Research Journal of Biology Sciences
E-ISSN: 1308-0261 13(2): 28-36, 2020
Research Article

INVESTIGATION OF ANTIMICROBIAL AND ANTIBIOFILM EFFECTS OF *Ginkgo biloba* L.

Funda Karakaya[1,a+], Birgütay Şahin[1,b+], Ali Savaş Bülbül[3,c,*], Yusuf Ceylan[2,d], Elifnur Kurt[2,e], Mehmet Faruk Tarakçı[2,f]

[1]*Bartın University, Institute of Science, Department of Biology, Bartın, TURKEY*
[2]*Bartın University, Faculty of Science, Department of Molecular Biology and Genetics, Bartın, TURKEY*
[3]*Kahramanmaraş Sütçü İmam University, Faculty of Science and Letters, Department of Biology, Kahramanmaraş, TURKEY*

**Corresponding Author:*
E-mail: asavasbubul@gmail.com
[+]*: Equalship Author*

(Received 14th September 2020; accepted 09th November 2020)

a+: ORCID 0000-0002-5113-256X, b+: ORCID 0000-0003-0584-6075, c: ORCID 0000-0002-2200-7348, d: ORCID 0000-0001-8186-7252, e: ORCID 0000-0001-7178-0460, f: ORCID 0000-0003-3157-9441

ABSTRACT. Plants have been used to cure many diseases from past to present and are also used for industrial and medical purposes today. Plants are very essential in Turkey as in all countries. In our country which is rich in plant types, various methods are used to make use of plants especially medically. In this study, it was aimed to analyze antimicrobial and antibiofilm activity of *Ginkgo biloba* extract. Leaves were extracted with three different solvents like water, methanol and chloroform to analyze antimicrobial activity of the extracts. In vitro antimicrobial effects of extracts were investigated using disc diffusion method. The effects of plant extracts against *Enterobacter aerogenes* ATCC 13048, *Salmonella infantis*, *Listeria monocytogenes*, *Klebsiella pnemoniae*, *Listeria innocua*, *Salmonella enteritidis* ATCC 13075, *Enterococcus durans*, *Salmonella typhimurium*, *Staphylococcus epidermidis* DSMZ 20044, *Escherichia coli* ATCC 259222, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* antimicrobial and antibiofilm formation against gram positive and gram negative bacteria were examined. The plant extracts dissolved in different solvent inhibited bacterial growth at different rates. Methanol extract has been shown strong activity against both gram-positive and gram-negative bacteria. There is no any effect in the antibiofilm test.

Keywords: *Gingko biloba, Antimicrobial activity, Antibiofilm, Plant extract*

INTRODUCTION

Ginkgo biloba L. (common name shrine tree [1]; family- Ginkgoaceace) is a traditional but also economically important plant that is grown in China, Japan, Korea, France, Germany and some regions of India, especially Uttarakhand province (Figure 1) [2]. Medicinal parts of Ginkgo (fresh or dried leaves and seeds separated from their fleshy outer layers) are known for their antioxidant wound healing, neuroprotective and antimicrobial properties [3,4]. The medicinal and antimicrobial properties of Ginkgo can be attributed to two important chemical components which are terpenes trilactone (ginkgolides and bilobalid) and flavonoid glycosides [5]. Other components have attention in medical research because of its various beneficial properties, including flavonoids, antiallergic, anti-inflammatory, antioxidants, antimicrobial and estrogenic activities, enzyme inhibition, and cytotoxic antitumor activities [6].

Flavonoids can occur the form of glycosides in various glycosidic combinations in plants. However, rise in the ratio of aglycons to glycosides in extracts is an indication of deterioration [7]. Ginkgo biloba flavonoid glycosides have been reported in the form of mono-, di- and tri sugar units of quercetin (Q), kaempferol (K) and isorhamnet (I) [8].

Extraction of bioactive compounds that give antimicrobial activity facilitates pharmacology studies leading to the synthesis of stronger drugs with reduced toxicity [9]. The term "antimicrobial" refers to the inhibition of growth relative to specific microorganism groups such as antibacterial, antifungal, antiviral and antiprotozoan. Most research on Ginkgo leaf extracts involves isolation of phyto-components or evaluation of pharmacological activities. However, there are few studies on its effects on antimicrobial and antibiofilm activities [10].

Biofilm layer is organized by gathering one or more microorganisms and consists of chemicals that absorb the extracellular matrix of the surface they adhered and the materials that make up it [11-13] Biofilms prevent antibiotics against bacteria and cause microorganisms to be more resistant to antimicrobial agents [14-18]. In this study, antimicrobial and antibiofilm activities are investigated in addition to evaluating the extraction efficiency in Ginkgo through different solvents. The results will help to determine the variation in the antimicrobial and antibiofilm activities of the *Ginkgo biloba*.

Fig. 1. *General view of the Gingko biloba (Shrine tree) plant* [1]

MATERIALS AND METHODS

Material

Herbal material

The aboveground parts of the plant used in the study were thoroughly washed in tap water and dried in a cool environment.

Plant Extractions

Dry plants were powdered with liquid nitrogen and weighed. 1/10 ethanol was used as solvent and plant extraction was applied in the soxhlet device. The solvent was removed by keeping the liquid at room temperature overnight. Dissolved extract was prepared at the proper concentration in dimethyl sulfoxide (DMSO) (Fig. 2)

Fig. 2. *Dried form of Ginkgo biloba plant and powdering with liquid nitrogen from the air*

Antimicrobial Activity

Microorganisms and media

Twelve bacterial strains (*Enterobacter aerogenes* ATCC 13048, *Salmonella infantis*, *Listeria monocytogenes, Klebsiella pnemoniae*, *Listeria innocua, Salmonella enteritidis* ATCC 13075, *Enterococcus durans, Salmonella typhimurium, Staphylococcus epidermidis* DSMZ 20044, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus subtilis)* used for antibacterial activities of plant extracts. Luria-Bertani Broth (LB) medium was used for bacterial subcultures and MIC test, and Nutrient Agar medium was used for minimum bactericidal/bacteriostatic concentration (MBC) test and disc diffusion method.

Disc Diffusion Method

Antimicrobial activities of plant extracts have been tested against gram positive and gram negative microorganisms. Kirby and Bauer disc diffusion test was used to analyze antimicrobial activity [19]. Identified microorganisms were grown on Luria-Bertani Broth (LB) medium. Test microorganisms were inoculated into Nutrient Agar using the Drigalski spatula. The plant extract was loaded on sterile discs with a diameter of 6 mm in different concentrations. The disks were then placed on a petri dish and allowed to incubate for 16 hours. At the end of the incubation period, the diameters of the inhibition regions occurring around the discs were measured, and the antimicrobial activity was examined and recorded. Each sample was tested with 3 replicates. Since the positive control is a broad-spectrum antibiotic, tetracycline (TE 30) antibiotic was used.

Minimum Inhibition Concentration (MIC)

The lowest antimicrobial agent concentration that a microorganism can visibly inhibit is determined as “Minimum Inhibitory Concentration (MIC)”. The minimum inhibition concentration values of plant extracts were determined using sterile 96-well microplates.

Sterile LB broth was placed in each well. An equal volume of plant extract was added to the first well with LB, and then the mixture was serially diluted. In the next step, bacterial culture was added to the wells. Negative and positive control was also used. After these steps, microplates were incubated for 18 hours at 37 ° C. After incubation, the absorbance values of the samples were measured according to the positive control at 600 nm in the spectrophotometer device to see the lowest concentration at which plant extracts inhibit microorganism.

Minimum Bactericidal Concentration (MBC)

After determining the minimum inhibition concentration (MIC) of the plant extracts used in the study against microorganisms, the minimum bactericidal concentrations (MBC) were examined. For this purpose, after determining the MIC values, wells in which bacteria cannot reproduce were determined. Samples taken from wells where no bacterial growth were seeded on Nutrient agar (NA) solid medium with a sterile loop and kept in an oven for 18-24 hours at 37^{o}C. As a result of this period, the minimum antimicrobial substance concentration, which killed 99.9% of the bacteria inoculated in the medium, was accepted as the MBC value.

Antibiofilm Activity

The cluster of microorganisms formed by biofilm bacteria by sticking to the surface or to each other is called biofilm. Like the MIC experiment, sterile LB broth, serial diluted plant extract and bacterial culture were inoculated into a 96-well microplate and then incubated at 37 °C for 48 hours. After incubation, all wells were completely emptied and washed with distilled water (dH20). The microplates were then allowed to dry for 15 minutes at room temperature. 130 µl of 95% methanol was added to the wells for fixation, and after incubation for 15 minutes, it was discharged and allowed to dry. The wells were loaded with 125 µl of 0.1% crystal violet solution and washed for 10 minutes at room temperature, then washed with dH20. Gram-positive and gram-negative bacteria-containing wells were loaded with 200 µl of glacial acetic acid and ethanol, respectively, and were incubated for 15 minutes at room temperature, and then measured at 600 nm on a spectrophotometer (Thermo Scientific Multiskane GO). The effect of the applied plant extracts on the antibiofilm layer was compared by comparing the data obtained from the positive control and % reduction in biofilm inhibition was calculated (Merrit ve ark., 2011). Formula:

% Decrease = (1- (C-S) / C) x100

According to this formula;

C: Contains good positive control (containing medium and microorganism only).

S: Test wells (well with microorganism vaccine, medium and plant extract).

RESULTS AND DISCUSSION

Disk Diffusion Results

Water extract prepared from *G. biloba* plant were found to show no inhibition zones against microorganisms. Extracts prepared with methanol and chloroform were found to be effective in all microorganisms. In addition, Tetracycline (TE), used as a positive control, was observed to act against all microorganisms (Table 1).

Table 1. *Measurements of inhibition zones (mm) in different concentrations and solvents against the test microorganisms of the extract of G. biloba*

Microorganism	Zone Diameters (mm)									
	Water Concentration (mg /mL)			Methanol Concentration (mg /mL)			Chloroform Concentration (mg /mL)		Positive Control Concentration (mg /mL)	
	50	100	200	50	100	200	50	100	200	TE 30
E. aerogenes	7	7	7	7	7	7	6	6	7	17
S. infantis	-	-	-	7	7	8	6	6	6	10
L. monocytogenes	-	-	-	7	8	7	6	6	6	23
K. pnemoniae	7	7	7	7	7	8	6	7	6	16
Listeria innocua	-	-	-	8	8	9	8	9	11	16
S. entiritidis	-	-	-	7	7	7	9	10	10	23
E. durans	7	7	7	7	7	7	6	6	6	16
S. typhimurium	7	7	7	7	7	7	6	7	8	14
S. epidermidis	7	7	7	7	8	7	6	6	7	18
E. coli	7	7	7	7	7	8	6	7	7	12
S. aureus	7	7	8	7	8	7	6	6	7	25
B. subtilis	6	6	7	7	7	8	6	7	8	28

(-): No inhibition
(TE 30): Tetracycline (30 mg/ml).

Minimum Inhibition Concentration (MIC) Results

Samples placed in microplates to find the minimum inhibitory concentration of *G. biloba* with antimicrobial effect by disc diffusion method were measured at 600 nm wavelength in the spectrophotometer after incubation and MIC results are shown in Table 3 for each plant. According to the MIC results shown in Table 2, it was observed that the plant extract used showed antimicrobial activity against 6 applied strains of bacteria.

G. biloba showed a minimum inhibitory effect against *E. aerogenes* ATCC 13048, *S. infantis*, *S. aureus* ATCC 25923, *S. epidermidis* DSMZ 20044, *B. subtilis*, *E. coli* ATCC 25922 bacteria strains at a concentration of 50mg / mL.

Table 2. *MIC and MBC values of plant extract (mg/mL)*

Microorganism	Plant Concentrations					
	200 mg/mL	100 mg/mL	50 mg/mL	25 mg/mL	12,5 mg/mL	6,25 mg/mL
E. aerogenes		*	M			
S. infantis		*	M			
S. aureus		*	M			
S. epidermidis		*	M			
B. subtilis		*	M			
E. coli		*	M			

M: minimum inhibitory concentration
*: bactericidal concentration

Minimum Bactericidal Concentration (MBC) Results

Whether there is a visible growth in microorganisms with minimum inhibition concentrations (MIC), microorganism strains from microplate wells are passaged with

antibiotic-free medium, and the minimum bactericidal concentrations are determined as shown in Table 2.

As shown in Table 2, the lowest bactericidal concentrations (MBC) that inhibit the growth of plant extract up to 99.9% of the test microorganisms after MIC are shown.

According to these results, Ginkgo biloba extract at the concentration of 100 mg /mL *E. aerogenes* ATCC 13048, *S. infantis*, *S. aureus* ATCC 25923, *S. epidermidis* DSMZ 20044, *B. subtilis*, *E. coli* ATCC 25922 *against CFAI* bacterial strains. It was found to have the lowest bactericidal concentration that inhibits (Fig. 3).

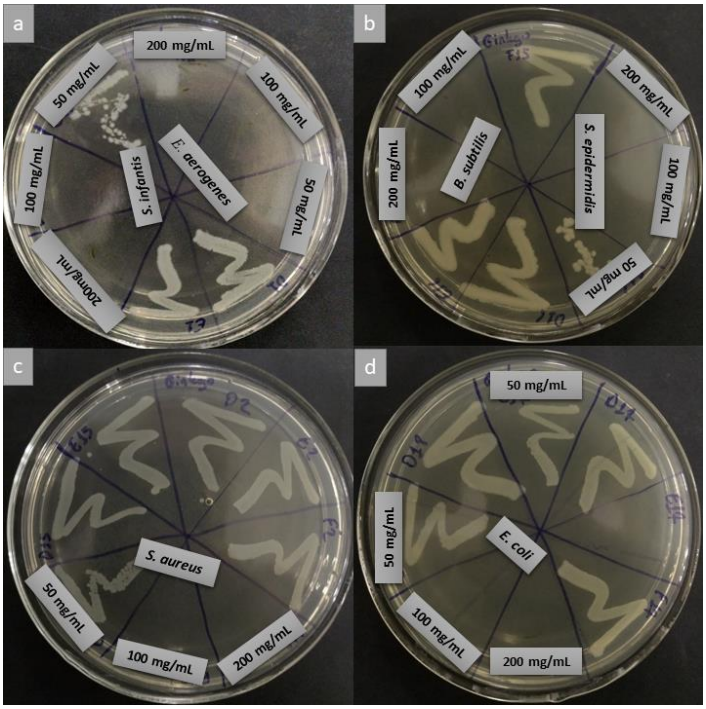

Fig. 3. *Antibacterial activity of ginkgo biloba extract against bacterial strains (a, b, c, d)*

Antibiofilm Results

As the antibiofilm effects of *G. biloba* extract against bacterial strains at different concentrations shown in Table 3, it was found that the concentrations applied to the extract did not inhibit the biofilm formation of all other microorganisms except *S. infantis*, *S. aureus* and *B. subtilis* strains.

Table 3. *Biofilm inhibition values (%) of Ginkgo biloba in different concentrations*

Micoorganism	**Plant Concentrations**					
	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	12,5 mg/ml	6,25 mg/ml
E. aerogenes	0	0	0	0	0	0
S. infantis	0	0	2,4	0	0	0
E. coli	0	0	0	0	0	0
S. aureus	17,27	0	0	0	0	0
S. epidermidis	0	0	0	0	0	0
B. subtilis	21,3	0	15,26	0	0	0

CONCLUSION

In this study, the antimicrobial activities of EGb (Ginkgo biloba's Extract) against common microorganisms were investigated. Among the microorganisms used according to the disc diffusion method, the maximum zone diameter was observed in Listeria innocua and Salmonella enteritidis bacteria. When looking at the solvents used, the most antimicrobial effect was observed in methanol followed by chloroform and finally in water. As a result of the minimum inhibition concentration (MIC) test, 6 bacterial strains at 50 mg/mL concentration were found to be effective. As a result of the minimum bactericidal concentration (MBC) test the lowest concentration that inhibits 99.9% of bacteria strains was found to be 100 mg/mL. When antibiofilm values were examined, it was observed that EGb against biofilm formation against *S. infantis, S. aureus* and *B. subtilis* strains, but had no effect on *E. coli* CFAI, *E. aerogenes* and *S. epidermitis*.

Bülbül et al. (2018) [20] studied the antimicrobial activity of extracts from *Acanthophyllum acerosum* and *Acanthophyllum microsepherium* (against *E. aerogenes* ATCC 13048, *K. pneumoniae, S. enteritidis* ATCC 13075, *S. typhimurium* SL1344, *S. aureus* ATCC 25923, *S. epidermidis* DSMZ 20044, *B. subtilis* DSMZ 1971, *E. coli* ATCC 25922, *E. coli* ATSS 2592, *S. infantis*, *S. kentucky*, *P. aeruginosa* DSMZ 50071, *E. faecium, E. durans, L. innocua, E. faecalis* ATCC 29212, *P. fluorescens* P1, *L. monocytogenes* ATCC 7644 and *C. albicans* ATCC 10231). They noticed that extract of *A. acerosum* had no antimicrobial activity *E. faecium*, *E. durans*, *L. innocua*, *E. faecalis*, *P. fluorescens, L. monocytogenes* and *C. albicans* while showing the activity against other microorganisms within the range of 1,83 and 6,67 mm. *A. acerosum* had MIC values at 10 mg/ml against *K. pneumoniae, S. epidermidis, E. coli* CFAI, *S. kentucky*, *P. fluorescens, E. faecalis* and *S. aureus*. They also found that although the extract of *A. microsepherium* had the effect (diameter in 6-7 mm) on *K. pneumoniae, S. enteritidis, S. typhimurium, S. epidermidis*, *S. kentucky* and *E. faecium*, it did not have MIC value against any microorganism. When compared with their findings, we observed the higher antimicrobial effect of *G. biloba* extract.

Lee et al. (2014) [21] observed the antibiofilm activity of Ginkgo biloba extract upon infection with Enterohemorrhagic *Escherichia coli* O157: H7 (EHEC). Antibiofilm screening of 560 purified phytochemicals against EHEC reported that the ginkgolic acids C15: 1 and C17: 1 at 5 μg / ml and Ginkgo biloba extract at 100 μg / ml significantly inhibited EHEC biofilm formation on polystyrene and glass surfaces and nylon membranes. . However, they observed that at their working concentrations, ginkgolic acids and *G. biloba* extract did not affect bacterial growth. Additionally, they reported that ginkgolic acids and *G. biloba* extract inhibited biofilm formation of three Staphylococcus aureus strains. The findings of this study showed that plant secondary metabolites are an important source of biofilm inhibitors.

Sati et al. (2012) [3] used the minimum inhibitory concentration (MIC) and disk diffusion test to determine the antimicrobial potential of leaf extracts of Ginkgo biloba. Species of Gram negative (*Escherichia coli 1, Escherichia coli 2, Pseudomonas corrugata, Pseudomonas putida, Serratia marcescens*), Gram positive bacteria (*Bacillus megaterium, Bacillus subtilis, Bacillus pumilus, Micwrococcus luteus, Micrococcus ruseus*), actinomycetes (*Nocardia sp., Rhodococcus sp., Streptomyces sp., Streptomyces griseobrunneus, Streptomyces griseoluteus*) and fungi (*Aspergillus niger, Fusarium oxysporum, Paecilomyces variotii, Trichoderma viride*) were used as test organisms. Leaf extracts were prepared in different organic solvents (methanol, ethyl acetate, and n-butanol) and distilled water. Leaf extracts exhibited antimicrobial activity against all the

3 groups of microorganisms, bacteria being most sensitive, followed by actinomycetes and fungi. While methanolic extract gave best results amongst the organic solvents used, aqueous leaf extract did not show any activity.

Considering the biological activity studies of *Ginkgo biloba* plant, which is important in our country and other countries in the light of the tests carried out, it can be used for different purposes in the fields of medicine, pharmacy, industry and food and as an antimicrobial agent in the treatment of infectious diseases caused by microorganisms. It is thought that this kind of studies with plants should be continued.

Biofilms where bacteria are formed are usually formed where mains water flows. The antibiofilm properties of plant extracts are important in the study conducted for the treatment of infectious diseases and many diseases and food establishments that may occur accordingly. Since biofilm-forming bacteria are more resistant to antibiotics than non-biofilm-forming bacteria. They pose a danger to living creatures on earth. For this reason, it is thought that such studies should be continued regarding the antibiofilm activity of Ginkgo biloba plant on certain bacteria strains.

Acknowledgement. This study and/or authors did not receive any direct support or funding from any person or organization. We also appreciate to Bartın University Central Research Laboratory for providing laboratory facilities, Bartın, Turkey.

REFERENCES

[1] Singh, B., Kaur, P., Gopichand Singh, R.D., Ahuja, P.S. (2008): Biology and chemistry of Ginkgo biloba. Fitoterapia, 79: 401-418.

[2] Mazzanti, G., Mascellino, M.T., Battinelli, L., Coluccia, D., Manganaro, M., Saso, L. (2000): Antimicrobial investigation of semipurified fractions of Ginkgo biloba leaves. J Ethnopharmacol, 71: 83-88.

[3] Sati, P., Pandey, A., Palni, L.M.S. (2012): Antimicrobial Potential of Leaf Extracts of *Ginkgo biloba* L.,Growing in Uttarakhand, India. Natl. Acad. Sci. Lett., 35(3): 201-206.

[4] Xu, S. L., Choi, R. C., Zhu, K. Y., Leung, K. W., Guo, A. J., Bi, D., Xu, H., Lau, D. T., Dong, T. T., Tsim, K. W. (2012): Isorhamnetin, A Flavonol Aglycone from *Ginkgo biloba* L., Induces Neuronal Differentiation of Cultured PC12 Cells: Potentiating the Effect of Nerve Growth Factor. Evid Based Complement Alternat Med. 2012;2012:278273. doi: 10.1155/2012/278273. Epub 2012 Jun 17. PMID: 22761636; PMCID: PMC3385709.

[5] Van Beek, T.A., Montoro, P. (2009): Chemical analysis and quality control of *Ginkgo biloba* leaves, extracts and phytopharmaceuticals. J Chromatogr A., 1216: 2002-2032.

[6] Cushnie, T. P. T., Lamb, A. J. (2005): Antimicrobial activity of flavonoids. Int J Antimicrob Agents, 26: 343-356.

[7] Sticher, O., Meier, P., Hasler, A., Van Beek, T. A. (2000): *Ginkgo Biloba*. The Analysis of Ginkgo Flavonoids. Amsterdam: Harwood, 179.

[8] Hasler, A., Sticher, O. (1992): Identification and determination of the flavonoids from Ginkgo biloba by high performance liquid chromatography. J Chromatogr A, 605: 41-48.

[9] Ebana, R. U. B., Madunagu, B. E., Ekpe, E. D., Otung, I. N. (1991): Microbiological exploitation of cardiac glycoside and alkaloids from Garcinia kola, Borreria ocymoides, Kolanitida and Citrus aurantifolia. J Appl Biotechnol., 71: 398-401.

[10] Bedir, E. T., Khan, R. A., Zhao, J. (2002): Biologically active secondary metabolites from Ginkgo biloba. J Agri Food Chem., 50: 3150-3155.

[11] Atalan, E., Bulbul, A., Ceylan, Y. (2020): *Cephalaria syriaca (L.)*: Investigation Of Antimicrobial, Antibiofilm, Antioxidant Potential And Seed Morphology. Freesenius Enviromental Bulletin, 29(7): 3641-3649.

[12] Oliveira, A., Cataneli Pereira, V., Pinheiro, L., Moraes Riboli, D. F., Benini Martins, K., Ribeiro de Souza da Cunha, M. L. (2016): Antimicrobial resistance profile of planktonic and biofilm cells of *Staphylococcus aureus* and coagulase-negative staphylococci. International Journal of Molecular Sciences, 17: 133-140.

[13] Stepanovic, S., Cirkovic, I., Ranin, L. (2004): Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol, 38: 428-432.

[14] Ulgen, H., Bulbul, A., Ceylan, K. B. (2020): Investigation Of Antimicrobial, Antibiofilm, Antioxidant Potential And Seed Morphology Of *Camelina sativa* L. Crantz. Freesenius Enviromental Bulletin, 29(7): 5121-5129.

[15] Bonsaglia, E. C. R., Silva, N. C. C., Fernades Ju´nior, A., Araujo Junior, J. P., Tsunemi, M. H., Rall, V. L. M. (2014): Production of biofilm by *Listeria monocytogenes* in different materials and temperatures. Food Control, 35: 386-391.

[16] Casarin, L. S., Brandelli, A., de Oliveira Casarin, F., Soave, P. A., Wanke, C. H., Tondo, E. C. (2014): Adhesion of *Salmonella enteritidis* and *Listeria monocytogenes* on stainless steel welds. Int J Food Microbiol, 191: 103-108.

[17] Tozyılmaz, V., Bulbul, A., Ceylan, Y., Armağan, M. (2020): Antibacterial, Antifungal, Antibiofilm And Antioxidant Activities Of Some Endemic Plants In Anatolian Flora. Freesenius Enviromental Bulletin, 29(6): 4338-4346.

[18] Corcoran, M., Morris, D., De Lappe, N., O'Connor, J., Lalor, P., Dockery, P., Cormican, M. (2014): Commonly used disinfectants fail to eradicate Salmonella enterica biofilms from food contact surface materials. Appl Environ Microbiol, 80: 1507-1514.

[19] Hudzicki, J. (2009): Kirby-Bauer disk diffusion susceptibility test protocol.

[20] Bülbül, A., Ceylan, Y., Armağan, M. (2018): Investigation of Antibacterial and Antifungal Properties of *Acanthophyllum acerosum* and *Acanthophyllum microcephalum*. Research Journal of Biological Sciences, 11(2): 14-17.

[21] Lee, J. H., Kim, Y. G., Ryu, S. Y., Cho, M. H., Lee, J. (2014): Ginkgolic acids and *Ginkgo biloba* extract inhibit Escherichia coli O157:H7 and *Staphylococcus aureus* biofilm formation. International Journal of Food Microbiology, 174: 47-55.