Süleyman Demirel Üniversitesi
Fen Bilimleri Enstitüsü Dergisi
Cilt 23, Özel Sayı, 147-154, 2019

Süleyman Demirel University
Journal of Natural and Applied Sciences
Volume 23, Special Issue, 147-154, 2019

DOI: 10.19113/sdufenbed.446772

Evaluation of Chemical and Mineralogical Structure of Davutoğlan Bird Sanctuary Clay for It's Antimicrobial Efficiency

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(Alınış / Received: 23.07.2018, Kabul / Accepted: 28.11.2018, Online Yayınlanma / Published Online: 15.03.2019)

Keywords
Natural clay,
Antimicrobial activity,
Clay minerology

Abstract: In this study we examined possible antimicrobial properties of clay specimen taken from Davutoğlan Bird Sanctuary which is located in Kıztepe skirts as a remote ecological environment to industrial areas. Antimicrobial effects of Gram (-) (*Escherichia coli, Pseudomonas aeruginosa*) and Gram (+) (*Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus*) pathogenic bacteria species on clay samples collected from the region were investigated. Between 0.5-1.5 g/mL concentration range clay has been shown to affect bacterial growth with the bacterial inoculations of 10^5 cfu/mL. X-ray diffraction (XRD), X-ray fluorescence (XRF) and thermogravimetric (TG) studies of clay minerals and chemical structure have been determined in parallel with the study of antimicrobial activity in order to support these results and to explain the findings. In addition, scanning electron microscopy (SEM) and Brunauer–Emmett–Teller (BET) methods were used to determine the pore structure and surface area of the clay. The obtained results were correlated with antimicrobial activity. According to XRD, XRF and TG analysis results, illite, dolomite and calcite were found to be major clay minerals from the samples taken from the area. It was found that they have a large surface area of 57,002 m^2/g according to BET results. It is also concluded that since the large surface area gives strong absorbent properties and supplies a suitable interface for oxidation, suppression of the bacterial growth is observed.

Davutoğlan Kuş Cenneti Kilinin Kimyasal ve Mineralojik Yapısının Antimikrobiyal Etkinliği Açısından Değerlendirilmesi

Anahtar Kelimeler
Doğal kil,
Antimikrobiyal aktivite,
Kil mineralojisi

Özet: Bu çalışmada endüstri bölgelerine uzak bir ekolojik ortam olan Davutoğlan Kuş Cenneti Kıztepe mevki eteklerinden alınan kil örneğinin olası antimikrobiyal özellikleri incelenmiştir. Bölgeden alınan kil örneklerinin Gram(-) (*Escherichia coli, Pseudomonas aeruginosa*) ve Gram(+) (*Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus*) patojen bakteri türleri üzerindeki antimikrobiyal etkileri araştırılmıştır. Kilin 0.5-1.5 g/mL konsantrasyon aralığında 10^5 cfu/mL bakteri inokülasyonlarına karşı bakteriostatik etkide olduğu gösterilmiştir. Bu sonuçları desteklemek ve bulguları açıklamak amacıyla antimikrobiyal aktivite çalışmasına paralel olarak kil örneğinde X-ışınları kırınımı (XRD), X-ışınları floresans (XRF), Termogravimetri (TG) çalışmaları ile kilin minerolojik ve kimyasal yapısı belirlenmiştir. Ayrıca kilin gözenek yapısı ve yüzey alanını belirlemek için taramalı elektron mikroskobu (SEM) ve Brauer-Emmet-Teller (BET) yöntemleri kullanılmıştır. Tüm bu analizlerden elde edilen sonuçlar antimikrobiyal aktivite değerleri ile ilişkilendirilmiştir. Sahadan alınan kil örnekleği XRD, XRF ve TG analiz sonuçlarına göre illit, dolomit ve kalsit içerdiği tespit edilmiş ve BET sonuçlarına göre kil 57,002 m^2/g gibi geniş yüzey alanına sahip olduğu bulunmuştur. Geniş yüzey alanı kuvvetli absorbans özellik kazandırdığından ve bu geniş yüzey alanı oksidasyon için uygun bir ara yüzey olduğundan bakteriyal üremeyi baskıladığı sonucuna varılmıştır.

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1. Introduction

Humankind has been using and preparing equipments made from clays since Neolithic or even Chalcolithic period [1]. Possibly because of the fact that the ceramic surface is not a suitable medium for bacterial or fungal growth, it is one of the widespread material in use today. The medicinal effects related with antimicrobial properties of the clays have already been known for a long time [2, 3].

Clays differ in terms of their chemical and physical properties and are classified into different classes according to these properties. However, the clay structure is mainly composed of alumina and silica, which is called as alumina silicate. The chemical properties of the non-clay minerals are enriched by structural participation, such as Ca, Na, Mg, K, quartz [4]. The other elements that enrich the micro-chemical structure of the clays are organic substances and salts. Unlike the expectation in industrial practice, this micro-chemical environment is the most important properties in medicinal and cosmetic use [4, 5].

Geophagia is an old and widespread behaviour, which is known as consumption of soil minerals and pure clay structures such as illite and smectite by human and animals [6, 7]. Clay minerals as a natural nano-structures as well are also good adsorbent, ion-exchanger material they are rich in mineral and therefore have a widespread healing effects mainly on skin and gastrointestinal diseases [8, 9]. One of the most striking researches about the therapeutic effects of clays has been conducted by Williams *et al.* on the antimicrobial efficacy of the French green clay [10, 11, 12]. Upon the finding of effective treatment of very dangerous necrotizing fasciitis type infectious disease called Bruli ulcer [11], the relationship between the mineralogical and chemical structures was investigated [10]. As a result, it has been stated that the illite-smectite feature of the French green clay is mainly effected by the reduced iron species present in the structure and by the pH buffering ability -by increasing the Fe^{2+} solubility- in the aqueous medium [10, 13]. Apart from the French green clay, various therapeutical features and antimicrobial effects have been studied and many clay types have been examined in terms of their mineralogical properties. [14, 15, 16] Disinfection of drinking water by clays reported for deactivation or killing of pathogenic microorganisms in water [17]. It is interesting that different clay species may have different microchemical properties and mineralogical structures with similar therapeutic properties. For example, some clay species seem to owe their effective antibacterial properties to the aluminum compounds they contain [18]. The conclusion that can be drawn from all these research above is the non-clay minerals are the most important factor, and the antimicrobial effect is originates from the multifactorial properties of the clay including microchemical structure (E.g. the pH buffering feature of the clay which increases the solubility of the active soluble mineral) [10, 19].

The identification of the antimicrobial natural minerals, such as different clays types, and the investigation of their effect spectra and mechanisms are important for us for two main purposes. An important advance in terms of medical use is the growing number of pathogenic microorganisms that the medical world has so far pointed out to develop antibiotic resistance to existing chemical agents. When the action mechanisms of clay and natural minerals are examined, it appears that they are caused by a number of physical and chemical interactions that cannot be specific to the metabolic processes and thus bacteria cannot easily become resistant [13, 20]. The second important issue is the environmental impact and environmental management. Natural minerals and clays of soil structure has controlling effect on the micro-fauna of the soil environment [20]. This indicates a mutual environmental interaction, that is, while the chemical and physical structure of the clay in the environment shapes the micro-fauna, the vice versa ~~is~~ also happens at the same time. The micro-fauna will have an impact on all other living species present in the environment. For this reason, the impact on micro-fauna, as a possible antimicrobial factor is important for the evaluation and management of environmental conditions. From the above-mentioned critics, in this study, the microbial activities of clay sample taken from the Davutoglu Bird Sanctuary near Davutoğlan Village, Çayırhan Settlement Unit of Nallıhan District of Ankara province, were investigated. Besides the discovering of potential therapeutic effects of Çayırhan clay the information that can be elucidated from clay samples collected from the region. In-vitro antibacterial effects of clay against different pathogenic bacteria (*E. coli, S. aureus, P. aeruginosa, E. faecalis, B. subtilis*) were studied. Possible antimicrobial effects of clay have been investigated on these bacterial strains. The reason for the selection of this territory is that being far from the industrial regions. Only Çayırhan Thermal Power Plant is located in the region, and the central bird's sanctuary is about 8.5 km away. Davutoğlan is a bird's sanctuary where is located in the place where the Aladağ Stream meets the Sarıyar Dam. Aladağ Stream Born in Bolu province borders from Büyük Kartal hill, it flows in the borders of Seben district and flows into Sakarya River in Sarıyar dam lake area. There is no industrial structure in the basin and a HES. For this reason, the river is among the least polluted rivers in Turkey and especially as one of the richest fauna areas of our country. However, Sakarya River is not at the same situation. Although the Sakarya River has a very large basin area and 3 different sources (Sakaryabaşı, Başkurt and Pınarbaşı sources), the river has a variable flow regime. The amount of water from these sources is not small, but the abundance of agricultural land around the river and especially the watering of these land in the river upstream, the withdrawal of water from the Sakarya River drastically reduces the river. The Porsuk and Ankara Streams that pass through the city center of Eskisehir and the Alpu district increase the BOD value of the river water. Especially the situation of Ankara Stream is a worrisome dimension, because Ankara's Stream is the water that is treated by Tatlar refining plant where the household waste of the large settlement unit such as Ankara is refined. In addition, these streams are mixed in the treated waters from the purification plants of Ayaş, Polatlı and Nallıhan districts. Because of this reason, Sakarya River is unfortunately polluted at the upper side of the basin and most of these pollution elements accumulate as sediments in Sarıyar Dam. The Aladağ Stream meets the Sarıyar Dam Lake and the area is protected by the Ministry of Environment, but still in the area clean water is contaminated with polluted stream. The vicinity of the Davutoğlan bird sanctuary is at the level of the river. There is green clay layer is seen up to 200 m and red clay is located upper side of the hills. The geological survey of the region has been known for a long time [21, 22].

In this study, antimicrobial examination of green clay specimens taken from Davutoğlan bird sanctuary was made. X-ray diffraction (XRD), X-ray fluorescence (XRF) and thermogravimetric (TG) studies have been carried out in parallel with the microbiological study in order to evaluate the results of antimicrobial examination together with clay structure. The purpose of the XRD study is to determine clay and other minerals in the green clay, the XRF study was carried out to know the major components and the amount of heavy metals. TG method was applied to support XRD studies and to determine the amount of clay moisture and amount of carbonate minerals. In addition, the clay surface was imaged with a scanning electron microscope at a micro level and the major components were once again identified using the XRF (Energy Dispersive X-ray Analysis) feature of the electron microscope, and the result was compared with the XRF. The Brunauer-Emmett-Teller method (BET) was used to determine the surface area of the clay, and the surface area was determined by N_2 gas absorption. The results of the antimicrobial activity were interpreted with the help of chemical structure of the clay.

2. Material and Method

2.1. Apparatus

The TG study was carried out with Shimadzu DTG-60H apparatus. The thermogravimetric analyses were carried out in Pt pans at 10 °C/min heating rate under nitrogen atmosphere. The temperature and heat calibrations of both devices were carried out using In and Pb metals.

The surface area of complexes and pyrolize product NiO were measured by BET method in Quantachrome Nova 2200e device using N2 with 18 hours degassing time at 110 °C equilibrium temperature.

The major and minor components of the clay were determined Spectro XLABII polarized XRF device and XRD pattern of the clay was obtained using Bruker D8 Advance diffractometer. The surface morphology was also confirmed by Scanning electron microscopy (SEM, FEI NOVANANOSEM 650).

2.2. Sampling of green clay

The green clay samples were collected from Davutoğlan bird sanctuary located in close proximity of Davutoğlan Village at the beginning of the spring season in which there was a semi dry environmental condition. The samples were collected from the sediment of the basin in the area on which migratory bird colonies hosting. The area at this part was consisting partly watery and majorly muddy and dry soil and clay zones at the season. The muddy and dry clay zones of the area was the most suitable part for collecting samples. Samples were dried and preserved in plastic bags at room temperature in a dry and dark environment until it will be used.

2.3. Antimicrobial activities

Bacterial cultures (*E. coli, P. aeruginosa, E. faecalis, B. subtilis, S. aureus*) used in this study are stored in Tryptic soy broth containing 20% glycerol at -80 °C. 24 hrs fresh cultures prepared in Hinton Mueller agar. For antimicrobial activity collected clay samples are suspended in Hinton Mueller Broth and these clay (0.5-1.5 mg/mL) containing media are inoculated at final bacterial concentration of 10^4 cfu/mL. Before suspending in the media the clay samples are well grinded with a mortar until it became a powdery dust and sterilized in the oven at 100 °C. Antimicrobial test was performed by pipetting 10 µL samples at 30 min, 1, 2, 3 and 4th hrs and was spread on agar and cultivated at same growth condition. The colonies on which occurred on the agar was observed and counted at 24th hours. To be able to observe the bacteriostatic effect it was necessary to check the bacterial growth from overnight culture as well.

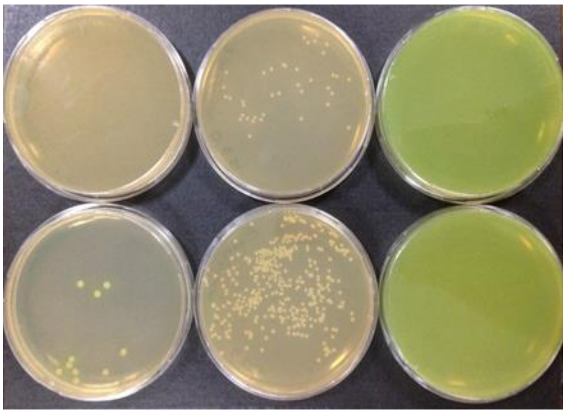
3. Results

3.1. Antimicrobial assessment of Davutoğlan clay

We employed two G(-) (*E. coli, P. aeruginosa*) and 3 G(+) (*E. faecalis, B. subtilis, S. aureus*) common pathogen bacterial species to evaluate the possible bacteriosidic or bacteriostatic effect of Davutoğlan clay. Bactericides function by impeding nourishment, disrupting essential metabolic activities, suffocation (precipitation of a solid phase rendering the cell wall impermeable), poisoning (delivery of a toxin), or physical disruption (cell lysis by bursting or penetration) [23]. Bacteriostatic antibiotics on the other hand limit the growth of bacteria by interfering with bacterial protein production, DNA replication, or other aspects of bacterial cellular metabolism. However, there is not always a precise distinction between them and bactericidal antibiotics; high concentrations of some bacteriostatic agents are also bactericidal, whereas low concentrations of some bactericidal agents are bacteriostatic [10].

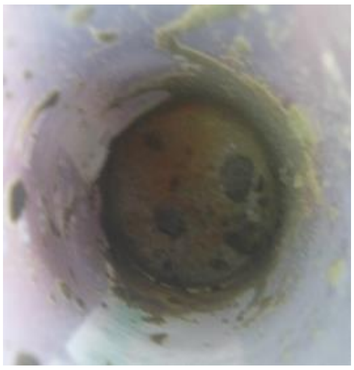
To show the antibacterial agent's bacteriosidic and bacteriostatic activity it is necessary to observe growth inhibition prolonged time (up to 24 hrs) of cultivation with antibacterial agent. According to our observation in all the bacterial strain that have been examined against Çayırhan clay were show suppression growth at first 1-4 hours. Table 1 shows that the clay slurry has a slight bacteriostatic effect at the given concentrations of clay (0.5-1.5 mg/mL) and bacterial strain 10^5 cfu/mL. Growth of control experiment was also weak at first hours but clay applied samples in most cases shows growth suppression by clay. Growth of samples taken from 24 hrs were confirming the observation that clay samples has bacteriostatic effect rather than bacteriosidic activity (Picture 1). As the control experiment clay slurry with no bacteria addition was not show growth. There was bacterial colony forming with no culture media addition to clay. Clay poultices prepared with de-ionized water incubated and growth over layer of poultices observed (Picture 2).

The results of the antimicrobial activity test indicate that the clay sample suppresses the growth of the bacterial population but after 24 hours there is inevitably an increase in the bacterial population. In recent studies in the literature, the inhibition of bacterial growth by clay specimens was reported [18, 19]. In these studies, it was indicated that soluble Al^{3+} and Fe^{3+} ions originating from the clay inhibited the bacterial population and that the population did not increase due to the stress which was caused by the Al^{3+} ion on the cell membranes. In another study, it was reported that the high surface area of clay is a suitable environment for oxidation and this results in the antimicrobial effect of oxidative stress [10]. The elemental analysis results of the clay samples taken from the Davutoğlan bird sanctuary basin, which were determined by XRF method, are given in Table 2 and XRD pattern obtained in order to determine the composition of clay is given in Graph 1 SEM image and EDX analysis of the clay was also performed and the results obtained with XRD was confirmed by EDX (Picture 3).



Picture 1. Growth of *P. aeruginosa* with clay (first row) and control experiments (second row) at 1, 4 hrs and overnight cultures

The growth was abundant single type colony greenish yellow colors. Usually this type of Fe rich clay minerals shows sulphur reducing bacterial growth [24].



Picture 2. Colony formation over the clay sample prepared with deionized water

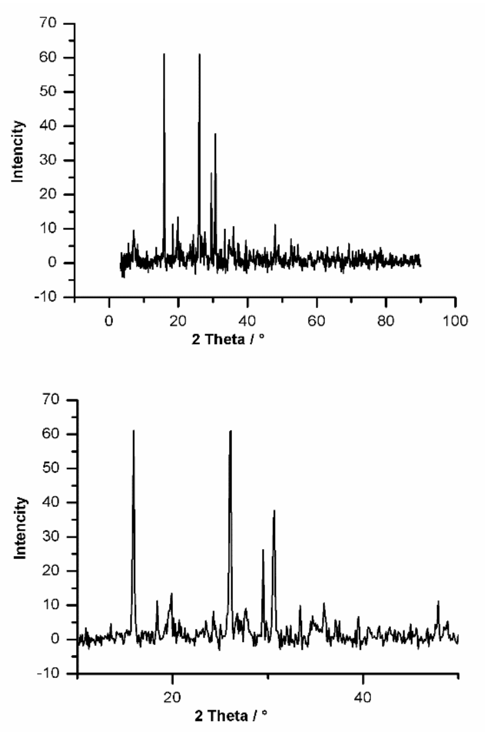
Table 1. Number of viable cells from the petri dishes of 1 hrs, 4 hrs and overnight cultures of clay applied and control experiment

	P. aeruginosa		***E. coli***		***B subtilis***		***E. faecalis***		***S. aureus***	
	Clay	**CG**	**Clay**	**CG**	**Clay**	**CG**	**Clay**	**CG**	**Clay**	**CG**
1 hrs	-	10	-	2	4	38	22	62	30	112
4 hrs	10	172	1	18	10	54	91	168	50	390
o/n	Biofilm	Biofilm	Biofilm	Biofilm	Biofilm	Biofilm	Biofilm	Biofilm	Biofilm	Biofilm

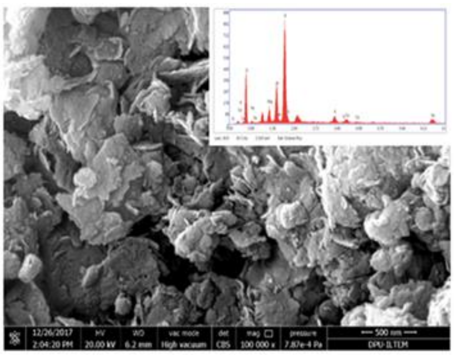
O/n, overnight culture; Clay, growth on clay added media; CG, control experiment

Table 2. Elemental analysis results found in Davutoğlan clay with XRF

Major Components (%)			**Minor elements (ppb=µg/kg)**								
	Component	**%**		**Component**	**ppb**		**Component**	**ppb**		**Component**	**ppb**
1	Na_2O	4.67	**1**	Co	34.3	**15**	Mo	3.0	**29**	Tl	0.8
2	MgO	4.54	**2**	Ni	53.4	**16**	Cd	0.9	**30**	Bi	0.6
3	Al_2O_3	14.19	**3**	Cu	21.2	**17**	In	0.9	**31**	Th	6.5
4	SiO_2	52.39	**4**	Zn	61.5	**18**	Sn	1.1	**32**	U	10.6
5	P_2O_5	0.0023	**5**	Ga	17.6	**19**	Sb	1.1			
6	Cl	0.02	**6**	Ge	0.8	**20**	Te	1.4			
7	K_2O	3.09	**7**	As	8.6	**21**	I	4.8			
8	Fe_2O_3	4.995	**8**	Se	0.2	**22**	Ba	401.9			
9	CaO	3.89	**9**	Br	1.4	**23**	La	30.1			
10	SO_3	0.12	**10**	Rb	93.1	**24**	Ce	42.2			
11	MnO	0.06	**11**	Sr	284.6	**25**	Ta	2.7			
12	TiO_2	0.51	**12**	Y	7.6	**26**	W	2.5			
13	V_2O_5	0.019	**13**	Zr	126.2	**27**	Hg	0.7			
14	LOI	11.47	**14**	Nb	16.0	**28**	Hf	4.1			



Graph 1. The XRD pattern of Davutoğlan clay

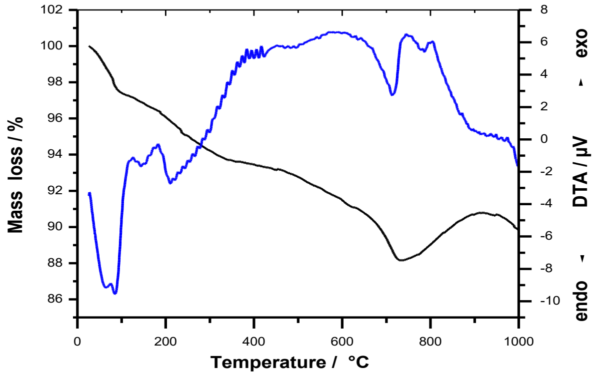

Picture 3. SEM analysis and EDX results of Davutoğlan clay

XRF analysis shows large clay minerals in the clay material and some carbonates of the IIA group elements

Existing of 4.54% MgO and 3.89% CaO in Davutoğlan clay indicates that some calcite + dolomite is also present in the mineral structure. Mineral mixtures coming from clay and limestone usually named as marls by geologist, in some geological literature sources this green clay in this region is also named as Davutoğlan-Çayırhan marls [21, 22]. The composition which is given in the Table 2 was also supported by XRD pattern of the clay (Graph 1). There are 4 significant diffractions observed in the XRD curve. They were diffracted at 2θ values of 15.593, 26.027, 29.495 and 30.480° respectively. The first intense peak is probably from the clay mineral called illite, the second peak from quartz or illite mineral. As it is known, the clay is dominated by aluminum oxides and hydroxides between the two-dimensional silicon dioxide plates. Part of the Al (III) ion in the clay can be replaced by Mg(II), Ca(II), K^+ and Na^+ ions. Because of this reason, it can be concluded that all of the amount of MgO and CaO calcite found in the clay can't be originated from dolomite mineral. Some Ca (II) and Mg (II), should be possibly coming from illite mineral. The intense peak at 26.027 ° can be attributed to SiO_2 platelets illite mineral or from free SiO2. The peaks observed at 29.495 and 30.480 ° show calcite and dolomite minerals existence in the clay.

XRF results show that there are sufficient amounts of Al^{3+} and Fe^{3+} in Davutoğlan clay. In this case, it was expected that to show we should have observed more effective antimicrobial activity on the bacterial species that have been tested according to the literature. Instead we observed a limited antimicrobial effect which can be expressed as growth suppression at the given bacterial inoculation and clay concentration. But in the literature, the pH value for growth inhibition is in the weak acidic region which is 4.6-4.7 [18,19]. Inhibition was observed in the acidic medium. At this pH value, the solubility of Al^{3+} ion for the clays is quite high [25]. At pH = 4.6, there is sufficient amount of Al^{3+} in the medium, which prevents the bacterial population from increasing. When it comes to our experimental condition we see an alkali pH (9.7-10). After Davutoğlan clay was mixed with distilled water and suspended, the pH value was measured as 9.7-10. The solubility of Al^{3+} and Fe^{3+} ions at this pH is too small to be measured. Considering that clay minerals and exchangeable ions also has a buffering effect, it would be cleared up why there were no significant antimicrobial effect although the sufficient amount of Al^{3+} and Fe^{3+} exist. For this reason, there is also no possibility to link the cause of the weak antimicrobial effect observed in this study to Al^{3+} and Fe^{3+} ions. The reason for the poor antimicrobial activity observed might possibly is the broad surface of the clay as a suitable interface for oxidation.

The calcite and dolomite limestone minerals of Davutoğlan clay structure are supported by the results of thermogravimetric analysis (Graph 2). According to the Graph 2, a mass loss of about 2% is observed in the sample heated to about 100 °C. It is because of the water absorbed by the clay, since this mass loss is endothermic. Then, as the temperature rises, a mass loss of about 11% from a temperature of 200 °C to a temperature of 800 °C is observed. These mass losses are probably due to dehydration of clay minerals in the material and decarboxylation of carbonate minerals, namely calcite and dolomite. The endothermic signal beginning at 630 °C and completed at 800 °C is the decarboxylation signal of a typical $CaCO_3$ mineral. The XRF analysis results are consistent with the TG given as the heating mass loss given as LOI (Lost of ignition). In TG operation, O_2 gas was supplied at a temperature of 800 °C and there were no exothermic signal was observed at this temperature range, indicating that there is no organic residue in the clay sample. As can be seen from the XRF results, the clay is a quite a poor in P element. In summary, only the potassium is present in the clay as nutritive element, the organic matter and the P in the clay are also very few. The solution obtained by treating the media obtained from clay by rinsing it with hot water and then mixing with H_2SO_4 and $KMnO_4$ found to be free of any organic material [26]. In this case, there are no organic or inorganic nutrients in the clay. Only K of NPK essential nutrients and Ca, Mg and Fe are essential elements [27]. But there was still bacterial colony forming was observed on the poultices obtained with no culture media addition to clay. Clay poultices prepared with de-ionized water incubated and growth over layer of poultices observed (Picture 2). The growth was seen as abundant single type colony greenish yellow colors. This might be the type of metal reducing bacterial colonies that can be absorbed by the clay [28].



Graph 2. The TG-DTA curve of Davutoğlan clay. Black, TG; blue, DTA curves

Davutoğlan Bird Sanctuary is located in the place where Aladağ Stream meets with the Sakarya River, more precisely in the Sarıyar Dam pond. Aladağ Stream is originated from the Kartalkaya region of the Köroğlu Mountains in the Bolu province of Turkey. It flows into the Seben county boundaries and merges with the Sarıyar dam at Kıztepe location 2 km away from Davutoglu village within the boundaries of Nallıhan district. There is no industrial facility on the road of river and it is not in a big settlement. There is a small irrigation pond just around Kartalkaya called Aladağ pond. There are 4 villages and 2 HES on the flow route and there are no treatment plants. In summary, Aladağ Stream is one of the rare rivers that can drift in the summer and winter, yet remain untouched by urbanization and industrial pollution. The surrounding area with rice farming and fruit gardens but still is not a region with high population density. It cannot be said that Aladağ Stream is affected too much by agriculture and industrial pollution. Due to this reason, it is not highly probable that the nutritional materials for microbial growth can be carried to Davutoğlan. The risk of aquatic pollution in Davutoğlan is actually coming from the Sakarya River. Especially in the spring season, the dam causes a lake formation in the bird sanctuary a few km in diameter. It is more probable that the pollutants that may come from the Sakarya River are likely to accumulate around the sanctuary because the clays and marl are strong absorbent materials. The poor antimicrobial activity of the Davutoğlan clay can be explained by the large surface area of the clay and the formation of a suitable interface for oxidation due to this large surface [10]. It would be possible to increase the bacterial population if there are appropriate nutrients for the bacteria in the environment. However, the results of the analysis show that for now, there is no dangerous accumulation on the clay. Otherwise, if the nutrient elements accumulate, it is obvious that the antimicrobial effect will be observed much less because the Davutoğlan clays do not have Al^{3+} and Fe^{3+} release due to the alkali aqueous environment. Also the natural microbial fauna would be effected and as a result the chemical properties of the clay and soil would be changed in return.

4. Discussion and Conclusion

Antimicrobial activity was determined by using five bacterial cultures on green clay specimens taken from the Davutoğlan Bird Sanctuary in the Nallıhan district borders. Initially, a weak antimicrobial activity was observed, but after 24 hours antimicrobial activity could not prevent the bacteria from proliferation. The situation was explained in the light of the literature. It was concluded that Davutoğlan's clay did not produce Al^{3+} and Fe^{3+} release due to their alkali environment in aqueous mixtures and therefore did not produce a strong antimicrobial effect. It is also concluded that the area is still protected from organic pollution which gives a balanced microbial fauna but still should be accepted as delicate for the chemical and biological environment of the bird sanctuary soil.

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