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Antioxidant and Antimicrobial Activities of Extracts from Some Selected Mediterranean Plant Species

Merve Deniz Köse, Oguz Bayraktar*, Ali Bora Balta

***Abstract*— In this study, *in vitro* antioxidant and antimicrobial activities of the plant extracts of four different plant species were evaluated. These plants are; *Olea europaea* (Olive leaf), *Liquidambar orientalis* (Turkish sweetgum), *Ziziphus jujuba* (Lotus) and *Juniperus communis* (Juniper) which are common in Mediterranean region and used in various application, especially for therapeutic purposes. The most efficient extraction yield was obtained for *Liquidambar orientalis* as 24.7 %, and followed with *Ziziphus jujuba* and *Olea europaea*. The extracts of *Liquidambar orientalis* exhibited the highest phenol content as 0.372 mgGAEq/g sample. Antioxidant activities of the extracts ranged between 8.009 and 0.527 TEAC (mmol/g sample). Extracts of *Liquidambar orientalis* had the highest antioxidant capacity with 8.009 TEAC (mmol/g sample). The antimicrobial activities of plant extracts were evaluated by using both disc diffusion and minimum inhibition concentration assays. Disc diffusion results were obtained for each plant extracts. The diameter of inhibition zones ranged between 20.5 and 7.8 mm. The extracts of *Liquidambar orientalis* had the highest inhibition activity with 20.5 and 19.2 mm inhibition zones against *Pseudomonas aeruginosa* and *Escherichia coli* respectively. The extract of *Olea europaea* had second highest antimicrobial activity against *E.coli* with a inhibition zone diameter of 13.6 mm. Both extracts of *Ziziphus jujuba* and *Juniperus communis* showed approximately the same inhibition activity. The minimum inhibition concentration (MIC) values of plant extracts ranged between 0.4 and 100 mg/ml. *Liquidambar orientalis* extract had the lowest MIC values against *Staphylococcus aureus* and *Staphylococcus epidermidis*, *Bacillus subtilis*. The extract of *Juniperus communis* had second highest antimicrobial activity against *S.epidermidis*, *S.aureus* and *B.subtilis* with minimum inhibition concentration value of 3.125 mg/ml.**

***Index Terms*— Extract, Juniper, Lotus, Olive leaf, Turkish sweetgum.**

I. Introduction

There is an increasing interest especially for plant-derived antimicrobial natural compounds. These natural compounds are playing a critical role and they possess health-beneficial components for human life. These natural compounds are preferred to be used for many industries all over the world [1]. They are known as plant secondary metabolites having antioxidative, antimicrobial and anti-inflammatory properties [2]. These bioactive natural compounds can potentially be used for pharmaceutical preparations, cosmetic products, food supplements and therapy applications. Today, many researchers try to isolate these bioactive compounds because these phytochemicals are being used in a great deal of area and have a good potential for medical applications [3]. Since ancient times, many plants and their parts such as leaves, seeds, roots and flowers have been used in various applications, especially for therapeutic usage [4]. Recently, the overuse of antibiotics has raised concern by health officials because of the rise of antibiotic resistant strains of bacteria. The continuing problem of development of resistance to existing antibacterial and antifungal agents will continue to motivate researchers for further screening studies. Natural compounds have been investigated by many researchers in order to find new antimicrobial agents which will be alternative to commonly used antibiotics [5]. Many plant products such as coffee, tea, spices and herbs have been shown to contain compounds that act as antimicrobial agents. [6].

The olive tree is one of oldest cultivated trees and the olive leaf has been used medicinally throughout history. Many researchers emphasized the presence useful phenolic compounds in olive leaf [7]. According to the studies, oleuropein and hydroxytyrosol were determined as major phenolic compounds in olive leaf extract [3, 8].

Turkish sweetgum with another name Anatolian Sığla tree exists only in certain locations of Turkey [9]. The antibacterial and antifungal activities of Turkish sweetgum crude extract were reported by several researchers in the literature [10, 11].

In Turkey, natural *Ziziphus jujuba* forests are distributed in Denizli region and researchers indicated that there is a various type of *Ziziphus* genotypes in the region [12]. Studies indicated that the active compounds in *Ziziphus* plant consist of triterpenes, saponins and glycosides [13]. However, recent studies revealed the presence of alkaloids, flavonoids, sterols, tannins and saponin in different species of the genus *Ziziphus* plant [14, 15]. Some studies also showed that extracts obtained from *Ziziphus* plant's parts had anticancer, antifungal, antibacterial, antiulcer, anti-inflammatory, antinephritic, imunositimulant and wound healing properties [16].

Juniper Seeds are rich in essential oil, organic acids, resin, glucose and sucrose. Juniper fruits are commonly used in herbal medicine as a household remedy and also in some commercial preparations. The major compounds present in

Merve Deniz Köse, Department of Chemical Engineering, Ege University, İzmir, Turkey
***Oguz Bayraktar**, Department of Chemical Engineering, Ege University, İzmir, Turkey
Ali Bora Balta, Spine Business Unit at Medtronic Spine & Biologics, İstanbul, Turkey.
*Corresponding author

juniper leaves are alkaloids, phenolics, flavonoids, tannins and terpenoids [17].

In this study, the crude extracts of four different plant species namely, *Olea europaea* (Olive leaf), *Liquidambar orientalis* (Turkish sweetgum), *Ziziphus jujuba* (Lotus) and *Juniperus communis* (Juniper) were investigated for their antioxidant and antimicrobial activities.

II. MATERIAL AND METHODS

A. Plant Materials and Chemicals

Fresh green olive leaves and Juniper seeds were collected from the trees which are grown in Izmir Institute of Technology campus (Urla) and Karaburun region respectively. Turkish sweetgum leaves were collected from natural sweetgum forests distributed in Muğla and Lotus leaves were collected from Ege University botanic garden. In all extraction experiments analytical grade ethanol was used and purchased from Merck (Germany). Folin-ciocalteu was used in order to determine total phenol content obtained from Sigma (USA). Sodium carbonate anhydrus (99.5%) was obtained from Fluka (Switzerland). Trolox (6-hydroxy-2,5,7,8,-tetramethylchroman-2carboxylic acid), ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) reagents and potassium persulfate ($K_2O_8H_8$) from Fluka (Germany) were used for antioxidant analyses. Glycerol was used to prepare the stock cultures and stored in -80°C. Nutrient broth and Nutrient agar were purchased from Fluka and used for bacterial reproduction. Penicillin, Gentamycin and Ampicillin antibiotics purchased from local pharmacy were used for comparison to evaluate the antimicrobial activities of plant extracts antimicrobial tests. Penicillin G (CTOO43B), Gentamicin (CTOO24B), Vancomycin (CTOO58B) and Streptomycin antibiotic discs were used in the standard disc diffusion assays and purchased from OXOID. Ultra-pure water was used for all experiments.

B. Extraction of Plant Material

Plant materials were dried at 35 ºC in dark. After grinding of samples, 70% aqueous ethanol solution was used as extraction solvent with a solid-liquid ratio of 1/10 at 37 ºC and 180 rpm in thermo shaker for 2 hours. The liquid extracts were then separated from solid plant material by filtration and then centrifuged at 5,000 rpm for 5 min. Rotary evaporator below 40 ºC was used to remove the ethanol in extract completely. After freeze-drying aqueous extracts, dried extracts were obtained and kept in the dark at +4 ºC until used in tests.

C. Determination of Total Phenol Contents

Total phenol content of four plant species was determined by using Folin-ciocalteu method with slight modification. Plant extracts were dissolved in ultrapure water in a ratio of 0.05 g extract in 1 ml water. Folin-ciocalteu reagent was diluted with deionized water in a ratio of 1:10 as a stock solution. 500 µl plant extract was mixed with 2.5 ml Folin-ciocalteu reagent and left to stand 2.5 min at room temperature. Then, 2 ml of sodium carbonate solution (7.5 % in deionized water) was added. After incubating 1 hour at room temperature in a dark place, the absorbance values were measured at 725 nm by UV spectrophotometer. Results were expressed as mg of Gallic acid equivalents (GAEq)/gr weight.

D. Determination of Antioxidant Activity

In order to determine antioxidant activity aqueous ABTS solution was used. 14 mM ABTS [light blue] and 4.9mM potassium persulfate ($K_2S_2O_8$) was mixed in a ratio of 1:1 and stand for 16 hours in a dark place at ambient temperature. After the reaction completed, solution forms $ABTS^+$ and its color turns into dark blue. While performing the experiment, first crude extract was dissolved in water. $ABTS^+$ solution was diluted with ethanol and absorbance was adjusted 0.7 (±0.03) at 734 nm. 10µl dissolved crude extract was added to 2 ml of $ABTS^+$ solution. Six kinetic readings were performed and absorbance was measured at every minute during six minutes with UV spectrophotometer. All samples were analyzed at least three times at different concentration (10, 20, 30 µl). The percentage inhibition of absorbance at 734 nm was calculated and the Trolox equivalent antioxidant capacity (TEAC) value was determined. Results were expressed as TEAC (mmol/g sample).

E. Determination of Antimicrobial Activity with Disc Diffusion

In order to determine the antimicrobial activities of the plant extracts; *Escherichia coli* and *Pseudomonas aeruginosa* were used as gram negative bacteria. *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Bacillus subtilis* were used as gram positive bacteria. *Candida albicans* was used as a fungus (a form of yeast).

Sterile cultures were prepared daily in 8 ml broth by transferring one loop of stock bacteria which are kept in -80 °C. These cultures were incubated for 18 hours and subcultures were obtained by transferring 80 µl from this 18 hour-incubated cultures to fresh broth (8 ml). Experiments were performed with these daily prepared subcultures which are standardized for inoculation on agar surface corresponding to certain numbers of CFU/ml. Log phases of growth curves were taken into account to reach approximate inoculation numbers also the standardized inoculums were confirmed by measuring OD values. In this study, six hours subcultures were taken in all experiments so as to use the same number of bacteria ($6x10^7$ CFU/ml). 100 µl of bacteria culture were inoculated onto agar surface. The agar depth was adjusted to 25ml for each plate. Inoculated culture was dispersed by streaking the sterile swab over the entire sterile agar surface by rotating the plate 60° each time to ensure the inoculum uniformly spread. The inoculated plates were allowed to sit for 5-10 minutes to let the broth absorb into agar. Sterile blank discs were applied after soaking with sterilized extracts. The concentration of extract solutions was determined as 100 mg extract/ml sterile ultrapure water for each plant

extracts. Only 4 discs were placed on each plate and then gently pressed to ensure contact with the agar surface. Plates were incubated for 24 hours at 37 °C. After 24 hours the inhibition zone diameters were measured by using a compass and results were expressed as millimeter (mm).

- *F. Determination of Minimum Inhibition Concentrations (MIC)*

The crude extracts were dissolved in sterile deionized water for a concentration of 100 mg/ml. Serial dilutions of each extract were carried out with a final concentration of 0.4 mg/ml by using sterile deionized water. 100 µl of each extract concentration and 95µl nutrient broth were added in each well of a 96 well plate. Each well was inoculated with 5µl of bacterial subculture suspensions incubated for 6 hours. This value was standardized by adjusting their optical densities at 640 nm by UV spectrophotometer. In all experiments, negative, positive and blank controls were carried out for each strain. Negative control well carried out 195µl of nutrient broth and 5µl of bacterial suspension. Positive controls were performed by using antibiotics. Blank control consisted of only 200 µl nutrient broth. Penicillin, Gentamycin and Ampicillin were used as a positive control. Initial concentration of antibiotics were adjusted to 1000 µg/ml. Serial dilutions of each antibiotic were carried out with a final concentration of 0.2 µg/ml by using sterile deionized water. 100µl of each antibiotic concentration and 95µl nutrient broth were added in each well of a 96 well plate. Each well was inoculated with 5µl of bacterial subculture suspensions. The assay plate was incubated at 37 °C for 24 hours and growth kinetic assays for each strain were performed by growth curves. MIC values of each extract and antibiotics were determined by a Varioskan microplate reader at 640 nm.

MIC results were reported as mg/ml for extracts and µg/ml for antibiotics. These spectrophotometric measurements of MIC values were carried out with a standardized protocol of Varioskan multiplate reader.

III. RESULTS AND DISCUSSION

- *A. Extraction Yields of Plants Materials*

As a result of the standardized extraction protocol, extraction yields were obtained for each plant material. Extraction yields ranging between 12.4 % and 24.7 % were determined for the plant materials. As seen in Table 1, *Liquidambar orientalis* had the highest extraction yield with 24.17 %. The extraction yields of *Ziziphus jujuba*, *Olea europaea* and *Juniperus communis* were determined as 21.5, 15.6 and 12.4 %, respectively.

Table 1. Extraction Yields of Plants

Plant and Codes		Part of Plant	Extraction Yield (%)
Liquidambar orientalis	**(A)**	Leaves	24.7
Ziziphus jujuba	**(B)**	Leaves	21.5
Olea europaea	**(C)**	Leaves	15.6
Juniperus communis	**(D)**	Seeds	12.4

These results were calculated on the basis of initial weight and expressed as a percentage. For each plant material, 10 gram dry powder was used and after extraction procedure, dry crude extracts were obtained. These extracts were weighted and the values were converted into percentage yield values.

- *B. Total Phenol Contents of Extracts*

Phenolic compounds are a class of antioxidant agents which act as free radical terminators. Phenolic compounds inhibit lipid oxidation by scavenging free radicals, chelating metals, activating antioxidant enzymes and inhibiting enzymes that cause oxidation reactions. Total phenol contents of plant extracts were analyzed with Folin-ciocalteu Reagent and obtained for each plant material. Total phenol contents of crude extracts ranged between 0.372 and 0.032 mgGA/g sample. The extract of *Liquidambar orientalis* was the most promising plant extract in terms of total phenol content. As seen in Table 2, *Liquidambar orientalis* had the highest phenolic compounds with 0.372 GAEq. Total phenol contents of *Ziziphus jujuba*, *Olea europaea*, and *Juniperus communis* were determined as 0.210, 0.159 and 0.032 GAEq, respectively.

Table 2. Total Phenol Contents of Plant Extracts

Plant and Codes		Part of Plant	Total Phenol Content GAEq [mgGA/g sample]
Liquidambar orientalis	**(A)**	Leaves	0.372
Ziziphus jujuba	**(B)**	Leaves	0.210
Olea europaea	**(C)**	Leaves	0.159
Juniperus communis	**(D)**	Seeds	0.032

- *C. Total Antioxidant Capacities of Extracts*

Total antioxidant capacities of plant extracts were determined with ABTS assay and obtained for each plant material. Antioxidant activities ranging between 8.009 and 0.527 TEAC [mmol/g sample] were determined. As seen in Tsble 3, *Liquidambar orientalis* had the highest antioxidant capacity with 8.009 TEAC [mmol/g sample]. Antioxidant activities of *Ziziphus jujuba*, *Olea europaea* and *Juniperus communis* were determined as 2.609, 1.387 and 0.527 TEAC, respectively.

Table 3. Total Antioxidant Activities of Plant Extracts

Plant and Codes		Part of Plant	Antioxidant Capacity TEAC [mmol/g sample]
Liquidambar orientalis	**(A)**	Leaves	8.009
Ziziphus jujuba	**(B)**	Leaves	2.609
Olea europaea	**(C)**	Leaves	1.387
Juniperus communis	**(D)**	Seeds	0.527

Among these four plant extracts, *Liquidambar orientalis* extract showed the significant antioxidant activity compared with the others. It was also obvious in results that there was a clear relationship between antioxidant capacities and total phenol contents for these extracts.

Especially, *Liquidambar orientalis* and *Ziziphus jujuba* were the most interesting ones that should be investigated for the identification of their bioactive phytochemical constituents.

- *D. Antimicrobial Activities of Extracts and Antibiotics*

In the disc diffusion assays, six microorganism species were chosen to determine the antimicrobial activities of plant extracts. *Escherichia coli and Pseudomonas aeruginosa* were used as gram negative bacteria. *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Bacillus subtilis* were used as gram positive bacteria. On the other hand, *Candida albicans* was used as a fungus. Disc diffusion results were obtained for each plant extracts. The diameter of inhibition zones ranging between 20.5 and 7.8 mm was observed. Extract of *Liquidambar orientalis* had the highest inhibition activity with 20.5 and 19.2 against *P. aeruginosa* and *E. coli* respectively. The other plant extract with relatively high antimicrobial activity was olive leaf extract. It showed the strong activity against *E.coli* with an inhibition zone diameter of 13.6 mm. Extracts of *Ziziphus jujuba* and *Juniperus communis* showed approximately the same inhibition activities. The results of antimicrobial screening of the crude extracts of all species by disc diffusion method are tabulated in Table 4.

Table 4. Disc diffusion zone diameters of plant extracts against different microorganisms

Microorganisms	Plants			
	A	B	C	D
Escherichia coli	19.2	9.6	13.6	8.5
Pseudomonas aeruginosa	20.5	9.8	9.6	7.8
Candida albicans	14.5	9.4	9.1	7.5
Staphylococcus epidermidis	14	14	13	10.6
Staphylococcus aureus	15.3	10.1	9.7	11.2
Bacillus subtilis	16.9	9	9.6	10.4

*Disc diffusion values were expressed as milimeter (mm)

Plant codes: (A) *Liquidambar orientalis*; **(B)** *Ziziphus jujuba*; **(C)** *Olea europaea*; **(D)** *Juniperus communis*

Although, some plant extracts inhibited one microorganism, some of them were effected more than one microorganism. For example, Extract of *Liquidambar orientalis* significantly inhibited all microorganisms including *Candida albicans*. Among the plant extracts, Extracts of *Liquidambar orientalis*, *Ziziphus jujuba* and *Olea europaea* demonstrated promising antibacterial activities against all tested microorganisms. The tested plant extracts were more active against gram positive bacteria than gram negative bacteria, depending on the different structural and inherited features of these two groups. Antibiotic controls were also performed in order to compare the sensitivity of tested microorganisms against antimicrobial agents. Inhibition zone diameters ranging between 21.1 and 9 mm were observed for different antibiotics. According to the results, penicillin had the highest inhibition activity with 40 mm against *S. aureus* but it did not influenced *P. aeruginosa*. Table 5 shows the inhibition zone diameters of antibiotics against microorganisms.

Table 5. Disc diffusion zone diameters of antibiotics against microorganisms

Microorganisms	Antibiotics			
	Streptomycin (STR)	Gentamicin (GEN)	Vancomicin (VAN)	Penicillin (PEN)
Escherichia coli	16	14	10	7.2
Pseudomonas aeruginosa	17	15.5	No Inhibition	No inhibition
Candida albicans	No Inhibition	No Inhibition	No Inhibition	No Inhibition
Staphylococcus epidermidis	9.5	9	16	21.1
Staphylococcus aureus	13	10	14.5	40
Bacillus subtilis	19	16.5	18	20.6

*Disc diffusion values were expressed as milimeter (mm)

Disc diffusion assays were performed in duplicate experiments for each species. *Bacillus subtilis* exhibited weak resistance against samples and antibiotic controls in disc diffusion assays. All plant extract samples that were used in the disc diffusion assays showed bactericidal activities. Bactericidal activity is defined as the transparently cleared zones around discs. On the contrary bacteriostatic activity is defined with cleared zones containing micro colonies around the discs. 'Bacteriostatic' means that the agent prevents the growth of bacteria (it keeps them in the stationary phase of growth). On the other hand, 'bactericidal' means that it kills bacteria.

- *E. Minimum Inhibition Concentration (MIC) Values*

In this study four plant extracts that have previously confirmed for their antimicrobial activities by disc diffusion tests were examined for their minimum inhibition concentrations (MIC). In order to determine the MIC, serial micro broth dilution method was performed by using Thermo 96 well microtiter plates. Dilution methods are known as quantitave, more repeatable and reliable assays when compared with other methods. When performing this study a microplate reader (Thermo Varioskan) was used. Varioskan

has several features that facilitate the continuous antimicrobial susceptibility testing. In similar systems there are various problems affecting the reliability of results such as temperature fluctuations and evaporation from microplate wells. This specific instrument enables a single system comprising a spectrometer, spectrofluorometer, incubator, shaker and microplate lid heater which prevents the evaporation problem. Minimum inhibition concentration results were obtained for each plant extract. Inhibition concentration values ranged between 0.4 and 100 mg/ml. According to the results, *Liquidambar orientalis* extract had the highest antimicrobial activity with the lowest minimum inhibition concentration values of 0.4, 0.8, 0.8 mg/ml against *S. aureus*, *S. epidermidis* and *B. subtilis* respectively. The second highest antimicrobial activity was shown by *Juniperus communis* extract. It showed the significant antimicrobial activity against *S. epidermidis*, *S.aureus* and *B. subtilis* with a minimum inhibition concentration value of 3.125 mg/ml. Extracts of *Ziziphus jujuba* and *Olea europaea* had approximately the same inhibition concentration values. Table 6. shows the minimum inhibition concentration values of plant extracts against different microorganisms.

Table 6. Minimum inhibition concentration (MIC) values of plants against microorganisms

Microorganisms	Plants			
	A	B	C	D
Escherichia coli	1.56	12.5	6.25	12.5
Pseudomonas aeruginosa	1.56	25	50	50
Candida albicans	6.25	50	100	25
Staphylococcus epidermidis	0.8	3.125	25	3.125
Staphylococcus aureus	0.4	6.25	25	3.125
Bacillus subtilis	0.8	12.5	12.5	3.125

*Minimum Inhibition Concentration values were expressed as mg/ml

Plant codes: (A) *Liquidambar orientalis*; **(B)** *Ziziphus jujuba*; **(C)** *Olea europaea*; **(D)** *Juniperus communis*

Minimum inhibition concentration results were also obtained for each antibiotic. Minimum inhibition concentration values between 0.4 and 1250 µg/ml were determined. According to the results, gentamycin and ampicillin had the highest inhibition activity but ampicillin did not inhibit P. *aeruginosa*. Minimum inhibition concentrations were determined for each antibiotic in order to compare with those of plant extracts. Table 7. shows the minimum inhibition concentration values of antibiotics against microorganisms.

Table 7. Minimum inhibition concentration (MIC) values of antibiotics against microorganisms

Microorganisms	Antibiotics*		
	Penicillin**	Gentamycin	Ampicillin
Escherichia coli	1250 (1500IU)	3.125	6.25
Pseudomonas aeruginosa	No Inhibition	3.125	No Inhibition
Staphylococcus epidermidis	50 (60IU)	12.5	0.8
Staphylococcus aureus	0.4 (0.48IU)	3.125	0.4
Bacillus subtilis	50 (60IU)	0.4	1.56

*Minimum Inhibition Concentration values were expressed as µg/ml
**1200IU Penicillin = 1000µg/ml

The entry of antibiotics as well as other complex molecules into gram negative bacteria requires a pathway through the lipopolysaccharide outer membrane. This pathway is provided by protein channels called porins. The ability of molecules to pass through these channels is influenced by their size, shape, and electrical charge. It has been demonstrated that porins serve as major entry gates for antibacterial compounds in these organisms. These membrane proteins were originally thought to be exclusively responsible for the inherently higher resistance of gram negative bacteria to antibacterial agents. Decreased entry of antibiotic into the bacterial cell is not important in gram positive bacteria because they lack a lipopolysaccharide outer membrane. Although the peptidoglycan layer of gram positive bacteria is thicker than that of gram negative bacteria, it does not pose a significant barrier to antibiotic entry.

IV. Conclusion

In this study extracts of four plant species namely olive leaf, Turkish sweetgum, Lotus and Juniper were examined in order to determine their relative total antioxidant, total phenol contents and also antibacterial activities. These bioactive natural compounds present in these extracts can further be isolated and potentially be used for pharmaceutical preparations, cosmetic products, food supplements and therapy applications.

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Merve Deniz Köse She has a BS degree in Chemical Engineering from Haccettepe University. Currently MS student at the Department of Chemical Engineering in Ege Univeristy. Her research interest is isolation and encapsulation of plant-derived natural compounds.

Prof. Dr. Oguz Bayraktar received his MSc. and Ph.D. degrees in Chemical Engineering from West Virginia University, USA. He worked as a faculty member in İzmir Institute of Technology between 2001-2014. Now, he has been working as a professor at the Department of Chemical Engineering in Ege University. In 2006, he has established a company (Natural Products R & D Ltd. Co.) at İzmir Technology Development Zone. He published more than 30 publications, and 40 conference proceedings. He has 200 citations and his h-index is 7. His research interests include: biopolymers, bioactivities of natural compounds derived from plants, drug delivery systems and nanotechnology.

Ali Bora Balta He has a BS degree in Biology from Adnan Menderes University. He received his MS degree in Biotechnology from İzmir Institute of Technology. Currently, he works for Spine Business Unit at Medtronic Spine & Biologics, İstanbul, Turkey.