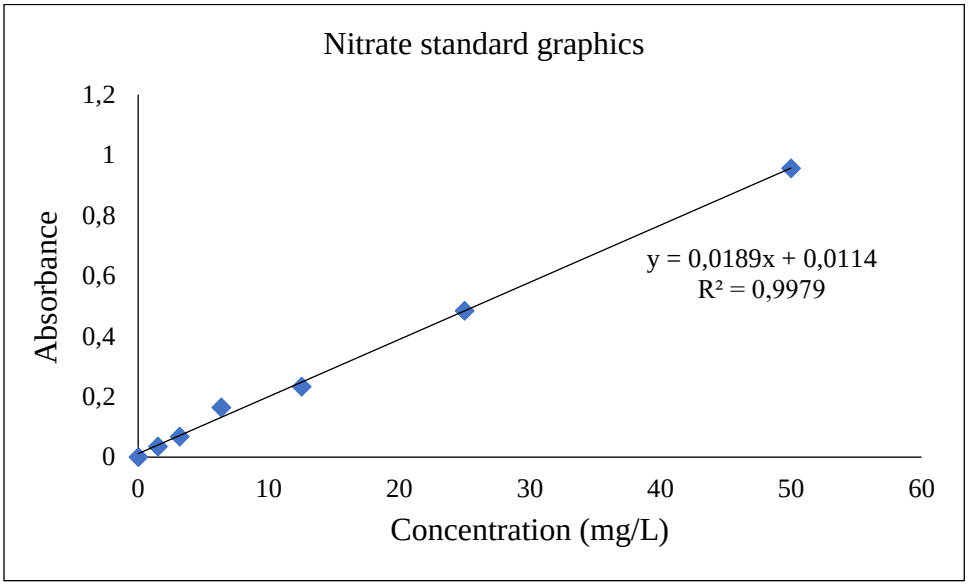


Figure 13. Potassium nitrate standard graph

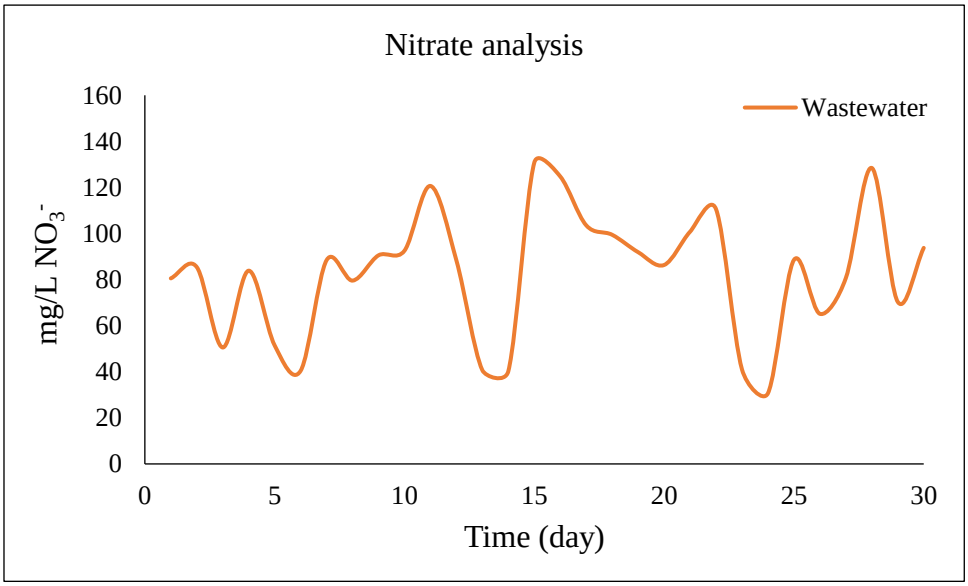


Figure 14. 30-day nitrate graph of wastewater

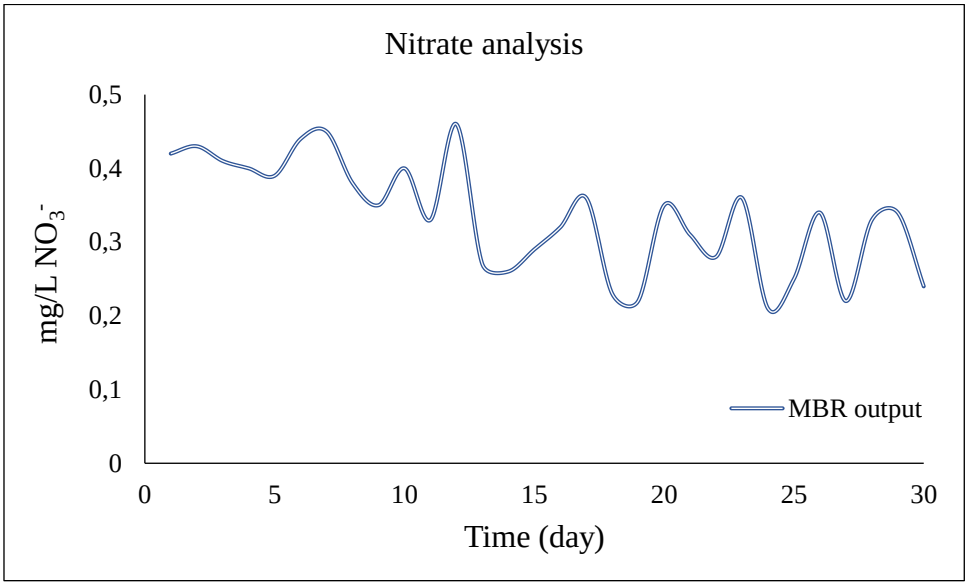


Figure 15. 30-day nitrate graph of MBR effluent

3.8. Nitrite Determination

The results showing the 30-day graphs of the nitrate analyzes of the inlet and outlet water samples taken from the wastewater pool are given in Figure 17. In addition, the sodium nitrite standard graph used to calculate the results is given in Figure 16. The standard graphic equation gives the equivalent nitrite value of sodium nitrite at different concentrations. It is seen that there is a linear change between these two variables. As a result of the analysis, it was observed that the nitrate of the wastewater inlet was 5.84±1.39 mg/L NO_2^- on average, while nitrite could not be detected in the MBR outlet water.

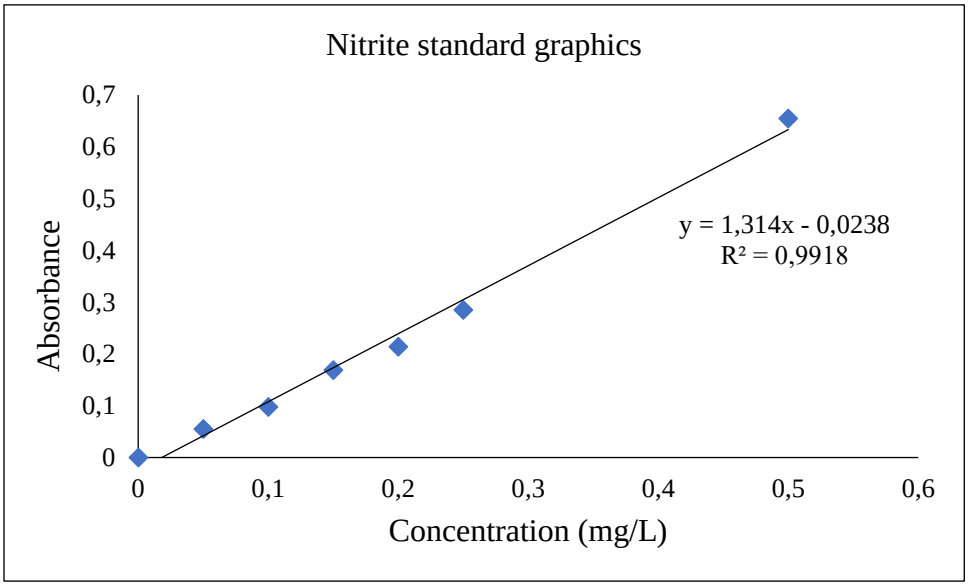


Figure 16. Sodium nitrite standard graph

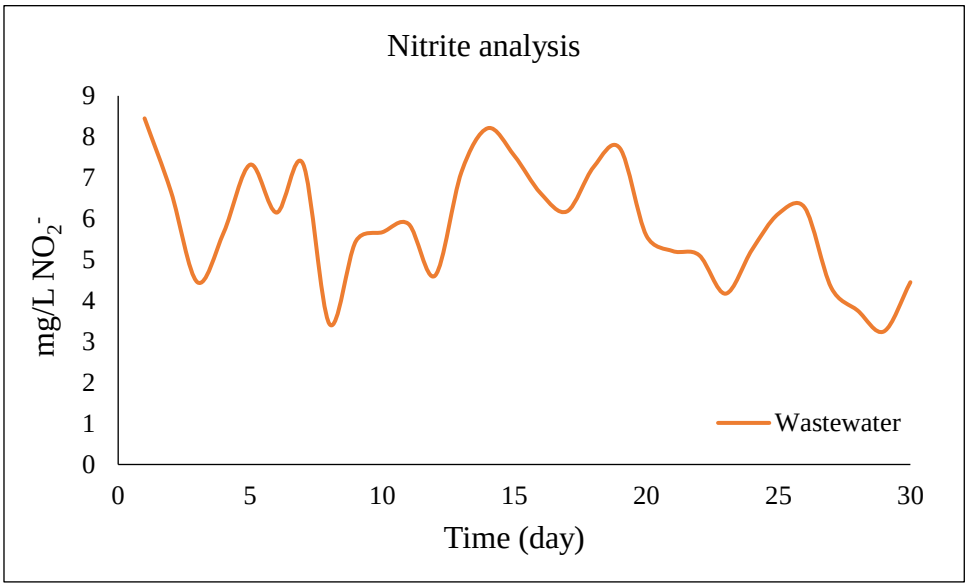


Figure 17. Wastewater inlet 30-day nitrite graph

3.9. Total Phosphate Determination

The results showing the 30-day graphs of the total phosphate analyzes of the inlet and outlet water samples taken from the wastewater pool are given in Figures 19 and 20. In addition, the potassium dihydrogen phosphate standard graph used to calculate the results is given in Figure 18. The standard graphic equation gives the equivalent total phosphate value of potassium dihydrogen phosphate at different concentrations. It is seen that there is a linear change between these two variables. As a result of the analysis, it was seen that the nitrate of the wastewater inlet was 55.34±8.42 mg/L PO_4-P on average, while the MBR outlet water was 1.01±0.25 mg/L PO_4-P.

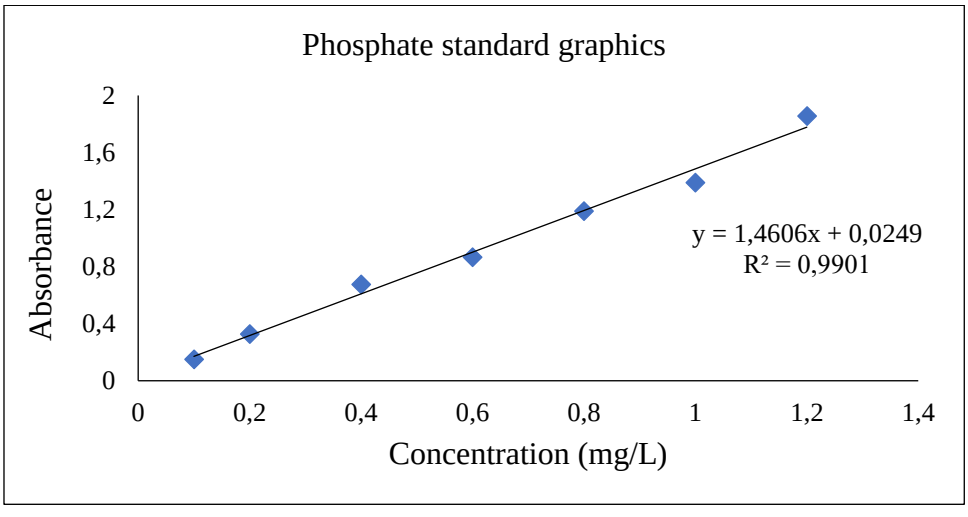


Figure 18. Potassium dihydrogen phosphate standard plot

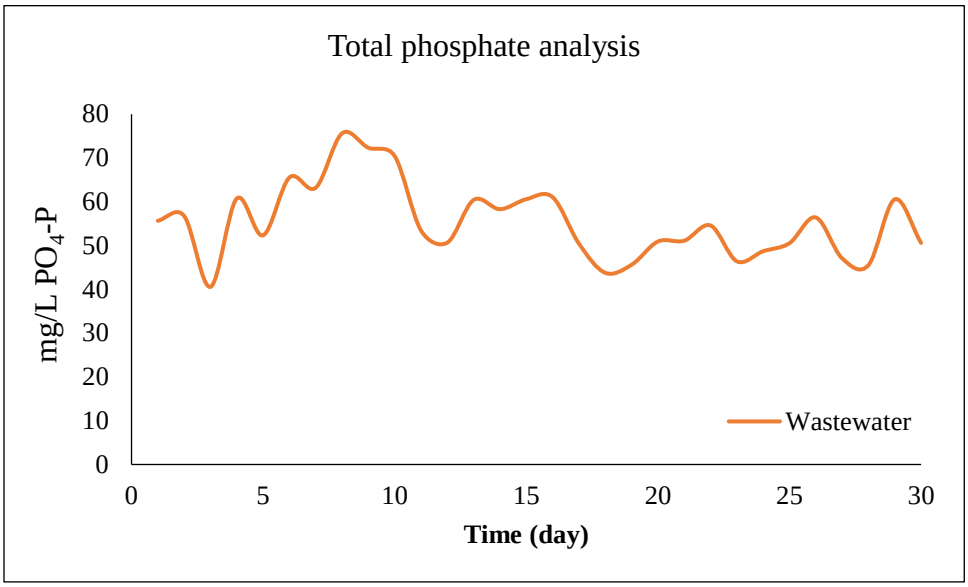


Figure 19. 30-day total phosphate graph of wastewater

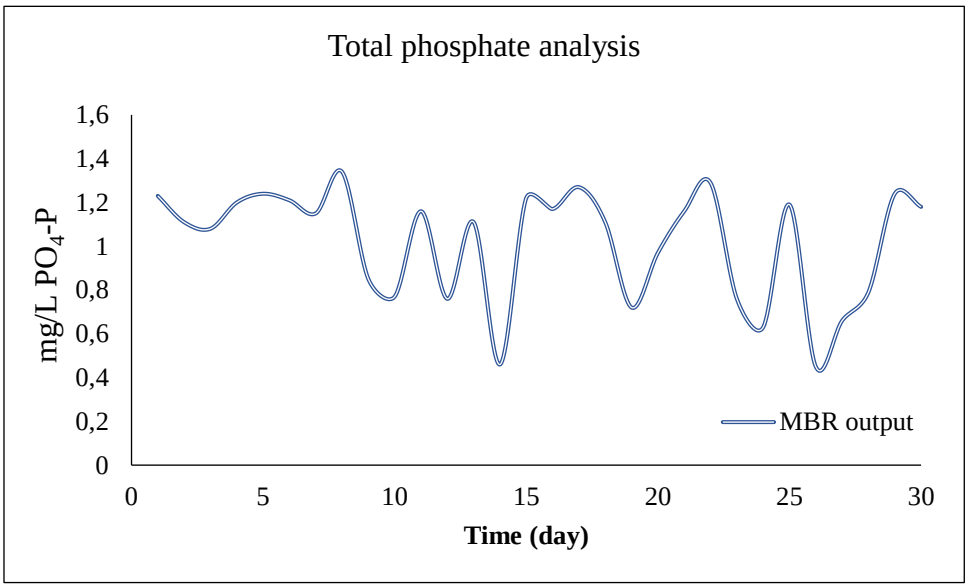


Figure 20. 30-day total phosphate graph of MBR output

4. CONCLUSION

In this study, the wastewater of the molasses industry was characterized and treated with activated sludge process. Firstly, wastewater properties were determined by taking wastewater samples from the inlet water for 30 days. Characteristics of wastewater vary between COD 2500-5500 mg/L, BOD 800-1800 mg/L, TSS 1.10-1.40 g/L, conductivity 1800-2600 µS/cm and pH 3.5-5.5. Then, the characteristics of the effluent after the activated sludge process were determined. After the activated sludge process, the COD and BOD values were determined

between 30-70 mg/L and 8-24 mg/L, pH 7.6-8.0 and conductivity 1100-1200 µS/cm, respectively. In addition, the properties and TSS of the activated sludge were measured for 30 days. In this way, the amount of suspended solids (TSS) in activated sludge pools was kept in the range of 4-9 g/L. In cases where the density of activated sludge is over 9 g/L, sludge swelling was observed in the pools and it was determined that the microorganisms in the sludge died. It was observed that adequate treatment could not be performed in pools with 4 g/L activated sludge density. In addition, values such as total nitrogen, nitrite, nitrate, and total phosphate of wastewater and treated water were determined and it was determined that they were in compliance with the communiqué. As a result, the discharge of highly polluted wastewater has become possible by using this activated sludge process.

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Therapeutic Potentials of Honey, Royal Jelly and Bee Venom on Testosterone Deficiency in Male Albino Rats Infected by $AlCl_3$

Erkek Albino Sıçanlarda $AlCl_3$ Maruziyetiyle İndüklenen Testosteron Eksikliği Üzerine Bal, Arı Sütü ve Arı Zehirinin Terapötik Potansiyeli

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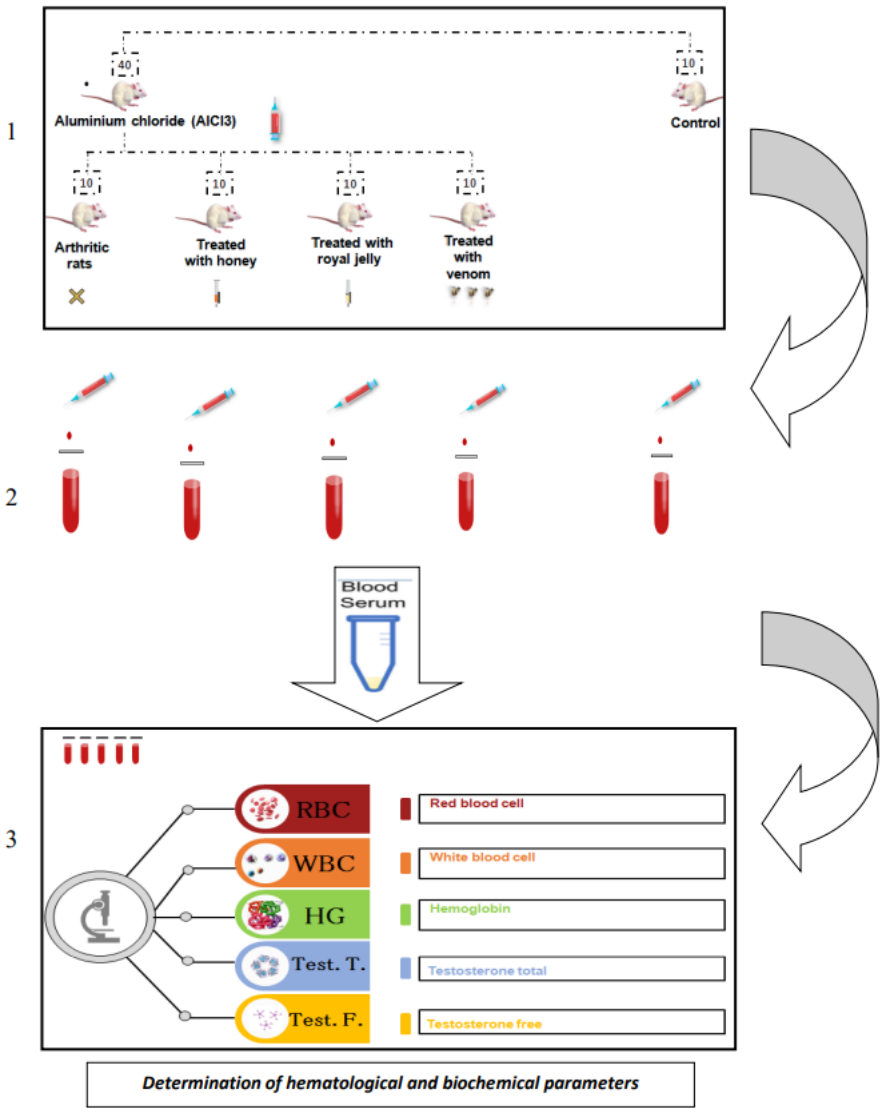
Graphical Abstract



Abstract

Honeybee products have several health benefits and claim toward various types of diseases in addition to being food. Thus, the therapeutic potentials of some bee products on testosterone deficiency in male albino rats were studied. This work was carried out from February to June 2019; fifty male rats were used and divided into five groups: 10 rats as controlled group, 10 rats were infected with Aluminium chloride ($AlCl_3$) intraperitoneally, 10 infected rats treated with honey as third group, 10 infected rats treated with royal jelly as fourth group, and 10 infected rats treated with venom as fifth group. From all groups, blood samples were collected, hematological parameters; red blood cells, white blood Cells, mean corpuscular volume, packed cell volume and hemoglobin concentration. Besides that, serum level of aldosterone hormone and blood glucose, liver functions and testosterone levels were measured. Data obtained showed significant differences with all parameters in defected rats cured by royal jelly, venom, and honey in RBCs, MCV, PCV, and Hb respectively, whereas WBCs showed more effect in infected rats treated with honey, venom, and royal jelly, respectively. Serum level of testosterone hormone, aldosterone hormone and blood glucose, superior effects on infected rats were caused by royal jelly, followed by venom and honey, respectively. On the other hand, ALP, AST and ALT were recorded high level in infected rats cured by venom, royal jelly, and honey, respectively. The results indicated that the symptoms of testosterone deficiency were alleviating in rats treated with royal jelly, venom, and marjoram honey, respectively.

Keywords: Apitherapy, Bee products, Testosterone hormone, Haematological

Özet

Bal arısı ürünlerinin sağlık üzerine birçok yararları vardır ve gıda olmanın yanında çeşitli hastalık türlerine yönelik iddiaları bulunmaktadır. Bundan dolayı erkek albino sıçanlarda bazı arı ürünlerinin testosteron eksikliği üzerindeki terapötik potansiyelleri çalışılmıştır. Bu çalışma Şubat-Haziran 2019 tarihleri arasında gerçekleştirilmiş olup elli erkek sıçan kullanıldı ve beş gruba ayrıldı: Kontrol grubu olarak 10 sıçan, intraperitoneal olarak alüminyum klorür ($AlCl_3$) ile enfekte edilen 10 sıçan, üçüncü grup olarak bal ile tedavi edilen 10 enfekte sıçan, dördüncü grup olarak arı sütü ile tedavi edilen 10 enfekte sıçan, ve beşinci grup olarak arı zehri ile tedavi edilen 10 enfekte sıçan şeklindedir. Tüm gruplardan kan örnekleri alınarak hematolojik parametreler; kırmızı kan hücreleri, beyaz kan hücreleri, ortalama korpüsküler hacim, paketlenmiş hücre hacmi ve hemoglobin konsantrasyonu, bunların yanında serum aldosteron hormonu, kan şekeri seviyeleri, karaciğer fonksiyonları ve testosteron seviyeleri ölçülmüştür. Elde edilen veriler, sırasıyla RBC'ler, MCV, PCV ve Hb değerlerinde arı sütü, arı zehri ve bal ile tedavi edilen kusurlu sıçanlarda tüm parametrelerde önemli farklılıklar gösterirken, WBC'ler sırasıyla bal, arı zehri ve arı sütü ile tedavi edilen enfekte olmuş sıçanlarda daha fazla etki göstermiştir. Testosteron hormonu, aldosteron hormonu ve kan şekerinin serum düzeylerinin, enfekte sıçanlar üzerindeki üstün etkileri sırasıyla arı sütü, ardından arı zehri ve bal tarafından sağlanmıştır. Öte yandan ALP, AST ve ALT sırasıyla arı zehri, arı sütü ve bal ile tedavi edilen enfekte sıçanlarda yüksek düzeyde bulunmuştur. Sonuçlar, sırasıyla arı sütü, arı zehri ve mercanköşk balı ile tedavi edilen sıçanlarda testosteron eksikliği semptomlarının hafiflediğini göstermiştir.

Anahtar kelimeler: Apiterapi, Arı ürünleri, Testosteron hormonu, Hematolojik

parameters, Biochemical parameters, Testosterone deficiency.
parametreler, Biyokimyasal parametreler, Testosteron eksikliği

Abbreviations: RBC, Red blood cells; WBC, White blood cells; MCV, Mean corpuscular volume; PCV, Packed cell volume; Hb, Hemoglobin Concentration; ALP, Alkaline Phosphatase; AST, Aspartate aminotransferase; ALT, Alanine Aminotransferase

1. INTRODUCTION

Apitherapy is the medicinal use of products collected from colonies of honeybee (Habryka et al., 2016; Stawiarz & Dyduch, 2014). Bee products are honey, beeswax, pollen grains, royal jelly, propolis and venom. The Egyptians ancient used bee products such as honey in very many different medicines (Hellner et al., 2008).

Bee honey is a sweet viscous substance, which is produced by worker honeybees from the sugary secretion parts of many plants (Osho & Bello, 2010). Honey is produced worldwide by more than 500 bee species and naturally presents some amounts of antioxidants (including flavonoids and phenolics), organic acids and amino acids in its composition, and specific sugar profile and acidity that bestow unique sensory characteristics (Singh et al., 2012).

Honeybee defends their colonies against enemies by a sting. The bee venom which collected from bee workers (*Apis mellifera* L.) contains several pharmacologically active substances (Bae et al., 2016) as follows; melittin, histamine, Dopamine and other peptides were used as medicine for a long time to cure the inflammatory diseases by direct sting or injections (Ali, 2014).

Royal jelly is a honeybee secretion that is used in the nutrition of worker and drone larvae, as well as larvae and adult queens (Vucevic et al., 2007), which is considered as one of the most important products of bee colonies as high nutritional, functional, and biological properties (Qu et al., 2007). It is secreted from two glands in the head of nurse bee workers: mandibular and hypopharingeal glands. Royal jelly was consisting of lipids, proteins, sugars, vitamins, amino acids, and complex vitamins; B1, B2, B6, and biotin. Moreover, it contains different minerals, trace elements with biological functions, and fatty acid (10-HDA), which play an important role in boosting the immune system and has anticancer activity (Ishmuratov et al., 2008). Recently, it can use the royal jelly to alleviating symptoms of many diseases such as testosterone deficiency (Suemaru et al., 2008).

Testosterone is the male sex hormone that is made in the testicles, testosterone hormone levels are most important to normal male sexual development and functions (Kok-Yang & Soelaiman,

2017). Testosterone levels generally decrease with age, so older men tend to have low blood testosterone levels which called testosterone deficiency, and it can be used the hematological parameters such as Red Blood Cells, White Blood Cells, mean corpuscular volume, packed cell volume and hemoglobin concentration to determine the testosterone hormone deficiency (Kok-Yang & Soelaiman, 2017). Whereas there are some conditions in which the level of the hormone testosterone decreases despite the young age, the reason may be due to men who have diabetes or overweight (Yakubu et al., 2017). There are many remedies that purport to ease this symptom; prescription pharmaceuticals and a wide range of alternative therapies such as bee products as Apitherapy (Abdel-Rahman et al., 2013).

Thus, the aim of this work was evaluating the therapeutic potentials of some bee products on male albino rats that are infected with testosterone deficiency by injection of Aluminium chloride ($AlCl_3$).

2. MATERIALS and METHODS

2.1. Preparing Sample of Bee Products

The present study was conducted from February to June months of 2019 at Giza governorate which planting the marjoram crop and Faculty of Agriculture apiary, Cairo University. Fifteen honeybee colonies of Carniolian first hybrid Apis mellifera carnica, which are equal in strength and exposed to the routine work during the experimental period, were used for this study to produce marjoram honey, royal jelly and bee venom.

2.2. Physiochemical Properties of Bee Product Samples

The honey quality was determined based on the presence of water content by the measurement of its refractive index value using ABBE WAY-IS refractometer at 20 °C. Quantity of sugars (glucose and fructose) was determined by HPLC according to the method of Bogdanov et al. (2007). The electrical conductivity was determined by the method of Nombre et al. (2010), by using EC meter model EN50081-1 at room temperature (2g of honey sample was dissolved in 10 mL of distilled water and the results were expressed as ppm). Optical density as well as color of the honey samples was measured by using the relation between optical density and USDA standard as indicated by the Association of Official Analytical Chemists (AOAC, 2016).

The royal jelly was produced from bee colonies by a grafted technique based on the method of Grout (Grout, 1992) and harvested after 3 days from grafting, and samples were kept in dark bottle and stored in the deep freezer for chemical analysis. The percentage of amino acids was

determined according to Vinas et al. (1997) as gm/100 gm using amino acid analyzer apparatus (AAA400, INGOS Ltd) and the percentage of fatty acids was determined by the method reported from AOAC (2016) using liquid chromatography as gm/100 gm.

Bee venom was collected according to the method of Pence (1981) and the venom samples were immobilized by quick freezing at −20 °C until analyzed. The samples were determined the amino acids and peptides composition using HPLC-Pico- Tag method according to Cohen and Bianchine (1995). Physiochemical and biochemical analyses of bee products were conducted in "Elements laboratory, Campus of research laboratories, FARP," Faculty of Agriculture, Cairo University Research Park.

2.3. Experimental Albino Male Rats

Fifty male Swiss albino rats (*Rattus norvegicus*) ranging in weight from 150–200 gm, acquired from Schistosoma Biological Supply Program (SBSP) Theodor Bilharz Research Institute, were housed in clear plastic cages (4 animals/cage) with wood chips as bedding and given pellet rodent diet, in addition of water *ad-libitum*. They were kept under controlled environmental conditions, including a temperature of 25°C, 70% humidity, and 12:12 H light/dark cycle according to AOAC (2016).

Two main groups of treated male rats were divided. The first main group was control rats (10 rats), served as negative control and kept under normal laboratory conditions during the whole period of experimentation and were fed on a standard diet, food and water were available ad *libitum* for 4 weeks, and the second main group was induced with testosterone deficiency by the Aluminium chloride ($AlCl_3$) (5 mg/ kg body weight) intraperitoneally for four weeks which was induced as described by Moselhy et al. (2012).

The second main group (infected rats induced by $AlCl_3$) was classified into four subgroups (10 rats per each): the first one of infected rats served as positive control. The second was infected rats treated daily dose of 10 ml marjoram honey/kg/5 ml of distilled water (Busserolles et al., 2012) through oral canola for 4 weeks; the third one was infected rats treated daily with royal jelly (1 g/kg b/wt, orally) for 4 weeks (Busserolles et al., 2012). The last one was infected rats treated daily with a direct sting in rats knee (three worker bees) used daily for 4 weeks (Choe et al., 1986).

2.4. Determination of Hematological and Biochemical Parameters in Albino Male Rats

Blood samples were collected from the two main groups of male rats by the orbital plexus by means of fine capillary glass tubes, with EDTA according to Lewis et al. (2006) for hematological and biochemical parameters and samples were centrifuged at 1,200 rpm for 5 minutes to obtain the serum for analyses.

The hematological parameters are count of white blood cells (WBC) x 10^3 mm^3, count of red blood cells (RBC) million/mm^3, hemoglobin concentration (Hb) (g/dl), mean corpuscular volume (MCV) (fl), packed cell volume (PCV) (%). Besides that, the serum levels of Aldosterone hormone (ng/l), testosterone total (pg/ml), testosterone free (pg/ml), blood glucose (mg/dl), as well as liver functions; Aspartate aminotransferase (AST) (IU/L), Alanine aminotransferase (ALT) (IU/L) and Alkaline phosphatase (ALP) (IU/L) were analyzed according to the standard techniques described by Baker et al. (1998).

2.5. Statistical Analysis

The two-way statistical analysis of variance (ANOVA), mean separation, and correlation required subprogram of MSTAT (1989) microcomputer statistical program. Simple and multiple linear regression analysis were applied and the Student "*t*"-test was used to express as the mean ± SE. Significance was considered at a level of $p < 0.01$.

3. RESULTS and DISCUSSION

3.1. Physiochemical Properties of Bee Product Samples

3.1.1. Marjoram Honey

As shown in Table 1, there were clear significant differences in all tested parameters. The moisture was 19.30% ± 0.18% in marjoram honey where the electrical conductivity (EC) was 0.02% ± 0.00%, whereas the pH value was 4.04 ± 0.13 in the tested samples of marjoram honey. On the other hand, the obtained data showed that glucose content was 28.04% ± 0.01% and the fructose content was recorded 36.90 ± 0.02, while the optical density (OD.) was 0.29 ± 0.01 OD in tested samples of marjoram bee honey.

The moisture values which obtained in this study are similar to those found in South Asia honey: from 15.3 to 21.7 g/100 g (Chuttong et al., 2016) and in North African honey (from 14.6 to 21.8 g/100 g) (28–29). EC values were in the same range as those reported by other authors in Burkina Faso honey (Escriche et al., 2016; Nombre et al., 2010; Schweitzer et al., 2013).

Table 1. Physiochemical parameters of the marjoram honey samples.

Physiochemical parameters (mean ± S.E)	
Moisture %	19.30 ± 0.18
pH	4.04 ± 0.13
Fructose %	36.90 ± 0.02
Glucose %	28.04 ± 0.01
Optical density	0.29 ± 0.01
Electrical conductivity %	0.02 ± 0.00

The sugar contents (glucose and fructose sugars) of bee honey samples are similar to those found with Escriche et al. (2017), which detected the levels of glucose (from 27.8 to 31.9 g/100 g) and fructose (38.3 and 42.7 g/100 g), while pH value agrees with that obtained by Rateb (2005).

3.1.2. Collected Royal Jelly

The analysis was done to determine the amino acids (essential and nonessential) and fatty acids percentage. As shown in Table 2, the essential amino acids means recorded 0.977% ± 0.02%, 0.965% ± 0.03%, 0.744% ± 0.02%, and 0.532% ± 0.02% in lysine, leucine, valine, and threnine, respectively, while the non-essential amino acids: aspartic, glutamic, serine, and glysine were scored 2.411% ± 0.23%, 1.101% ± 0.16%, 0.982% ± 0.03%, and 0.789% ± 0.04%, respectively.

Table 2 The essential, non-essential amino acids, and fatty acids of royal jelly samples.

Essential amino acids %	
Valine	0.744±0.02
Leucine	0.965±0.03
Threnine	0.532±0.02
Lysine	0.977±0.02
Non-essential amino acids %	
Serine	0.982±0.03
Glysine	0.789±0.04
Aspartic	2.411±0.23
Glutamic	1.101±0.16
Fatty acids %	
10-hydroxy-2-decenoic acid	3.177±0.16
Eicosanoic acid	0.201±0.03
Tetracosanoic acid	0.312±0.02

The data obtained were similar with Nabas et al. (2013) who mentioned that the amino acids: valine, leucine, and lysine were recorded 0.734%, 0.965%, and 0.986%, respectively, while the fatty acids were recorded 10-hydroxy-2-decenoic acid and tetracosanoic acid reached 3.158% and 0.298%, respectively. The main fatty acid present in RJ is 10-hydroxy-trans-2-decenoic acid (10-HDA); it plays an important role in boosting the immune system, anticancer activity (Yang et al., 2010).

3.1.3. Collected Venom

The chemical composition of bee venom was analyzed to determine the amino acids and protein fraction. Table 3 illustrates that the major amino acids were histadin 12.69% ± 0.18%, alanine 8.01% ± 0.27%, and cysteine 7.12% ± 0.16%, followed by glutamic and tyrosine, which recorded 4.77% ± 0.33% and 3.87% ± 0.16%, respectively. Furthermore, the protein and peptides components were scored in dry weight of venom 49.7% ± 0.80%, 2.7% ± 0.40%, and 1.2% ± 0.51% of melittin, apamine, and adolapin, respectively.

Table 3 Amino acids and peptides of the collected bee venom samples.

Percentage of amino acids	
Alanine	8.01±0.27
Glutamic	4.77±0.33
Tyrosine	3.87±0.16
Histadin	12.69±0.18
Cysteine	7.12±0.16
Percentage of peptides in dry weight venom	
Apamine	2.7±0.40
Adolapin	1.20±0.51
Melittin	49.70±0.80

Obtained data were similar with Rady et al. (2017) who stated that the melittin, a major peptide component of bee venom, which accounts for 40%–50% of dried bee venom, is an attractive candidate for therapy of many diseases. The broad bioactive potential of bee venom includes antioxidant, anti-inflammatory, and cytotoxic activities. Despite the identification of the most abundant molecules in bee venom, some other minor compounds, together with synergistic/antagonistic effects at specific concentrations, could be involved in the reported bioactivities (Sobral et al., 2016).

3.2. Determination of Hematological and Biochemical Parameters in Treated Albino Male Rats with Bee Products

All of the infected treated rats were shown significant testosterone deficiency as compared with normal control group manifested as a significant increase in all infected hematological and biochemical parameters. As shown in Figure 1, the red blood cells count was affected with

$AlCl_3$ treatment, as well as curing by bee products; the RBC was recorded 8.57 ± 0.33 million/ mm^3 in the control rats, while it reached to 6.28 ± 0.16 million/mm^3 in infected rats. On the other hand, the count of RBC in the curing rats with honey was recorded 7.55 ± 0.24 million/mm^3, whereas in curing with royal jelly was scored 8.28 ± 0.20 million/mm^3 and with bee venom was recorded 8.11 ± 0.23 million/mm^3.

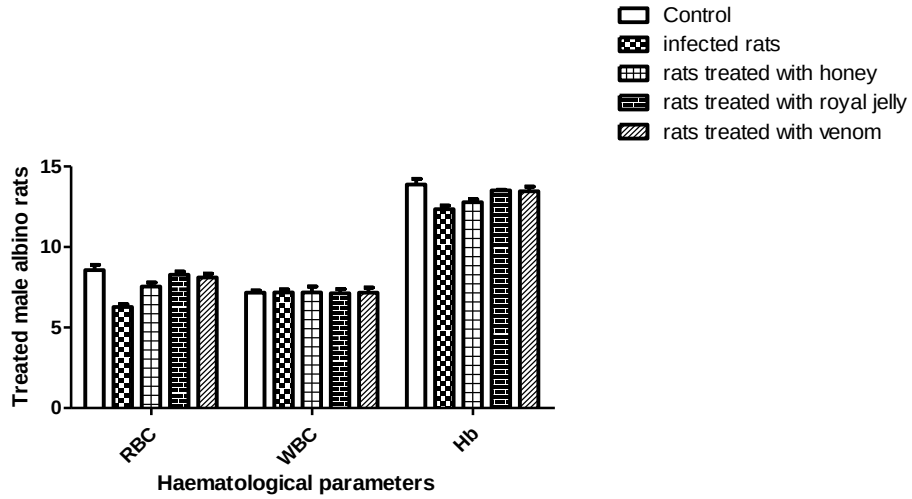


Figure 1. Effects of marjoram honey, royal jelly and venom on the haematological parameters of RBC (million/mm^3), WBC (×103 mm^3) and Hb (g/dl) in infected male albino rats induced by Aluminium chloride ($AlCl_3$).

The WBC count indicated clear significant differences; it was recorded 7.16 ± 0.14 × 10^3 mm^3 in control rats, while in infected rats reached to 7.18 ± 0.18 × 10^3 mm^3, and furthermore, the count of WBC in the curing rats with bee products scored 7.18 ± 0.36, 7.13 ± 0.26, and 7.16 ± 0.32 × 10^3 mm^3 with honey, royal jelly, and bee venom, respectively.

On the other hand, the concentration of hemoglobin was recorded 13.88 ± 0.35 g/dl in control rats, while in infected rats, the Hb concentration was reached to 12.35 ± 0.22 g/dl, whereas in the treated albino rats with bee products, the Hb scored 12.78 ± 0.19 g/dl with honey, 13.51 ± 0.05 g/dl with royal jelly, and 13.46 ± 0.29 g/dl with curing by venom.

Figure 2 illustrates the differences between the controlled, infected, and curing albino rats on the serum level of the Mean Corpuscular Volume (MCV) and Packed Cell Volume (PCV). The MCV was recorded 49.91 ± 1.99 % in controlled rats, while in infected albino male rats reached 69.17 ± 0.55 %. On the other hand, MCV was recorded 56.31 ± 1.99 % in cured rats with marjoram honey and in rats which were cured by royal jelly, the MCV has scored 51.31 ± 0.55 %, while with cured rats by venom, it was reached to 52.41 ± 1.30 %.

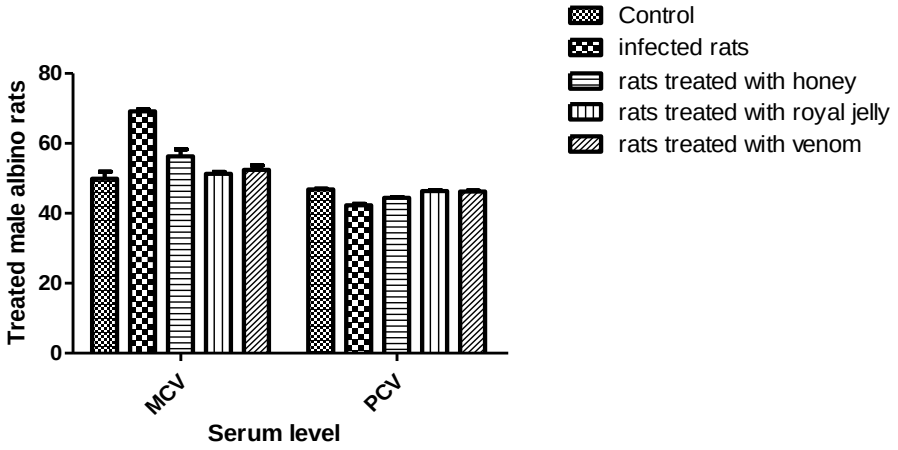


Figure 2. The effects of marjoram honey, royal jelly and venom on the serum levels of MCV (fl) and PCV (%) in testosterone deficiency male rats.

The PCV showed significant differences between controlled rats with all treated rats; in control male rats, the PCV was recorded 46.83 ± 0.22 fl while in infected rats, it was reached to 42.27 ± 0.41 fl, whereas in curing rats, it was scored 44.42 ± 0.24, 46.39 ± 0.26, and 46.22 ± 0.29 fl with honey, royal jelly, and venom, respectively.

Data obtained in Figure 3 illustrates that the serum level of Aldosterone hormone in control male rats was recorded 306.40 ± 0.67 ng/l, while it was reached to 272.33 ± 0.32 ng/l in infected rats. With therapeutic by honey, royal jelly, and bee venom, the serum level of Aldosterone hormone was scored 283.33 ± 3.33, 300.00 ± 0.00, and 295.23 ± 6.67 ng/l, respectively. Whereas the serum level of glucose showed significant differences between controlled rats with all treated rats; in control the glucose level reached to 166.30 ± 0.33 mg/dl, while in infected male rats recorded 157.29 ± 1.20 mg/dl. On the other hand, glucose was recorded 165.11 ± 0.24 mg/dl in cured rats with marjoram honey and in rats which were cured by royal jelly has scored 165.26 ± 0.33 mg/dl, while with cured rats by venom; it was reached to 164.89 ± 1.20 mg/dl.

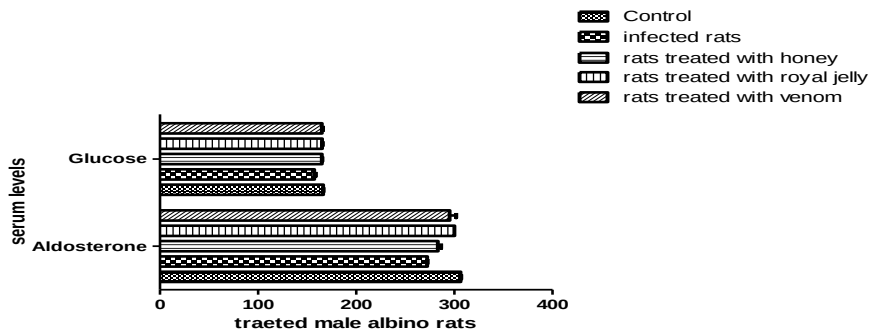


Figure 3. Effects of honeybee products on the serum level of aldosterone hormone (ng/l) and blood glucose (mg/dl) in infected male rats induced by $AlCl_3$.

Figure 4 shows that the level of liver functions; AST, ALT and ALP in the serum of control rats were 114.00 ± 0.57, 226.00 ± 0.58 and 222.31 ± 0.67 IU/L, respectively. Whereas, in infected male rats, there was reached to 116.12 ± 0.58, 252.00 ± 1.15 and 256.01 ± 1.10 IU/L, respectively. In cured rats with honey the level of liver functions scored 115.24 ± 0.33, 230.01 ± 0.33 and 228.23 ± 0.33 IU/L, respectively. Whereas with royal jelly were recorded 114.22 ± 0.33, 226.26 ± 0.25 and 225.34 ± 0.37 IU/L, respectively. While the AST, ALT and ALP in the serum of cured rats with venom scored 114.20 ± 0.38, 226.22 ± 0.32 and 225.12 ± 0.42 IU/L, respectively.

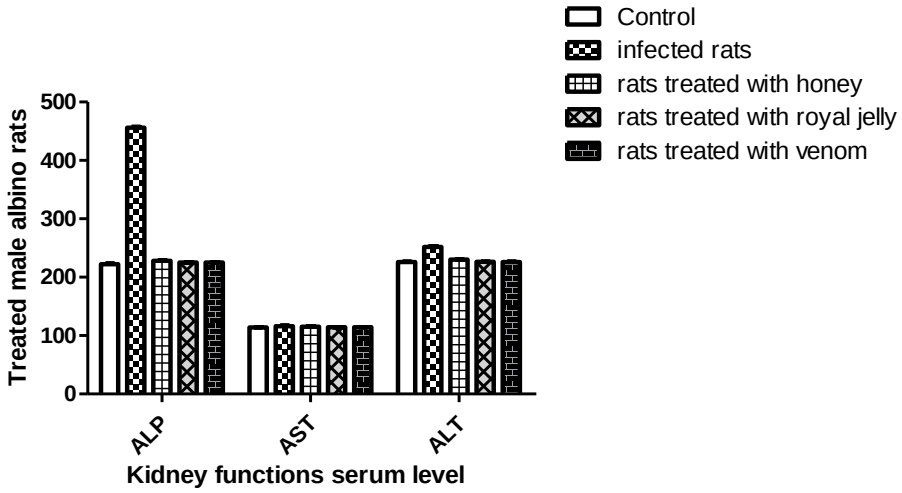


Figure 4. The serum levels of kidney functions; ALP (IU/L), AST (IU/L), and ALT (IU/L) in testosterone deficiency male rats cured by bee products.

As shown in Figure 5, the serum level of total testosterone hormone in the control male rats was scored 2.56 ± 0.08 pg/ml. While it was reached in infected rats 0.81± 0.08 pg/ml, whereas in cured rats with honey, royal jelly and venom the level of total testosterone hormone recorded 2.25 ± 0.22, 2.56 ± 0.06 and 2.46 ± 0.28 pg/ml, respectively. On the other hand, the serum level of free testosterone hormone in the control rats was scored 1.06 ± 0.08 pg/ml. While it was reached in infected rats 0.42± 0.00 pg/ml, whereas in cured rats with honey, royal jelly and venom the level of free testosterone hormone recorded 0.53 ± 0.04, 1.04 ± 0.03 and 1.01 ± 0.03 pg/ml, respectively.

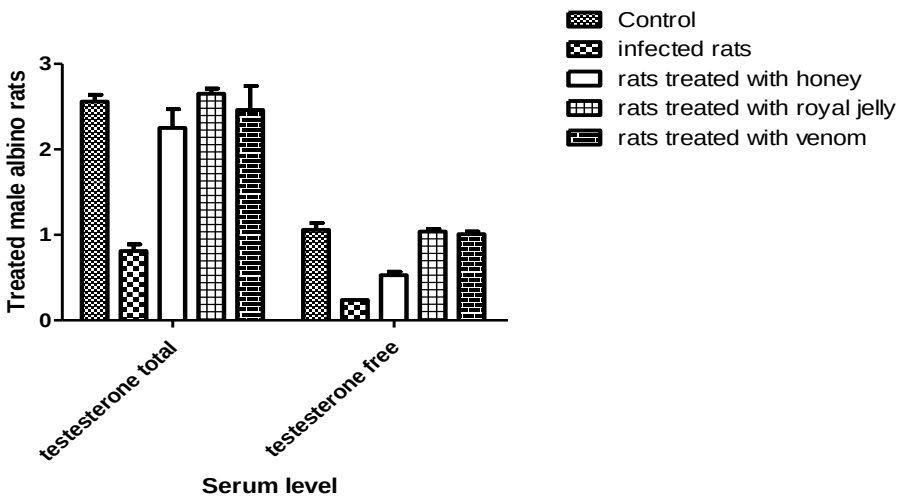


Figure 5. The serum levels of total and free testosterone hormone in testosterone deficiency male albino rats treated with marjoram honey, royal jelly and bee venom.

The present results were strengthened by those of (Nuhair, 2015; Yakubu et al., 2017) who reported that oral administration of Aluminium chloride resulted in a significant decrease in the serum testosterone in adult rats. $AlCl_3$ caused an increase in oxidative damage to sperm members, proteins and DNA (Aziz et al., 2007). This was associated with alterations in signal transduction mechanisms that affected male fertility (EbischI et al., 2006).

The organization of World Health Organization (WHO) stated that, levels of Reactive Oxygen Species (ROS) production in semen were negatively correlated with the percentage of normal sperm forms, these supported the results of the present study which indicated that there was a relationship between oxidative stress induced by Aluminium chloride ($AlCl_3$) and decrease in testosterone level (Geeta & Jain, 2017). Aluminium chloride was able to induced oxidative stress by produced a remarkable significant decrease in the ALP, AST and ALT by worsening liver functions (Huang, 2006).

From the obtained data, we suggested that honey, royal jelly, and bee venom have active pharmacological ingredients that alleviate the symptoms of many diseases; honey contains boron which avoids the hormonal unbalance that lead to testosterone deficiency, and royal jelly was rich with amino acids. Venom contains at least 18 active components, including enzymes, peptides, and biogenic amines, which have a wide variety of pharmaceutical properties.

4. CONCLUSION

The apitherapy is a branch of alternative medicine that deals with the use of honeybee products for the therapeutic and prevention of various diseases. Obtained results illustrated that the royal

jelly acts as a superior effect on curing of testosterone deficiency rats in several parameters: RBC, MCV, PCV, Hb, Aldosterone hormone, blood glucose, free and total testosterone hormone than other products under this study followed by bee venom and honey, respectively. On the other hand, other parameters: ALP, AST, ALT and WBC showed active curing of the infected rats with bee venom, royal jelly and honey, respectively.

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Volatile Oil Composition of Anzer Thyme

Anzer Kekiğinin Uçucu Yağ Bileşenleri

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Abstract

The Black Sea region belongs to one of the richest regions of Turkey regarding biological diversity. Totally 2239 species are distributed in the East Black Sea region. 514 species are endemic and the endemism ratio is ca. 23 %. Rize displays more than half of the plants distributed at the East Black Sea region. It can be stated that almost 70 % of these plants are of medicinal and aromatic value. One of them is Anzer Thyme. Totally 24 volatile oil components were detected corresponding to 99.88 % of total volatile oil. The biggest chemical group was oxygenated monoterpenes (77.83 %). Specially thymol, carvacrol and linalool were detected in high amounts (respectively 20.45 %, 14.83 % and 13.89 %).

Keywords: Anzer thyme, Volatile oil, Medicinal plant

Özet

Karadeniz bölgesi biyolojik çeşitlilik açısından Türkiye'nin en zengin bölgelerinden biridir. Doğu Karadeniz Bölgesi'nde toplam 2239 tür bulunmakta olup, bunların 514'ü endemiktir ve endemizm oranı yaklaşık %23' tür. Doğu Karadeniz bölgesinde yayılış gösteren bitkilerin yarısından fazlası Rize ilinde bulunmakta olup, bitkilerin yaklaşık %70'i tıbbi ve aromatik değere sahiptir. Bunlardan biri de Anzer Kekiğidir. Toplam uçucu yağın %99.88'ine tekabül eden toplam 24 uçucu yağ bileşeni tespit edilebilmiştir. En büyük kimyasal grup oksijenli monoterpenlerdir (%77.83). Özellikle thymol, carvacrol ve linalool yüksek miktarlarda (sırasıyla %20.45, %14.83 ve %13.89) tespit edilmiştir.

Anahtar kelimeler: Anzer kekiği, Uçucu yağ, tıbbi bitki

1. INTRODUCTION

Medicinal and aromatic plants have use in in traditional and modern medicine. They are helpful in the prevention, improvement and maintenance of diseases. These plants have use in nutrition as nutritional supplements, herbal tea, spices and condiments. Besides their use

inbody care products, perfumery and cosmetics, these plants are useful in the pesticide and brightening industry. Different parts of these plants (root, rhizome, tuber, stem or woody structure, bark, leaf, flower, fruit, seed and herb) are used as drug leaves. The use of medicinal plants as medicine for therapeutic purposes dates back to ancient times. In particular, Ibn Sina's work called El-Kanun Fi-t Tıbb, which he wrote in 930-1037, contains a lot of information about the use of many plants as medicine, which organs and which diseases they have therapeutic properties. The mentions that thyme has dissolving and disintegrating properties, breaking even frozen blood, preventing shivering from cold when drunk in winter, destroying warts, expectorant, relieving rib pain, improving eyesight and appetite, its use as digestion facilitator, dewormer and diuretic can be seen in the literature (Kahya, 2017).

The genus *Thymus* represents one of the most important genera of the Lamiaceae family, containing over 100 species (Tohidi et al., 2017). This medicinal plant has perennial behaviour and is distributed naturally in different regions of the world. The origin of this plant was was assumed as the Mediterranean regions (Pirbalouti et al., 2013). In Turkey this genus is represented by 38 species and altogether 64 taxa, 24 of them are endemic (Manou et al., 1998).

The Black Sea region is one of the richest regions of Turkey concerning biodiversity. 2239 plant species are present in the East Black Sea region. The number of endemic species are 514 and the endemism ratio is ca. 23 %. Over 50 % of the plants present at the East Black Sea region can be seen also in the Rize province. The percent of plants with potential medicinal and aromatic value are about 70 % (Yurteri et al., 2017).

Thymus praecox Opiz subsp. *caucasicus* (Wild. ex Ronniger) Jalas var. *caucasicus* was collected from the Anzer Plateau in Rize and investigated regarding its volatile oil composition.

2. MATERIALS and METHODS

The Anzer Plateau, where the plant material was collected, is located in Rize. Rize is located in Northeastern Anatolia, East of the Eastern Black Sea coastline. It is surrounded by Of and Trabzon from the west, İspir of Erzurum from the south, Yusufeli and Arhavi of Artvin from the east, and the Black Sea from the north, and its area is 3922 km^2 excluding the lakes. The plant material *Thymus praecox* Opiz subsp. *caucasicus* (Wild. ex Ronniger) Jalas var. *caucasicus* was collected from the Anzer plateau and its active ingredients were investigated.

2.1.1. Sample Preparation and Volatile Oil Analysis

Sample preparation and volatile oil analysis using GC-MS was done as described by Yurteri et al. (2021).

3. RESULTS and DISCUSSION

The volatile oil composition of collected Anzer Thyme can be seen in Table 1. Totally 21 volatile oil components were detected corresponding to 99.88 % of total volatile oil. The biggest chemical group was oxygenated monoterpenes (77.83 %). Specially thymol, carvacrol and linalool were detected in high amounts (respectively 20.45 %, 14.86 % and 13.89 %). Also monoterpene hydrocarbons (5.17), sesquiterpene hydrocarbons (4.59 %) and oxygenated sesquiterpenes (1.77 %) were detected as chemical groups. Isovaleric acid (1.26 %), α-terpinyl acetate (5.18), myristic alcohol (2.35 %), isoeugenyl phenylacetate (0.41) and pentadecanolide (1.32) were constituents of investigated volatile oil.

Sekeroglu et al. (2007) detected thymol (47.45%), γ -terpinene (8.73%), p-cymene (8.30%), terpinyl acetate (4.88%) and carvacrol (4.66%) as major components in the same species. Yurteri et al. (2017) investigated the same species and detected 39-42.12 % thymol, 20.76-53.57 % carvacrol and 11.65-31.37 % α-terpinyl acetate in Anzer thyme populations collected from different altitudes.

The reason for the difference in terms of volatile components among given literature can be explained by ecological conditions of the region grown on volatile components, the harvest times of the plant, the maturation stage, the different plant parts, species diversity and genetic diversity (Hazzit & Baaliouamer, 2009; Lukas et al., 2015; Toncer et al., 2010; Tümen et al., 1995).

Table 1. Volatile Oil Composition of (*Thymus praecox* Opiz subsp. *caucasicus* (Wild. Ex Ronniger) Jalas var. *caucasicus* collected from Anzer/Rize

Chemical Classes	RI	Chemical Formula	%
Monoterpene hydrocarbons			
p-Cymene	1025	$C_{10}H_{14}$	0.75
γ-Terpinene	1067	$C_{10}H_{16}$	4.42
Total			**5.17**
Oxygenated monoterpenes			
Linalool	1099	$C1_0H_{18}O$	13.89
trans-Sabinene hydrate	1101	$C10H_{18}O$	0.67
Eucalyptol	1032	$C_{10}H_{18}O$	0.32
Menthone	1158	$C_{10}H_{18}O$	4.76
Isoborneol	1165	$C_{10}H_{18}O$	6.74
α-Terpineol	1187	$C_{10}H_{18}O$	1.41
Pulegone	1241	$C_{10}H_{16}O$	1.27
Carvone	1246	$C_{10}H_{14}O$	9.11
(Z)-Jasmone	1248	$C_{11}H_{16}O$	4.35
Thymol	1304	$C_{10}H_{14}O$	20.45
Carvacrol	1313	$C_{10}H_{14}O$	14.86
Total			**77.83**
Sesquiterpene hydrocarbons			
α-Himachalene	1449	$C1_5H_{24}$	4.02
α-Humulene	1454	$C_{15}H_{24}$	0.57
Total			**4.59**
Oxygenated sesquiterpenes			
Caryophyllene oxide	1587	$C1_5H_{24}O$	1.77
Total			1.77
Others			
Isovaleric acid	820	$C_5H_{10}O_2$	1.26
α-Terpinyl acetate	1349	$C_{12}H_{20}O_2$	5.18
Myristic alcohol	1380	$C_{14}H_{30}O$	2.35
Isoeugenyl phenylacetate	1680	$C_{18}H_{18}O_3$	0.41
Pentadecanolide	1827	$C_{15}H_{28}O_2$	1.32
Total			10.52
Totally			99.88

4. CONCLUSION

Lamiaceae species are very popular in folk medicine to treat various health problems such as throat infections, stomach disorders, ulcer, spasm, cold, hemorrhages and skin problems. The family is also famous for the presence of essential oils. Their constituents have been found to

be anti-inflammatory, hemostatic, cicatrizing, stomachic, sedative, spasmolytic, diuretic, expectorant, cardiac, hypotensive.

Medicinal and aromatic plants form a numerically large group of economically important plants which provide basic raw materials for medicines, perfumes, flavours and cosmetics. These plants and their products not only serve as valuable source of income for small landholders farmers and entrepreneurs but also earn valuable foreign exchange by way of export facilities. Anzer Thyme was investigated regarding its valuabe volatile oil composition during this study.

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Some Characteristics of Honey and Propolis and Their Effects on Covid-19

Bal ve Propolisin Bazı Özellikleri ve Covid-19 Üzerine Etkileri

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Abstract

The new type of coronavirus disease (COVID-19) caused by coronavirus-2 (SARS-CoV-2) has recently led to a global pandemic due to severe acute respiratory syndrome. The novel coronavirus (COVID-19) is the part of the coronavirus family that contaminates individuals after SARS coronavirus and MERS. Currently, there is no specific medicine, treatment, or vaccine for coronavirus disease. In addition, studies on the use of foods that can be defined as alternative medicine continue in this process. In this context, the consumption of honey bee products in the treatment process, which is called apitherapy, has attracted great interest. Even though the antimicrobial property and immune-strengthen effect of honey are clear, there are limited studies available on its effectiveness against coronavirus outbreaks. In vivo and in vitro studies have been conducted on the potential effects of honey against COVID-19 based on previously investigated antiviral effects and phytochemical components. Despite some bioactive compounds in honey (such as methylglyoxal, chrysin, caffeic acid,

Özet

Koronavirüs - 2'nin (SARS - CoV - 2) neden olduğu yeni koronavirüs hastalığı (COVID - 19), şiddetli akut solunum sendromu sebebiyle son zamanlarda küresel pandemiye neden olmuştur. Yeni tip koronavirüs (COVID-19) SARS koronavirüsü ve MERS sonrası insanları enfekte eden koronavirüs ailesinin üyesidir. Halen koronavirüs hastalığı için belirlenmiş kesin bir ilaç, tedavi veya aşı bulunmamaktadır. Bunun yanısıra bu süreçte alternatif ilaç olarak tanımlanabilen gıdaların kullanımı üzerine araştırmalar devam etmektedir. Bu kapsamda apiterapi adı verilen ve bal arısı ürünlerinin tedavi sürecinde kullanılması büyük ilgi çekmiştir. Bal için tanımlanan antimikrobiyal özellik ve bağışıklığı kuvvetlendirici etkisine rağmen, bugüne kadar koronavirüs salgınlarındaki etkinliğiyle ilgili mevcut sınırlı çalışmalar bulunmaktadır. Balın daha önce araştırılan antiviral etkilerine ve fitokimyasal bileşenlerine dayanarak COVID-19'a karşı potansiyel etkisi üzerine in vivo ve in vitro çalışmalar yapılmıştır. Baldaki bazı biyoaktif bileşikler (metilglioksal, krisin, kafeik asit, galangin ve

galangin, and hesperidin) have shown potential antiviral effects and strengthened the immune system, more studies are needed to understand the mechanism of action of these compounds. Propolis, a material produced by honey bees from disparate resinous substances collected from plants, has been utilized as a traditional herbal source for a long time. It is also commonly consumed as an immune system booster. In this case, with the COVID-19 outbreak, the interest in propolis products has increased even more in the world. Various aspects of the SARS-CoV-2 infection mechanism are potential targets for propolis compounds. SARS-CoV-2 entry into host cells is characterized by viral spike protein interaction with cellular ACE-2 and TMPRSS2. This mechanism involves high expression of PAK1, a kinase that mediates coronavirus-induced pneumonia, fibrosis and immune system suppression. There are various in vitro and in vivo antiviral activity studies investigating the inhibitory effects of propolis components on ACE-2, TMPRSS2 and PAK1 signaling pathways. The purpose of this research is to bring together the studies on COVID-19 with the health effects of honey and propolis, to contribute to the in vivo and clinical studies that are still required.

Keyword: Apitherapy, Honey, Propolis, Antiviral effect, COVID-19, Antimicrobial effect

hesperidin gibi) potansiyel antiviral etki göstermiş veya bağışıklık sistemini kuvvetlendirmiş olsa da, bu bileşiklerin etki mekanizmaları için yeni çalışmalara ihtiyaç duyulmaktadır. Arıların bitkilerden topladıkları farklı reçineli maddelerden ürettiği bir materyal olan propolis, geleneksel şifalı bitkisel kaynak olarak uzun süredir kullanılmaktadır. Aynı zamanda bağışıklık sistemi güçlendiricisi olarakta tüketilmektedir. Bu durumda, COVID-19 salgını ile beraber, dünya çapında propolis ürünlerine olan ilgi daha da artmıştır. SARS-CoV-2 enfeksiyon mekanizmasının çeşitli yönleri propolis bileşikleri için potansiyel hedef olmaktadır. Konakçı hücrelere SARS-CoV-2 girişi, ACE-2 ve TMPRSS2 ile viral spike protein etkileşimi karakterize edilmektedir. Bu mekanizma, koronavirüsün neden olduğu pnömoni, fibroza ve bağışıklık sistemi baskılanmasına aracılık eden, bir kinaz olan PAK1 yüksek ekspresyonunu içermektedir. Propolis bileşenlerinin ACE-2, TMPRSS2 ve PAK1 sinyal yolakları üzerinde önleyici etkisinin araştırıldığı çeşitli in vitro ve in vivo antiviral aktivite çalışmaları mevcuttur. Bu araştırma kapsamının amacı bal ve propolisin sağlık üzerine etkileri ile beraber COVID-19 üzerine yapılan çalışmalarını bir araya getirerek hala ihtiyaç duyulan in vivo ve klinik çalışmalara bir katkı sağlamaktır.

Anahtar Kelimeler: Apiterapi, Bal, Propolis, Antiviral etki, COVID-19, Antimikrobiyal etki

Abbreviations: Coronavirus-2 (SARS-CoV-2); Severe Acute Respiratory Syndrome (SARS); Middle East Respiratory Syndrome (MERS); Angiotensin-converting enzyme 2 (ACE-2); Transmembrane protease serine 2 (TMPRSS2); RAC/CDC42-activated kinase (PAK1); transmembrane protease serine 2 (TMPRSS2)

1. INTRODUCTION

Coronaviruses are RNA viruses with broad-enveloped that cause a wide variety of respiratory disorders in humans, such as upper respiratory tract infections and severe pneumonia. Seven forms of coronavirus, known as a human coronavirus (CoVh), have been identified that infect humans. Two are alpha coronaviruses (229E and NL63), whereas the other five are beta coronaviruses (OC43, HKU1, SARS-CoV, MERS-CoV and SARS-CoV-2) (Anonymous, 2020 (a); Lim et al., 2016). It has been reported that coronavirus, which mainly targets the lungs, can be transmitted through droplets, aerosol microparticles and as well as asymptomatic infection (Yoshikawa et al., 2009).

Three outbreaks caused by this human coronavirus have been reported. One of them began in China in 2002 with acute and severe respiratory syndrome (SARS-CoV). The second one began in Saudi Arabia in 2012 with the respiratory tract syndrome (MERS-CoV) in the Middle East, and the third one was the COVID-19 pandemic (SARS-CoV2), which was reported in December 2019 in China. The most recent outbreak was the novel type of coronavirus (2019-nCov) in Wuhan city (Hubei province, China), which was found to be associated with a beta coronavirus and started with the same symptoms as the case of pneumonia. Afterward, the International Virus Taxonomy Committee named 2019-nCov as SARS-CoV2 because the 2019-nCov genome sequence indicated 89% resemblance to SARS-CoV (Lima et al., 2021). SARS-CoV-2 is spread through saliva droplets or nasal discharge when infected people cough or sneeze (UNICEF, 2020).

The recent coronavirus disease (COVID-19) maintains to spread around the world and becomes an emergency of concern on a global level. However, it is known that even after a decade of research on the coronavirus that has already emerged, licensed vaccines or therapeutic agents to heal coronavirus infection still have not been discovered (Lima et al., 2021).

At the stage of this review, it is seen that the COVID-19 pandemic has spread rapidly, vaccine and drug studies for COVID-19 are ongoing, and a definitive treatment method has not yet been developed for the treatment of the disease. People are searching the alternative prevention and treatment methods as well as medical treatment. One of these methods is the consumption of honey bee (*Apis mellifera*) products and the increase in demand for these products. The reason for this is that bee products have been used in the treatment of many illnesses in alternative medicine and the production of some drugs in pharmacology in recent years, moreover these products are the subject of many studies.

Until today, various biological therapeutic, nutraceutical and pharmaceutical properties of honey bee products have been determined and studies have been conducted on their use as cosmetic components (Viuda-Martos et al., 2008). Honey, as a complementary and alternative medicine product, has long attracted the attention of researchers (Al-Hatamleh et al., 2020).

Apitherapy is a type of complementary and alternative medicine that involves the therapeutic use of various bee products, including apilarnil (atomized drone larvae) to prevent and treat diseases (Nitecka-Buchta et al., 2014).

Apitherapy is also defined as the science and art of using products obtained from the honey bee hive to sustain health (Fratellone et al., 2016). Apitherapy, which offers treatments that depend on honey and other bee products against many diseases, has been developed as an alternative medicine branch. Apitherapy (using products produced by honey bees for treatment and pharmacological purposes like pollen, honey, royal jelly, bee venom and propolis) usage by Egyptians has been documented. Recently, some countries in the world, like New Zealand and Australia, have accepted the use of honey and honey products in wound treatment. Researchers conducting both in vivo and clinical studies on the healing of illnesses with honey have determined that honey is beneficial as a wound dressing through different mechanisms such as an antibacterial agent, a debriding agent, and an anti-inflammatory agent (Salcido, 2008).

Apitherapy is also said to be promising for the healing and prophylaxis of COVID-19 as an alternative medicine product combining the field of pharmacology with nutraceutical agents (Lima et al., 2021). In the earlier studies, various bee products such as honey, pollen, propolis, royal jelly and beeswax have been reported to show high antiviral activity against pathogens such as CoVh that cause severe respiratory syndromes (Brown et al., 2016). The benefits of these natural products to the immune system are striking, and it is said that most of them play a key role in the induction of antibody production, maturation of immune cells, and stimulation of innate and adaptive immune responses (Lima et al., 2021) (Figure 1).

Figure 1. Schematic representation of the main effects of bee products that can be utilized against the new coronavirus (SARS-CoV-2) (Lima et al., 2021)

This review, it is aimed to bring together in vivo, in vitro and clinical studies about some physicochemical properties of honey and propolis, which are bee products, and various use of these products as an alternative to the coronavirus pandemic (COVID-19), which continues to be a major and widespread threat to the world, causing various respiratory diseases and even deaths.

1.1. Honey and Covid-19

Honey is stated as "the natural sweet material produced by honey bees from the nectar of plants or the secretions of living parts of plants" (Codex Alimentarius Commission, 2001). Honey, which is naturally produced by bees and is the most common honey bee product, is obtained by digesting the nectar taken from flowers and stored in honeycomb cells. In other words, honey is a supersaturated aqueous solution that is produced at the acidic pH of the stomach of honey bees with the activities of invertase, diastase and amylase enzymes and contains mainly fructose and glucose, small amounts of sucrose, maltose and other sugars. Honey is often marketed for its nutritional benefits, but it has also been utilized as a folk cure since ancient times and recently has been used in pharmaceutical clinical applications (Cornara et al., 2017).

The use of honey, which is stated to date back to the Stone Age and has existed since the beginning of human history, was widely utilized as the only sweetening material until it started to be replaced by refined sugar with industrial sugar production after the 1800s (Bogdanov et al., 2008). Ancient societies such as the Romans, Greeks, and Egyptians have been reported to use honey as a type of sugar or to protect seeds, fruits and stems of plants (Chaven, 2014). In addition, it has been stated that throughout history, honey was used not only as food but also as a medicine (Jones, 2001).

With the increase of the human population, honey production also increases. According to FAO, (2019) data, it has been reported that the biggest honey production in the last ten years was in the Asian continent with approximately one thousand tons, followed by Europe and America. In addition, there are data that the amount of honey consumed per person is mostly in the Central African Republic, followed by New Zealand and Slovenia.

More than 200 substances have been found in honey, and among these components, carbohydrates (fructose, glucose, maltose, sucrose) are the most. Minerals, vitamins, organic acids, flavonoids, phenolic acids, enzymes, proteins and other phytochemicals are also among the key components of honey (Gomes et al., 2011; Iglesias et al., 2012). The color, minerals, vitamins and taste of honey are based on the types of flowers whose nectar is collected (Yaghoobi et al., 2008), geographical origin and seasonal differences, additionally harvest, processing and storage conditions (Gomes et al., 2011; Iglesias et al., 2012). For this reason, it is reported that honey collected from different honey sources has beneficial effects on health in different aspects (Iglesias et al., 2012). Naturally derived honey contains 82.4% carbohydrates, (38.5% fructose, 31% glucose, 12.9% other types of sugars), 17.1% water, 0.5% protein, organic acids, multi-minerals, amino acids, vitamins, phenolic substances, many other compounds and phytochemicals. Honey, which is known as a supersaturated sugar product, also consists of small amounts of bioactive components such as phenolic acid, flavonoid, and α-tocopherol (Shapla et al., 2018). Adaškevičiūtė et al. (2019) determined various mineral amounts of honey and other bee products, and it was reported that these products contain the most potassium. It has been reported that the ingredients of honey, which have positive benefits on health, containing ascorbic acid, carotenoids, as well as certain enzymes like glucose oxidase and catalase (Moniruzzaman et al., 2012). Many of honey's therapeutic characteristics, including anti-oxidant, anti-bacterial, anti-fungicidal, anti-inflammatory, hypotensive, anti-proliferative, anti-mutagenic, anti-diabetic, anti-tumoral (Khan et al., 2018; Terzo et al., 2020). It has been reported that the antioxidant capacity of honey is strongly correlated not only with

the concentration of total phenolic components but also with color. The color of honey changes from light brown to dark, and it is stated that dark honey has high total phenolic content and hence high antioxidant capacity (Terzo et al., 2020).

Honey has an antiviral effect due to its contained components (Bogdanov, 2020; Hashemipour et al., 2014; Shahzad & Cohrs, 2012; Zeina et al., 1996). Zeina et al. (1996) investigated the antiviral properties of honey solutions at varying concentrations in their study in vitro and it was determined that honey has antiviral activity. Hashemipour et al. (2014) investigated the antiviral effects of honey and its different products on Herpes simplex virus type 1 (HSV-1), in a study of honey samples used at different concentrations (5, 10, 50, 100, 250, 500, and 800 µg/mL). It has been reported that the highest antiviral effect is seen at 500 µg/mL concentration. Shahzad and Cohrs (2012), determined that honey has an important antiviral effect in vitro. Honey can be effective in chronic inflammatory processes, as it contains significant amounts of compounds such as flavonoids and other polyphenols that can act as anti-inflammatory agents (Terzo et al., 2020). Miryan et al. (2020) reported that honey significantly reduced neutrophil uptake and inflammatory behavior at the wound site in a dose-dependent manner below the cytotoxic limit. Owoyele et al. (2014) investigated the anti-inflammatory properties of honey in vivo. In the research conducted with the application of tamsulosin, propranolol, atropine and hexamethonium as autonomic blockers, it was reported that honey reduced the perception of pain, especially inflammatory pain, and the application of tamsulosin and propranolol preserved the effect of honey. Hexamethonium also preserved the effects of honey in the early and late stages of the test, while atropine only inhibited the early stage of the test. However, atropine and hexamethonium retained the anti-inflammatory effects of honey, but tamsulosin abolished the effects, while propranolol only abolished anti-inflammatory effects at the peak of inflammation. The study determined that autonomic receptors are involved in the anti-nociceptive and anti-inflammatory effects of honey, depending on the receptor types with different participation levels.

Escuredo et al. (2013) found that a hundred and eighty-seven different honey obtained from a certain region on the Atlantic side of Europe, heather, blackberry, polyfloral and eucalyptus honey had the highest content of carbohydrate, while honey extract and chestnut honey had the lowest content of carbohydrate, as well as they determined that there are crucial differences between honey types depending on their presence of some components. They noted that the contents of protein and mineral were significantly higher in honey extract and chestnut honey, and in conjunction with the presence of various antioxidant compounds, heather honey had the

highest content of phenolic, while honey extract and chestnut honey had the highest content of flavonoid. In addition, by using the multivariate analysis method, they found that minerals, flavonoids, proteins and phenols were fundamentally correlated with antioxidant activity.

So far, a few studies have been handled observing the effects of honey on SARS-CoV-2. Likewise, there are recently very limited clinical studies on the effectiveness of honey and its active components in COVID-19 patients (Ashraf et al., 2020b; Mustafa et al., 2020; Shaldam et al., 2021). Ashraf et al. (2020a) In a clinical study conducted on COVID-19 patients with different severity of the disease in Pakistan, they reported that the combination of honey and black seed is a healing, safe and effective treatment. In addition, according to this study, it was stated that honey and black seed, as a nutraceutical that does not require much cost, can be used alone or in combination with other drugs to have an additional effect.

Shaldam et al. (2021), in a study investigating the benefits of honey and propolis on COVID-19, among the bioactive components investigated in honey and propolis, ellagic acid, p-coumaric acid, kWaempferol and quercetin have been stated to have the ability to affect the active sites (RdRb and Mpro) of COVID-19. However, they stated that more in vivo study is required to evaluate the predicted affinity of chosen components against target enzymes of the novel coronavirus (COVID-19).

Ashraf et al. (2020a) studied the placebo effect of honey and black seed in randomly selected COVID-19 patients and found a significant reduction in the severity of clinical symptoms. They also reported that honey and black seed can be used alone or in combination with other treatments to achieve treatment-enhancing effects.

Mustafa et al. (2020), reported that honey can be considered as a functional food to complement the treatment of a patient infected with COVID-19. In another study, it was stated that a comprehensive clinical study was initiated that examined the efficacy of natural honey on many patients compared to current standard care in the healing of patients infected with COVID-19 (Anonymous, 2020b).

Hashem (2020) used the molecular modeling method that can estimate the activity of various active compounds from honey bee and propolis to hinder the main protease of SARS-COV2 (COVID-19) from honey, they determined that in which the binding energy of six compounds to the active site of the receptor in the main protease of COVID-19 was high. The study reported that caffeic acid phenethyl ester (CAPE), galangine, chymotrypsin-like and caffeic acid

substances can inhibit the enzyme cysteine protease (3CLpro or 3C-Like Protease), so honey may have the potential to inhibit viral replication.

Wu et al. (2020) stated that hesperidin, a flavonoid derived from plant extracts, and rosmarinic acid, which is also reported to be discovered in honey, can inhibit SARS-CoV2 3CLpro (3C-Like Protease). In the research, they reported that hesperidin is the only natural substance that may bind to the S protein receptor binding site (RBD) and therefore can neutralize ACE-2 and increase RBD binding. Al-Hatamleh et al. (2020) noted that honey containing hesperidin can have an important effect in preventing the virus from adhering to target cells.

In addition to the studies completed so far, more laboratory and clinical studies are required to fully determine the therapeutic effects of honey against COVID-19.

1.2. Propolis and Covid-19

Propolis is the general name of the mixture of different resinous materials obtained by bees from plants, used to cover the interior walls of the beehive, defend the entrance against intruders, and inhibit the growth of mold and bacteria (Burdock, 1998) (Figure 2). Propolis is reported to be one of the methods honey bee colonies preserve their immunity, forming an envelope that acts as a crucial antimicrobial layer in the nest (Simone-Finstrom et al., 2017).

(A) **(B)**

Figure 2. (A). Propolis (**B).** Propolis was collected from the hive and brought into pellets (Anonymous, 2020c)

Propolis, one of the most important honey bee products, contains mainly resin (50%), wax (30%), essential oils (10%), pollen (5%) and other organic compounds (5%) (Gómez-Caravaca et al., 2006). Propolis consists of important vitamins (B_1, B_2, B_6, C and E vitamins), minerals

(potassium, magnesium, calcium, sodium, copper, zinc, manganese and iron). Various enzymes have been found in propolis like glucose-6-phosphatase, succinic dehydrogenase, adenosine triphosphatase and acid phosphatase (Lotfy, 2006). The essential oil, which is the main active compound of propolis, is also responsible for the specific smell of propolis (Ribeiro et al., 2021).

To date, more than 300 various components have been found in propolis. The chemical content of propolis can be affected by its botanical origin, geographical conditions, collection season and bee species (Chi et al., 2020). Phenolic compounds, esters, flavonoids, amino acids, fatty acids, terpenes, diterpenes, lignans, beta steroids, aromatic aldehydes and alcohols are significant organic components discovered in propolis (Braakhuis, 2019). According to the study, twelve flavonoids, containing acacetin, pinosembrine, seizin, rutin, luteolin, kaempferol, myricetin, apigenin, catechin, galangin, naringenin and quercetin, two phenolic acids, cinnamic acid and caffeic acid, a stilbene derivative, resveratrol, were found in the extracts of propolis (Volpi, 2004). Woźniak et al. (2019) analyzed the chemical content of propolis extracts obtained in three seasons of the year. In this study, which is known that Polish propolis extracts, obtained in three seasons throughout the year, are rich sources of phenolic compounds, it was stated that chrysin, pinocembrin, galangin and coumaric acid are the fundamental phenols found in all extracts of propolis. When the concentrations of the compounds studied in all propolis samples were compared, just seven (Apigenin, Chrysin, Myricetin, Galangin, Kaempferol, Pinocembrin, Vanillic acid) of the fifteen components determined were found to be significantly different ($p < 0.05$).

The protective effect of propolis on the immune system, its pharmaceutical functions such as antioxidant, antibacterial, antiviral, anti-inflammatory, local anesthetic, antioxidant and anticancer properties, is due to its rich bioactive phytochemical components (Braakhuis, 2019; Chi et al., 2020).

Many studies have been conducted on the antiviral activity of propolis in particular (Kujumgiev et al., 1999; Lemos et al., 2020; Liao et al., 2021). Kujumgiev et al. (1999), investigated the antibacterial (against *Staphylococcus aureus* and *Escherichia coli*), antifungal (against *Candida albicans*) and antiviral (against *Avian flu virus*) activities of propolis samples taken from different geographical origins. It was stated that all the samples studied were active against mold and gram-positive bacteria strains and most of them showed antiviral activity. In addition, they found that the samples showed similar antiviral properties, although their chemical contents were different. Liao et al. (2021), investigated the antiviral properties of propolis water

extracts (PWE) and propolis ethanol extracts (PEE) against noroviruses and their application in fresh-cut products. As a result of the research, they reported that PEE, which is a polyphenol-rich extract, showed better antiviral activities than PWE.

To date, the antithrombotic property of propolis has not been clinically studied. However, in the study of Ohkura et al. (2020) on the antithrombotic property of propolis, it was stated that the concentrations compiled from different studies will contribute to future clinical studies. In addition, it has been stated that antithrombotic properties of propolis, similar to other biological properties, should be directly related to its chemical configuration and will vary according to regional vegetation, pollen collection season, collection techniques and bee species. It has also been stated that the active components of propolis and their effects on blood coagulation factors, platelets and the fibrinolytic system will require further research before propolis can be applied clinically.

Propolis samples collected from Turkey to determine the phenolic profile and origin of the plant have been analyzed using the High-Performance Thin Layer Chromatography method (HPTLC). In their study, Degirmencioglu et al. (2019), *Populus nigra L*. from the botanical origin O-type as specified propolis has revealed that the most abundant propolis species in Turkey. In addition, they defined 3MQ (3-O-methyl quercetin) rich propolis as a new type of propolis for the first time. Principal component analysis (PCA) showed that 3MQ-type propolis is different from O-type, and antioxidant activity studies have reported that O-type propolis has a higher antioxidant effect than other forms of propolis studied. They also determined that among others, caffeic acid, quercetin, CAPE, and galangin contributed significantly to the antioxidant capacity of O-type propolis. In their study, Chan et al. (2013), stated that the two main immunopotent chemical compounds of propolis, CAPE and artepilin C, exert a summative immunosuppressive function on T lymphocyte subsets, but paradoxically activate macrophage function. On the other hand, it has been reported to have potential antitumor properties with different putative mechanisms, such as suppressing the proliferation of cancer cells through its anti-inflammatory effects. In addition, it was emphasized that propolis could be an ideal adjuvant agent for future immunomodulatory or anticancer regimens, in the form of good bioavailability when taken orally.

It is known that propolis has been utilized in the healing of many illnesses and also in the production of food products and cosmetics since ancient times (Burdock, 1998). However, it has been reported that the activity of propolis as a scientifically therapeutic agent can be proven in the last century. The Egyptians are known to use propolis to embalm the dead after realizing

its anti-rotting properties. In Greece and Rome, propolis is known to be used as a natural antiseptic and treatment agent in the healing of wounds and as an oral disinfectant, in the same way, that the Incas used propolis as an antipyretic agent. It is also known that propolis was included in the British pharmacopeia official medicines list in the 17th century (Cauich-Kumul & Campos, 2019).

There is intense research on propolis among natural medicine alternatives and it is currently consumed widely in many countries (Chan et al., 2013; Kuropatnicki et al., 2013). Studies in China, Japan, Russia and Korea show that propolis has an important place in alternative medicine. These important studies are reflected in the number of patents registered for propolis-containing products by 2013, about 1200 by China and about 300-400 each for Japan, Russia and Korea (Toreti et al., 2013). Propolis has a wide range of pharmacological activities and is a dietary supplement generally used by healthy people both as a preventative measure and to treat patients (Furtado Júnior et al., 2020; Gajek et al., 2020). Furthermore, propolis is also used in veterinary studies due to its anti-bacterial, anti-fungal, anti-viral, anti-parasitic, hepatoprotective and immunomodulatory activities (Chan et al., 2013; Santos et al., 2020; Scorza et al., 2020).

To date, many large-scale scientific studies have been conducted on the bioactivity and beneficial effects of propolis on health (Lan et al., 2016; Almuhayawi, 2020). It has been determined in studies that different types of propolis exhibit various biological properties containing anti-bacterial, anti-fungal, anti-protozoal, anti-oxidant, anti-tumor, anti-inflammatory, anesthetic, wound healing, immunomodulatory, anti-proliferative and anti-cariogenic activities (Chan et al., 2013; Cauich-Kumul & Campos, 2019; Jalali et al., 2020). While positive effects of propolis on reducing inflammation in diabetic people were observed in some studies (Afsharpour et al., 2017; Fukuda et al., 2015; Zakerkish et al., 2019; Zhao et al., 2016). To combat SARS-CoV-2, studies on drug and vaccine development are carried out intensively worldwide. At this stage, it is stated that natural molecules isolated from medicinal and other plants show significant inhibitory antiviral activity against SARS-CoV-2 (Orhan and Deniz, 2020). Based on this clinical reason, the use of propolis as an available complementary therapy in infected people with SARS-CoV-2 has been proposed (Scorza et al., 2020).

Alcohol or water-derived propolis extracts have been presented in studies to have a strong and broad-spectrum antiviral property against a diverse panel of viruses such as Influenza virus type A and B, HSV-1, HSV-2, Parainfluenza virus Adenovirus, HIV and avian rheovirus, bovine

rotavirus, pseudorabilia virus, feline calicivirus, Newcastle virus disease, canine adenovirus type 2 and bovine viral diarrhea virus (Anjum et al., 2019).

While there are in vitro researches on the effect of propolis flavonoids on various DNA and RNA viruses, including the previous coronavirus (Bachevski et al., 2020; Pagani, 1990), insufficient studies are evaluating the effect on the new form of coronavirus (COVID-19). (Bachevski et al., 2020; Berretta et al., 2020; Burger, 2020; Guler et al., 2021; Khayrani et al., 2021; Miryan et al., 2020; Refaat et al., 2021; Sahlan et al., 2021; Silveira et al., 2021; Scorza et al., 2020; Tort and Acartürk, 2020). Debbiaggı (1990) studied the in vitro effect of five propolis flavonoids on various RNA and DNA viruses, containing coronavirus, using the viral plaque reduction technique. They reported that acacetin and galangin did not have any effect on the infectiousness or spread of any of the viruses studied, and chrysine and kaempferol were quite active in hindering replications.

Refaat et al. (2021), in their study on the development of an optimized liposomal formulation to increase the antiviral activity of propolis against COVID-19, are used as standard antivirals against both COVID-19 3CL-protease and S1 spike protein for certain components of propolis. They performed placement studies using Avigan, Hydroxychloroquine and Remdesivir. As a result of the study, they found that the optimized liposome formula of propolis had a significant inhibitory effect against COVID-3CL protease (IC50 =1.183 ±0.06) when compared with propolis extract (IC50 =2.452 ±0.11).

Sahlan et al. (2021) investigated whether Sulawesi propolis components produced by Tetragonula sapiens prevent the enzymatic activity of the primary protease SARS-CoV-2, as targeting the SARS-CoV-2 main protease to inhibit the COVID-19 virus is thought to be a promising treatment. As a result of the study, it was determined that two compounds (glycerine A and broussoflavonol F) are suitable for use as a potential drug for COVID-19. Both compounds were reported to show the desired effect profiles on the SARS-CoV-2 major protease with 63% and 75% similarities, respectively, compared to the positive control.

Guler et al. (2021) found the potential inhibitory properties of ethanol-extracted propolis against ACE-2 receptors in the treatment of COVID-19. In the study, the binding potential of some flavonoids in propolis to ACE-2 receptors was calculated. The binding constants of ten flavonoid compounds such as caffeic acid, chrysin, pinocembrin, galangin, rutin, caffeic acid phenethyl ester, myricetin, hesperetin, luteolin and quercetin were measured. As a result, it was reported that myricetin, caffeic acid phenethyl ester, hesperidin and pinocembrin, respectively, had the best inhibition potential and high binding energy among the molecules studied. It has

been stated that the flavonoids found in extracts of propolis have a high potential to bind to ACE-2 receptors, this natural bee product has a high potential for the treatment of COVID-19, yet this should be supported by experimental studies.

Khayrani et al. (2021) studied the effect of Sulawesi propolis components, a natural medicinal product produced by Tetragonula sapiens, which hinders ACE-2, which is the SARS-CoV2 receptor, in the human body. In the study, the interaction profiles of binding affinity and propolis compounds with ACE-2 were analyzed by representative molecular insertion. As a result, they determined that broussoflavonol F, glisperin A, sulabiroins A, (2S)-5,7-dihydroxy-40-methoxy-8-prenylflavanone and isorhamnetine, which are propolis compounds, may have binding properties. They reported that glycerine A and izorhamnetin compounds can have the highest effect with 85% and 77% binding properties.

In a large-scale study conducted by Burger (2020), the promising effects of propolis, which has a strong physical and electrostatic mechanism of action (detergent-like), to understand the presence of cationic antimicrobial peptide and the use of propolis extract through inhalation in the treatment of COVID-19 were examined. As a result of the research, it was stated that the compounds found in propolis can combine the expected effects of various therapeutic agents and are a potentially viable and promising alternative healing for COVID-19, especially compared to conventional therapy.

Silveira et al. (2021) studied the effectiveness of propolis as an additional therapy for COVID-19 patients who were hospitalized. In the study of adult hospitalized COVID-19 patients, a standardized propolis product (EPP-AF) was used and a randomized, controlled and single-center trial was conducted. They gave patients an oral dose of standard maintenance plus 400 mg/day (n=40) or 800 mg/day (n=42) propolis for seven days and standard care (n=42) alone as a control group. They found that the duration of hospital stay, which was stated as the recovery period of the patients, was shorter in the patients who were given propolis plus. Compared to the control, it was stated that the patients given 400 mg/day propolis stayed in the hospital for 7 days, those given 800 mg/day stayed in the hospital for 6 days, and the controls stayed in the hospital for 12 days. Briefly, they found that propolis given in different doses and for different durations could have a positive effect on patients.

Tort and Acartürk (2020) investigated disinfectants containing propolis and boron against COVID-19 and compared them with previous formulations. Tissue profile analysis, spreadability and bioadhesion tests of the formulations were evaluated. The disinfectant gel formulation containing propolis and boric acid has been reported to contain lower alcohol than

the World Health Organization (WHO) recommended formulation, but its effects on bacteria and mold are similar to the formulation recommended by the WHO. As a result, they stated that disinfectant gel formulations containing propolis and boric acid were successfully produced.

Interest in quercetin, which is a flavonol found in propolis, increased after researchers who researched SARS determined that quercetin along with vitamin C was an aminopeptidase inhibitor. Quercetin and its derivatives have been determined to hinder SARS-CoV and MERS-CoV major protease in vitro. In addition, quercetin is known to modulate the cellular UPR (unfolded protein response). In this case, coronaviruses can use UPR to complete all replication cycles, and quercetin can have an anti coronavirus effect because it can modulate this pathway (Polansky & Lori, 2020).

Propolis has proven antiinflammatory and immunoregulatory effects, also inhibition of active kinase (PAK-1). It is also reported that the host cell binding of the SARS-CoV-2 virus to ACE-2, which is the main target, is inhibited by propolis. Propolis components like quercetin, CAPE, rutin, kaempferol and myricetin show a strong interaction with ACE-2 in silico. For instance, kaempferol is reported to have decreased expression of TMPRSS2, also propolis does not interact with main liver enzymes or other key enzymes. The World Health Organization reported that propolis can be used as a supplementary food concurrently with medications without the risk of fortifying or inactivation (Berretta et al., 2020).

Although propolis is one of the safest natural remedy, it is expected propolis or its components may have adverse effects in rare cases, especially an allergic reaction. Therefore, more caution should be exercised in individuals with hypersensitivity when used in the prevention or healing of COVID-19 (Kurek-Górecka et al., 2020).

2. CONCLUSION

In the COVID-19 pandemic, which has affected the whole world, bee products help treatment in this period when the definitive treatment method has not yet been found. This review was made to bring together the studies investigating the physicochemical properties of honey and propolis and their effects on COVID-19. In the literature review conducted within the scope of the review, it has been reported that honey and propolis have a mechanism that can provide an inhibitory effect on the COVID-19 virus and these products provide a healing effect on patients, although in a limited number of clinical studies. Therefore, more experimental and clinical studies are needed to determine the therapeutic effects of both honey and propolis for COVID-19.

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The Isolation and Identification of Bacteria with the Remediation Potential of Calcerous Soil

Kireçli Toprağı Islah Etme Potansiyeline Sahip Bakterilerin İzolasyonu ve Tanılanması

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Abstract

Soil salinity and sodicity is a negative stress widely observed for agricultural production in the world. In saline and sodic soils, inter particulate distances increases by enhancing the repulsive forces because of high Na^+ ion concentration. As a results, this undesirable situation results in dispersion of the soil, loss of porosity, reduction of water permeability in the soil profile. Besides, the crop production of this soil is very poor because of high pH, exchangeable Na^+, deficiency of plant nutrients, high concentration of carbonates and bicarbonates. Considering the big need for the arable lands in the world, the remediation of these soil with effective methods will be the right approach. In this study, it was aimed to isolate and identify the bacteria with carbonate dissolution ability, which had potential to remediate calcerous soils from Dereli/Giresun/Turkey. According to the results, two isolates (*Bacillus* sp. and *Chromohalobacter* sp.) were determined that they were capable of $CaCO_3$ dissolution. Only one isolate

Özet

Toprak tuzluluğu ve sodikliği dünyada tarımasal üretim için yaygın gözlemlenen negatif bir strestir. Tuzlu ve sodik topraklarda, yüksek Na^+ iyon konsantrasyonu nedeniyle itici güçlerin etkisiyle partiküllerin arasındaki mesafe artar. Sonuç olarak, istenmeyen bu durum toprağın dağılmasına, gözeneklerin kaybolmasına, toprak profilinde su geçirgenliğinin azalmasına neden olur. Ayrıca, topraktaki tarımsal üretim yüksek pH, değişebilir Na^+, besin elementlerindeki eksiklik, karbonat ve bikarbonatın yüksek konsantrasyonu nedeniyle çok düşüktür. Dünyada ekilebilir arazilere olan büyük ihtiyaç göz önüne alındığında, bu toprakların etkin yöntemlerle ıslahı doğru bir yaklaşım olacaktır. Bu çalışmada, Dereli/Giresun/Türkiye'den kireçli toprakları ıslah etme potansiyeline sahip olan ve karbonat çözme potansiyeline sahip bakterilerin izolasyonu ve tanılanması amaçlanmıştır. Sonuçlara göre iki izolatın (*Bacillus* sp. and *Chromohalobacter* sp.) $CaCO_3$ çözme yeteneğinde olduğu

(*Halomonas* sp.) exhibited potential of both $CaCO_3$ and $MgCO_3$ dissolution.

belirlenmiştir. Sadece bir izolat (*Halomonas* sp.) hem $CaCO_3$ hem $MgCO_3$ çözme potansiyeli göstermiştir.

Keywords: Calcerous soil, Carbonate, Bacterial dissolution

Anahtar kelimeler: Kireçli toprak, Karbonat, Bakteriyel çözünme

1. INTRODUCTION

The global population is expected to reach 10 billion in 2070 according to the international reports. This rapid population growth has led to an increase in the need for food on a global scale (Goswami et al., 2014). Therefore, not only the effective use of arable land in the world but also the remediation of non-arable land has gained more importance. The total land area of the world is estimated as 149 million square kilometers and only about 27 million square kilometers can be used as arable land because of erosion, slope, excessive pesticide pollution, wrong product selection, and salinity (Sklenicka & Salek, 2008). Salinization and sodification, which are caused by low rainfall, low quality of irrigation water and high evaporations are major problems of agriculture in arid and semi-arid regions of the world. They cause huge losses in crop production all over the world (Ahmad et al., 2006; Shabala & Cuin, 2008). The saline and sodic soils include sodium (Na+) and carbonates which are undesirable and toxic for plants. Salinization and sodification reduce the quality, productivity, water of soil and emergence of seedlings, penetration of root and plant growth (Qadir & Schubert, 2002). These soils, especially found in arid and semi-arid regions, generally contain high amount of calcium carbonate ($CaCO_3$), that are poorly soluble (Tamilselvi et al., 2018). There are three polymorphs form of $CaCO_3$ as calcite, aragonite and vaterite. Aragonite is mainly found in shells of aquatic organisms, calcrete profiles and pendant coatings, but rare in soil profiles (Courty et al., 1994). Vaterite is found in shells of aquatic organisms, but as rare in soil profiles as aragonite (Courty et al., 1994). The third polymorphs form of $CaCO_3$, calcite, is most abundant and thermodynamically stable. Magnesian calcite and dolomites are the most known types of calcites, that include magnesium and calcium with carbonates (Tribble et al.,1995; Warren, 2000). Saline and sodic soils exhibit calcareous soil characteristics including magnesian calcite and dolomites (Whipkey et al., 2002).

The researchers study many applications to remediate these saline-sodic-calcareous soils. The remediation tehcniques are application of chemicals such as gypsum, elemental sulfur and phytoremediation by using different plants (Ahmad et al., 2006; Abdel-Fattah, 2012;

Hasanuzzaman et al., 2014; Murtaza et al., 2009). However, these techniques have some disadvantages such as high cost, difficulty of suitable plant selection, and time consumption. Therefore, there have been still a great need for alternative remediation techniques (Tamilselvi et al., 2018). According to the literature, the carbonates are slowly and continually dissolved by microorganisms in the nature (Efe et al., 2020; Farrag et al., 2021; Friis et al., 2003; Orhan et al., 2017; Yanmis et al., 2015). Therefore, the studies about soil remediation with microorganisms dissolving carbonates have been recently gained great attention.

In this study, it was aimed to isolate and identify the soil bacteria with the potential to remediate saline-sodic- calcareous soils by dissolving calcium carbonate.

2. MATERIALS and METHODS

2.1. Soil Samples

The soil samples used for bacterial isolates were collected from Pınarlar Köyü/Dereli/Giresun in sterile glass bottles as the soil of the village (Pınarlar Köyü) was significantly calcareous. The soil samples were supplied from subsurface layer (-5 to -20 cm) and they were transferred in sterile glass bottles to the laboratory.

2.2. Isolation and Purification of Halophilic Bacteria with Carbonate Dissolution Ability

1 g of soil samples were diluted with sterile saline water (0.9 %) by using ten-fold serial dilutions. The samples were shaken for 30 min at 150 rpm, and plated on medium. The isolation of bacterial strains was performed at 25-30 °C on moderate halophile (MH) medium. MH medium include NaCl (8.1 %), $MgCl_2$ (0.7 %), $MgSO_4$ (0.96 %), $CaCl_2$ (0.036 %), KCl (0.2 %), $NaHCO_3$ (0.006 %), NaBr (0.0026 %), peptone (0.5 %), yeast extract (1.0 %), glucose (0.1 %) and agar (1.5 %) (Orhan et al., 2017). In this study, 17 bacterial strains were chosen taking their morphological difference (colonies that differ in their size, shape texture and colour) into account and were stored at –86 °C in 15% glycerol and Luria Bertani Broth (LB) for further studies. The screening of the bacterial isolates for $CaCO_3$ and $MgCO_3$ dissolution potential was performed with Deveze-Bruni's $CaCO_3$ (DBC) agar and Deveze-Bruni's $MgCO_3$ (DBM) agar media, respectively. The medium used for $CaCO_3$ dissolution potential was as follows (per liter): 20 g glucose, 10 g NaCl, 3 g $MgCl_2$, 0.5 g $MgSO_4.7H_2O$, 0.4 g KCl, 0.2 g $(NH_4)_2SO_4$, 15 g agar, and 15 g $CaCO_3$. The medium employed to determine $MgCO_3$ dissolution potential of the bacteria was used which modified by Orhan et al. (2017). The medium used for $MgCO_3$ dissolution potential was same as the medium used for $CaCO_3$ dissolution potential, however,

$MgCO_3$ was added instead of $CaCO_3$. The bacterial strains were incubated at 25 °C for 2 weeks on DBC agar and DBM agar media for $CaCO_3$ and $MgCO_3$ dissolution potential, respectively. The presence of $CaCO_3$ and $MgCO_3$ in the medium results in a blury appearance of the medium. When the bacterial strain dissolves the $CaCO_3$ and $MgCO_3$ a clear zone may form. The clear zone around the colonies was considered as positive for $CaCO_3$ and $MgCO_3$ dissolution capabilities (Orhan et al., 2017).

2.3. Amplification of 16 S rDNA Gene Region by PCR

Genomic DNA extractions of the bacterial isolates with carbonate dissolution ability were performed with a DNA extraction kit (Promega). Then, amplification of 16S rDNA region were performed by PCR using universal primer [UNI16S-L: (5'-ATTCTAGAGTTTGATCATGGCTCA-3') and the reverse primer UNI16S-R: (5'-ATGGTACCGTGTGACGGGCGG TGTGTA-3')] (Orhan et al., 2017). The reaction mixture contained genomic DNA (50 ng) (3 µL), water (15.3 µL) of, 10X buffer (100 mM Tris–HCl, pH 8.30; 500 mM KCl) (3 µL), $MgCl_2$ (25 mM) (1.8 µL), dNTP's mixture (dATP, dCTP, dGTP, dTTP at 10 mM concentration) (0.6 µL), each primer (20.0 pmoles/µL) (3.0 µL) and Taq DNA polymerase (0.30 µL). The PCR was carried out with the reaction steps as a primary heating step (for 2 min at 95 °C); 36 cycles of denaturation (for 1 min at 94 °C), annealing (for 1 min at 54 °C), and extension (for 2 min at 72 °C); a final extension step (for 5 min at 72 °C). The PCR products were screened on 1% agarose gel including ethidium bromide (0.5 mg/mL) and 1 kb DNA molecular weight marker. The PCR products were sequenced by Macrogen (Seoul, Republic of Korea). The sequence data of all the isolates were blasted with the NCBI GenBank sequence database and the bacterial isolates were identified.

3. RESULTS and DISCUSSION

The United Nations declared 17 sustainable development goals (SDGs), under the 2030 Agenda for Sustainable Development and emphasized the importance of environmental sustainability dimensions for socioeconomic development. Especially, according to the 2030 Agenda it has been aimed '*to combat desertification, restore degraded land and soil, including land affected by desertification, drought and floods, and strive to achieve a land degradation-neutral world*' (Toth et al., 2018). Soil degradation due to salinity and sodicity is one of the most important environmental problems that has negative effects on agricultural productivity and sustainability in arid and semi-arid regions (Suarez, 2001). In this context, it is of great importance to remediate saline and sodic lands for agricultural production. The saline and sodic soils are

mostly calcareous in arid and semiarid regions of the nature (Qadir et al., 2007). The researchers have developed many strategies to use these soils for agricultural production. The strategies such as chemical application, phytoremediation have some limitations. Therefore, carbonate dissolution with microorganisms have gained acceptance to ameroliate the soil, which is unsuitable for agriculture (Tamilselvi et al., 2016). There are many studies about isolation and identification of bacteria with carbonate dissolution ability (Gulluce et al., 2014; Li et al., 2007; Lian et al., 2008; Orhan et al., 2017; Orhan et al., 2021). In this study, it was decided to isolate bacteria with carbonate dissolution ability, as the application of halophilic bacteria would alleviate soil salinity by increasing decomposition of organic matters and dissolving carbonates ($CaCO_3$ and $MgCO_3$) and these bacterial activities may result in improved soil quality and replacement of Na^+ on exchange site. In this study 17 bacterial strains were isolated from soils of Dereli/Giresun/Turkey. The isolates were evaluated in terms of $CaCO_3$ and $MgCO_3$ dissolution potential by using Deveze-Bruni's carbonate medium (Figure 1). According to the results, three isolates exhibited ability to dissolve $CaCO_3$ (D1, D4, D8) and only one isolate exhibited ability to dissolve both $CaCO_3$ and $MgCO_3$ (D1).

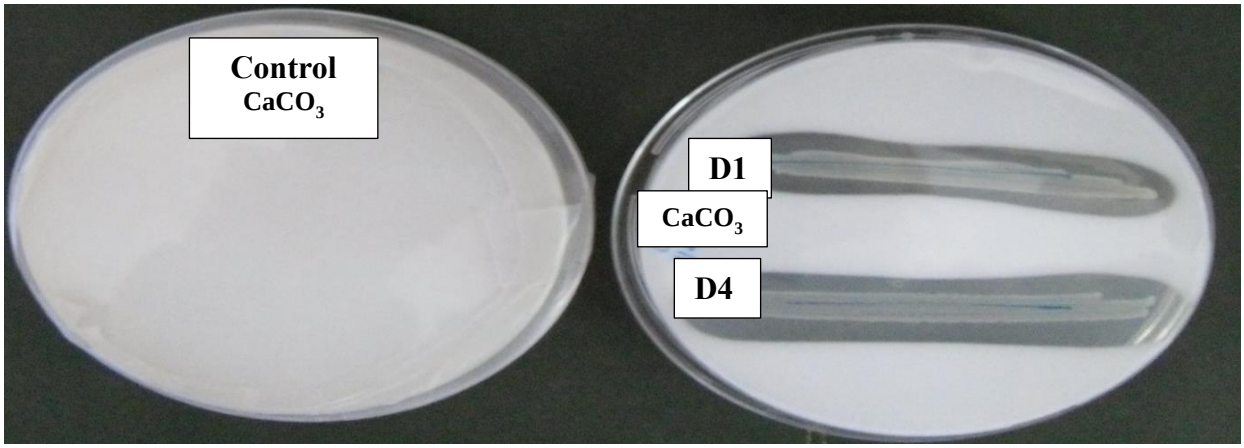

Figure 1. Screening of the bacteria in terms of $CaCO_3$ dissolution ability

These three isolates (D1, D4, D8) were identified by using 16 S rDNA gene region. As a result, they were identified as *Halomonas* sp. (D1), *Bacillus* sp. (D4), and *Chromohalobacter* sp. (D8).

Table 1. Identification Results and Genbank Numbers of Bacterial Isolates

Isolation Number	Identification Results	Genbank Acession Number	Similarity Rate (%)	Base Length (nucleotide)
D1	*Halomonas* sp.	OL913979	99	906
D4	*Bacillus* sp.	OL913980	100	952
D8	*Chromohalobacter* sp.	OL913981	100	884

It was previously reported that *Halomonas* sp. with the dissolution ability of was isolated from from salt-affected soil $CaCO_3$ and $MgCO_3$ (Orhan et al., 2017). According to the literature, there were many *Bacillus* species including *Bacillus subtilis* (Fris et al., 2003; Tamilselvi et al., 2018), *Bacillus* sp. (Gulluce et al., 2014; Orhan et al., 2017) with $CaCO_3$ dissolution ability. In another study, it was reported that two species of *Chromohalobacter* sp. with $CaCO_3$ dissolution ability were isolated from magnesite ore. The results of this study were in accordance with the literature. It is thought that the bacteria isolated and identified in this study could have remediation potential for calcerous soil. The bacteria dissolve carbonate compounds due to their metabolism products such as inorganic acids, organic acids, and some extracellular enzymes (Efe et al., 2020). In further study, it is planned to determine the mechanisms of carbonate dissolution, plant growth promoting (PGP) properties of the bacterial isolates.

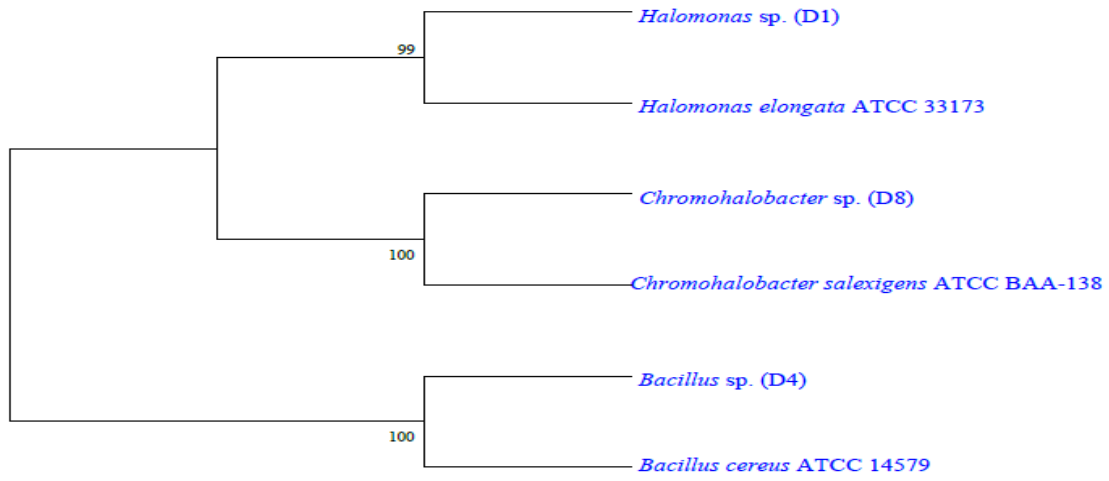

Figure 2. Evolutionary relationships of bacterial isolates. The evolutionary history was inferred by using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA7. The type strains are *Halomonas elongate* ATCC 33173, *Choromohalobacter salexigens* ATCC BAA-138, *Bacillus cereus* ATCC 14579.

4. CONCLUSION

Considering the increasing demand for agricultural production, it has gained significant importance to ameroliate saline and calcerous soils, which are non-arable. The halophilic bacteria isolated and identified in this study, which have ability of carbonate dissolution, can provide significant output for soil remediation. Since, adding a new salt to soils that already have excess salts can be prevented due to these bacteria. However, field application of these

bacteria should be investigated in order to determine their potentials for salt-affected soil amelioration and soil fertility improvement.

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