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1 **_In vitro_ activity of plant extracts against biofilm-producing food-related bacteria**

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17 **Keywords:** Plant extracts, Antibacterial activity, Biofilm, Antioxidant activity, Bioautographic

18 analysis, HPLC

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20 **Running title:** Plant extracts and bacterial biofilm

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36 **Abstract**

37 The identification of effective antimicrobial agents also active on biofilms is a topic of crucial
38 importance in food and industrial environment. For that purpose methanol extracts of Turkish
39 plants, *Ficus carica* L., *Juglans regia* L., *Olea europaea* L., *Punica granatum* L. and *Rhus coriaria*
40 L., were investigated. Among the extracts, *P. granatum* L. and *R. coriaria* L. showed the best
41 antibacterial activity with minimum inhibitory concentrations (MIC) of 78-625 μg/ml for *Listeria*
42 *monocytogenes* and *Staphylococcus aureus* and 312-1250 μg/ml for *Escherichia coli* and
43 *Pseudomonas aeruginosa*. SubMICs produced a significant biofilm inhibition equal to 80-60% for
44 *L. monocytogenes* and 90-80% for *S. aureus*. The extracts showed also the highest polyphenols
45 content and the strongest antioxidant activity. Bioassay-guided and HPLC procedures demonstrated
46 the presence of apigenin 4′-O-β-glucoside in *P. granatum* L. and myricetrin and quercitrin in *R.*
47 *coriaria* L. Antigenotoxicity of plant extracts was also observed The present findings promote the
48 value-adding of *P. granatum* L. and *R. coriaria* L. leaves as natural antimicrobial/antioxidant agents
49 for control of food-related bacterial biofilms.

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62 **1. Introduction**

63 Most of the pathogens involved in foodborne diseases are capable to adhere to and form
64 biofilms on different surfaces (Le Magrex-Debar et al., 2000). The biofilm development on the
65 food-product or food-contact surfaces is a potential source of contamination that may compromise
66 food quality or cause pathogen transmission with health hazards (Bridier et al., 2015; Gibson et al.,
67 1995). Once firmly established, a biofilm can be very difficult to eradicate because the bacteria
68 embedded in a self-produced polymeric substance exhibiting poor susceptibility to conventional
69 antimicrobial agents (Olsen, 2015). Alternative strategies or more effective agents are needed. An
70 interesting approach to limit the formation of bacterial biofilms could involve the use of natural
71 products (Nostro et al., 2012; Buommino et al., 2014; Di Giulio et al. 2015; Nostro et al., 2007).
72 The acceptance of traditional medicine as an alternative form of health care has led to an increased
73 use of medicinal plants. The World Health Organization estimates that plant extracts or their active
74 constituents are utilized in traditional medicine in ~ 80% of the world's population (Anonymous,
75 1993). In this context, it is worth emphasizing the importance of scientific research in the
76 identification of new natural compounds with a proven antimicrobial-antibiofilm activity. Literature
77 already reports experimental evidences of the use of secondary plant metabolites, such as alkaloids,
78 flavonoids, tannins, terpenes and terpenoids, for their potential antimicrobial role in biomedical,
79 industrial and food fields (Cowan, 1999).

80 Turkey is known as one of the richest country of plants in the world. The number of flora
81 species is estimated to be around 3.000-5.000 in the 1960s and 8.500-9.000 today (Guner et al.,
82 2000; Turker and Koyluoglu, 2012). Around 3.500 of these species are endemic plants (Yeşilada et
83 al., 1993; Yeşilada et al., 1995). Owing to their biological properties, several plants belonging to the
84 flora of Turkey are used in the traditional medicine. However, only a small proportion of these
85 plant species have been thoroughly studied and investigated for their antimicrobial activity and very
86 few studies on biofilm production have been published (Marino et al., 2010).

87 The first aim of this study was to study the polyphenols content and the biological properties
88 such as antibacterial**,** antibiofilm and antioxidant activities of methanolic extracts derived from
89 Turkish *Ficus carica* L. (Incir), *Juglans regia* L. (Ceviz), *Olea europaea* L. (Zeytin), *Punica*
90 *granatum* L. (Nar) and *Rhus coriaria* L. (Sumac). The most active extracts were successively tested
91 by antibacterial and antioxidant High Performance Thin Layer Chromatography (HPTLC)
92 bioautographic assay in order to determine the active fractions and characterize the compounds. In
93 addition the antigenotoxic potential was also assessed.
94

95 2. Materials and methods

96 2.1. Plant materials and extraction procedures

97 The selected plants *Ficus carica* L. leaf, *Juglans regia* L. leaf and fruit peel, *Olea europaea*
98 L. leaf, *Punica granatum* L. leaf and *Rhus coriaria* L. leaf were bought from the local market in
99 Konya, Turkey. The leaves from all samples and fruit peel from *J. Regia* L. were air dried at room
100 temperature for two weeks and aliquots of 20 g were then extracted with methanol using Soxhlet
101 apparatus for about 8 h at a temperature not exceeding the boiling point of the solvent. The extracts
102 were filtered by using a Whatman® quantitative filter paper, ashless, Grade 589/2 white ribbon and
103 the residues were evaporated to dryness using rotary evaporator at not more than 40 ºC. Than the
104 extracts were lyophilized and stored until the analysis. The total yield from extraction of 20 g
105 sample was reported in Table 1.

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107 2.2. Determination of total phenolics
108 The determination of the total polyphenolic content of plant extracts were performed using a
109 ThermoSpectronic Helios-y spectrophotometer, according to previously described methods (Rossi
110 et al., 2011). The content of total polyphenols was expressed as g of gallic acid equivalent

1111 (GAE)/kg of extract and as mean of three independent experiments.
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113 2.3. Radical scavenging activity: spectrophotometric DPPH assay

114 Stock solutions of all extracts (25 mg/ml of pure methanol, ≥99.9%) were diluted 100, 300, 115 500, 700, 850, 1000-fold with methanol. An aliquot of 100 µl of each solution were added to 2.9 ml 116 of 1,1-diphenyl-2-picrylhydrazyl (DPPH; $1 x 10^{-4}$ M in ethanol), shaken vigorously and kept in the 117 dark for 30 min at room temperature. Sample absorbance was measured at 517 nm with UV–vis 118 spectrophotometer (ThermoSpectronic Helios-y, Cambridge, UK). A blank was assessed as the 119 solution assay described above without extract, instead of which methanol was employed. Trolox 120 (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, purchased from Sigma-Aldrich) was 121 used as positive control and prepared with testing solutions as described above for dried extracts. 122 The radical scavenging activities of each sample were calculated according to the following formula 123 for inhibition percentage (Ip) of DPPH:

124
$$Ip\ DPPH\% = (A_B - A_A) / A_B \times 100$$

125 where A_B and A_A are the absorbance values of the blank sample and of the test sample respectively, 126 after 30 min. Extracts and Trolox antiradical activity was considered as the concentration providing 127 DPPH 50% inhibition (IC_{50}), calculated from inhibition curves obtained by plotting inhibition 128 percentage against extract concentration (Rossi et al., 2012). Data are presented as mean ± SD of 1291 three independent experiments.
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131 2.4. Antibacterial activity

132 Microrganisms used for this study were: *Staphylococcus aureus* ATCC 6538P, *Listeria* 133 *monocytogenes* ATCC 7644, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 134 9027. Cultures for antimicrobial tests were grown in Mueller-Hinton Broth (MHB, Oxoid, 135 Basingstoke, United Kingdom) at 37°C for 24 h.

136 For antibacterial testing, the agar disc diffusion method was used. Briefly, each extract was

137 dissolved in pure dimethylsulfoxide (DMSO; BDH, Milan, Italy) to obtain a stock solution at a

138 concentration of 250 mg/ml. Overnight broth cultures, adjusted to yield approximately 5 x 10^8

139 colony forming units (CFU)/ml were streaked with a calibrated loop on plates containing Mueller
140 Hinton Agar (MHA, Oxoid). Filter paper discs (6 mm diameter; Oxoid) were placed on the
141 inoculated agar surfaces and impregnated with 10 µl of stock solutions. Pure DMSO (10 µl) was
142 used as a negative control while vancomycin disc (30 µg/disc), for *S. aureus* ATCC 6538P,
143 ampicillin disc (10 µg/disc; Oxoid), for *L. monocytogenes* ATCC 7644 and for *E. coli* ATCC
144 25922 and gentamicin (10 µg/disc), for *P. aeruginosa* ATCC 9027, were used as positive
145 controls. The plates were observed after 18 h at 37 °C. All tests were performed in duplicate and
146 the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced by the
147 plant extracts
148 The minimum inhibitory concentration (MIC) of plant extracts was determined in MHB,
149 using a broth dilution micromethod in 96-well round-bottomed polystyrene microtiter plates
150 according to the Clinical Laboratory Standards Institute (2009) guidelines, with some
151 modifications. The stock solutions of each extract were serial twofold diluted in MHB and the final
152 concentration ranged from 5000 to 39 µg/ml (the maximum value of DMSO was 2% and this final
153 concentration appeared to be not toxic for all studied bacteria). The final inoculums was 5 x 10^5
154 CFU/ml and MHB with DMSO were included as control. The MIC was considered as the lowest
155 concentration of each extract giving a complete inhibition of visible bacterial growth in comparison
156 with a control well after incubation at 37 °C for 18 to 24 h. The minimum bactericidal concentration
157 (MBC) was determined by seeding 20 µl from all clear MIC wells onto MHA plates and was
158 defined as the lowest extract concentration that allowed no microbial growth after incubation at 37
159 °C for 18 to 24 h. The data from at least three replicates were evaluated, and modal results were
1601 calculated. For the internal quality control, *S. aureus* ATCC 6538P was tested against vancomycin.
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162 2.5. Effect on growth an biofilm formation

163 The extracts of *J. regia* L. leaf, *O. europaea* L., *P. granatum* L. and *R. coriaria* L. were
164 selected as the best antibacterial extracts for evaluation of the effect on bacterial growth and biofilm
165 formation. The effect of different concentrations of plant extracts (ranging from 1/2 to 1/16 MIC)
166 on biofilm-forming ability was tested on 96-well polystyrene flat-bottomed microtitre plates
167 (Costar, Corning) as described by Cramton et al., (1999) with some modifications. Briefly, bacterial
168 cultures were grown overnight in 10 ml of Tryptic Soy Broth (TSB) for *L. monocytogenes, E. coli*
169 and *P. aeruginosa* and TSB+1 % glucose for *S. aureus*, diluted in growth medium to 5×10^5 CFU/ml
170 and dispensed (100 µl) into each well of microtiter plate in presence of sub-MIC concentrations
171 (100 µl) of plant extracts or control medium. TSB or TSB with DMSO were included as controls.
172 After incubation 24 h at 37 °C, the effect on (a) planktonic bacterial growth and (b) biofilm
173 formation was evaluated as follows:
174 (a) planktonic bacterial growth was estimated by measuring the optical density (OD) at 492
175 nm using a spectrophotometer EIA reader (Bio-Rad Model 2550, Richmond, CA, USA).
176 (b) biofilm formed on the polystyrene well was washed twice with sterile phosphate-
177 buffered saline (PBS; pH 7.4), dried, stained for 1 minute with 0.1% safranin and then
178 washed with water. The stained biofilms were resuspended in 200 µl of 30% (v/v) acetic
179 acid and OD was measured by spectrophotometry at 492 nm using a spectrophotometer EIA
180 reader.
181 The growth or biofilm reduction was calculated as:
182 $$100 - [\text{mean } OD_{492} \text{ of treated well / mean } OD_{492} \text{ of control well}] \times 100.$$
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184 2.6. HPTLC -bioautographic assay
185 The extracts of *P. granatum* L. and *R. coriaria* L. were selected for HPTLC -bioautographic
186 assay with the aim to determine the most active radical scavenging and antibacterial compounds
187 (Rossi et al., 2011).

188 For DPPH- HPTLC bioautographic assay, sample solutions of 25 mg/ml of *P. granatum* L.
189 and *R. coriaria* L. extracts were prepared. Negative control was set up with methanol (chosen
190 solvent), and positive with Trolox. For *R. coriaria* L., 15 µl of solution were applied in triplicate to
191 HPTLC plate of silica gel (Merck, silica gel 60, with fluorescence indicator F254) as 15 mm wide
192 bands with Linomat IV (Camag Muttenz, Switzerland) and then, eluted in a chromatographic
193 chamber at first step with a solvent solution composed as ethyl acetate/acetic acid/formic acid/
194 water 100/11/11/20 for 4 cm and at second step with toluene/ethyl acetate/acetic acid 100/90/10 for
195 8 cm. For *P. granatum* L., 15 µl of solution were applied in triplicate to HPTLC plate as 15 mm
196 wide band and the eluted with ethyl acetate/methanol/acetic acid/water for 8 cm. After plate
197 development (three chromatograms on the same plate), the first chromatogram was sprayed with an
198 ethanolic solution of 2,2-diphenyl- 1-picryl-hydrazyl radical (DPPH, 20 mg/100ml) to detect the
199 antioxidant fractions, while the other two were visualized at 254 nm (in the case of *R. coriaria* L.,
200 sprayed with NP/PEG reagent) to identify the active constituents spots and be able to scratch it out,
201 and extract it, from the eluted silica gel. The spot extract was directly analyzed by HPLC.

202 The antibacterial- HPTLC bioautographic assay was performed employing *S. aureus*. The
203 strain was cultured according to the method previously described (Rossi et al., 2011). Stock
204 solutions of *P. granatum* L. and *R. coriaria* L. were applied in triplicate to HPTLC plate, processed
205 and eluted ad described in the above paragraph. Negative control was set up with methanol, and
206 positive with chloramphenicol. The developed plates were dried at room temperature for 30 min for
207 complete removal of the solvent and a chromatogram was separated from the other two and treated
208 with *S. aureus* (10^7 CFU/ml) in proper agarized medium distributed over HPTLC plate. An aqueous
209 solution of triphenyl tetrazolium chloride (TTC) (20 mg/ml) was added to the medium as growth
210 indicator. The HPTLC plates, prepared as described above, were then transferred to Petri dishes as
211 support and incubated overnight at 37°C. Antimicrobial compounds appeared as clear yellow spots
212 against a red coloured background. The other two chromatograms were used to separate and
213 analyze the active compounds as described in the previous paragraph.

214 2.7. HPLC analysis

215 The active fractions revealed with DPPH and antibacterial bioautographic assay were
216 scratched out and extracted from silica gel in methanol solution was injected in HPLC. The analysis
217 were performed using a JASCO modular HPLC system (Tokyo, Japan, model PU 2089) coupled to
218 a diode array apparatus (MD 2010 Plus) linked to an injection valve with a 20 μl sampler loop. The
219 column used was a Eclipse-PLUS-C18 (25 μm × 0.46 cm, i.d., 5 μm) at a flow rate of 1.0 ml/min.
220 The mobile phase consisted of solvent solution A (methanol/acetonitrile = 50:50) and B
221 (water/formic acid = 99.5:0.5). The gradient system adopted was chosen according to the molecule
222 to analyze. The gradient was characterized by five steps: 1, starting point at 90:10 v/v (A/B); 2,
223 gradual changing to 50:50 v/v in 30 min; 3, B progressive raise to 0:100 v/v in 5 min; 4, isocratic
224 (0:100 v/v) for 5 min and 5, back to starting point (90:10 v/v) in 5 min. Injection volume was 40 μl
225 (Tacchini et al., 2015). The mass experiment were carried out on a FinniganMAT LCQ
226 (ThermoQuest Corp./FinniganMAT; San Jose, CA) mass spectrometer module, equipped with an
227 ion trap mass analyzer and an ESI ion source electrospray, in negative ion mode. For ESI-MS and
228 MS^2 experiments, the parameters were set as follows: the capillary voltage was 3.5 kV, the
229 nebulizer (N_2) pressure was 20 psi, the drying gas (N_2) temperature was 300 °C, the drying gas flow
230 was 9 L/min and the skimmer voltage was 40 V. The mass spectrometer was operated in the
2312 negative ion mode in the m/z range 100–1500.
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233 2.8. SOS-Chromotest

234 Genotoxicity and antigenotoxicity assays were performed as described by Prencipe et al.,
235 (2014) using exponential-phase culture of *E. coli* PQ37. The induction factor (IF), value that
236 establish the genotoxicity degree of the tested matrix, was obtained by comparing β-galactosidase
237 and alkaline phosphatase activity in treated and untreated cells. The protective effect of *P. granatum*

238 L. and *R. coriaria* L. extracts was, instead, expressed as percentage of inhibition of genotoxicity

239 induced by 4NQO and was evaluated following the formula reported by Bouhlel et al., (2007):

240 $$\text{inhibition (\%)} = 100 - ((IF_1 - IF_2)/(IF_2 - IF_0)) \times 100$$

241 where IF_1 is the induction factor in presence of both test compound and mutagen; IF_2 is the
242 induction factor of the mutagen in absence of the tested compound ; and IF_0 is the induction factor
243 of the untreated cells. All the data collected for each assay are the average of three determinations in
244 three independent experiments.

245 2.9. Statistical analysis

246 All the experiments were performed in triplicate. All values are expressed as the mean ± SD.
247 The significance of the results was analyzed by one way analysis of variance (ANOVA), with a p
2482 value <0.05 considered significant.
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2502 **3. Results**
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252 3.1. Determination of total phenolics

253 The content of total polyphenols of Turkish plant extracts is reported in Table 1. The most
254 relevant results were obtained for *P. granatum* L. and *R. coriaria* L. extracts: the content of
2552 polyphenols were respectively 17.63±0.43 and 25.38±1.03 g $_{\text{gallic acid}}$ /100 g $_{\text{plant extract}}$
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257 3.2. Radical scavenging activity: spectrophotometric DPPH assay

258 The samples tested by spectrophotometric DPPH assay showed different antioxidant activity

259 (Fig. 1). The most relevant result was exhibited by *R. coriaria* L. extract, which showed an IC_{50}

260 value better than positive control Trolox, followed by *P. granatum* L. extract.

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263 3.3. Antibacterial activity

264 The antibacterial activity of plant extracts is listed in Table 2. Specifically, disc diffusion

265 testing reported the higher activity of *P. granatum* L. and *R. coriaria* against Gram-positive bacteria

266 (inhibition diameters ranged from 12 to 21 mm) than Gram-negative bacteria (inhibition diameters
267 of 8-18 mm). The other extracts showed slight activity according to following order: *O. europaea*
268 L. > *J. regia* L. leaf > *F. carica* L. and *J. regia* L. fruit peel. The DMSO negative control showed no
269 inhibiting effect. The positive controls showed inhibition diameters of: 13.4 mm for vancomycin (*S.*
270 *aureus* ATCC 6538P), 12 mm for ampicillin (*L. monocytogenes* ATCC 7644) and 20 mm
271 ampicillin (*E. coli* ATCC 25922) and 29 mm for gentamicin (*P. aeruginosa* ATCC 9027).
272 The higher efficacy of *P. granatum* and *R. coriaria* was confirmed by the broth dilution
273 method. The MIC values ranged from 78 µg/ml (*R. coriaria*) to 156 - 625 µg/ml (*P. granatum*) for
274 *S. aureus* and *L. monocytogenes* and from 312 µg/ml (*R. coriaria*) to 625 - 1250 µg/ml (*P.*
275 *granatum*) for *E. coli* and *P. aeruginosa.* However, the inhibitory effect of the extracts was
276 bacteriostatic rather than bactericidal except for *S. aureus,* in fact the MBC values were 625 µg/ml
277 (*P. granatum*) and 2500 µg/ml (*R. coriaria*). The MIC value for vancomycin for *S. aureus* ATCC
278 6538P, the internal quality control, was 0.3 µg/ml.
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280 3.4. Effect on growth and biofilm formation
281 Despite that, the biofilm production was differently formed in accordance with the strain, *P.*
282 *granatum* L. and *R. coriaria* L. leaves extract were able to reduce biofilm formation more readily
283 than other extracts. The results revealed that 1/2, 1/4, 1/8 and 1/16 x MIC of *P. granatum* L. and *R.*
284 *coriaria* L. extracts poorly interfered with the planktonic growth, causing a slight and no significant
285 decrease equal to 20-10% respect to the control (Fig. 2) while produced a significant ($p<0.05$)
286 inhibition of biofilm formation equal to 80-60 % and 90-80 % for *L. monocytogenes* and *S. aureus*
287 respectively (Fig. 3). In contrast, a reduced biofilm biomass inhibition of *E. coli* (40-25 %) and *P.*
288 *aeruginosa* (30-20 %) was observed. SubMIC doses of *J. regia* leaf and *O. oleuropea* showed a
289 lower inhibiting activity with reductions of biofilm formation equal to 50-25% for Gram positive
290 bacteria and 40-10% for Gram negative bacteria than the other extracts.
291 3.5. HPTLC -bioautographic assay and HPLC analysis

292 The bioautographic analysis demonstrated the most active fractions at R_f= 0.7 for *P. granatum* and at R_f= 0.5 for *R. coriaria* (Fig. 4). Based on the previous literature data, as well as by direct comparison of standards UV spectra obtained by HPLC-DAD and mass spectra obtained by HPLC-MS (Table 3), we confirmed the presence of myricetrin and quercitrin in the active fraction at R_f=0.5 of *R. coriaria* L. extract and the apigenin 4′-O-β-glucoside as main active constituent in the active fraction at R_f=0.7 of *P. granatum* L. extract.

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299 3.6. SOS-Chromotest

300 A earlier series of experiments carried out on the antigenotoxic activity evaluation pointed out the absence of influence of the different concentrations of *R. coriaria* L. extract on the viability of the *E. coli*. The same phenomenon was not exhibited by *P. granatum* L. extract, that showed an effect on viability at the highest concentrations tested. The SOS chromotest genotoxicity assay examined the ability of the two plant extracts to produce a SOS response. According to Kevekordes et al., (1999), a compound is classified non-genotoxic if its Induction Factor (IF) is <1.5, slightly genotoxic if the induction factor ranges between 1.5 and 2, and genotoxic if the IF value exceeds 2. The test revealed, for both plant, the very low effect of non-citotoxic concentrations on the induction factor of the SOS chromotest, because IF values are lower than 1.5 (Table 4). Considering the experimental data and the literature data, *P. granatum* L. and *R. coriaria* L. extracts were considered non-genotoxic. Dose of 2.5 μg/assay of the directly acting mutagen 4-nitroquinoline-1-oxide (4NQO) was chosen for the antigenotoxic activity evaluation, since this dose induce a significant SOS system activation and was not toxic. Every concentration of *R. coriaria* L. extract decreased the activation of the SOS system induced by 4NQO, showing inhibition of genotoxicity in a dose dependent manner except for 0.6 and 0.3 μg/ml. Concerning *P. granatum* L., the highest concentration of the extract interfered with *E. coli* viability and the remaining concentrations tested showed antigenotoxic potential in a non-dose-dependent manner (Table 4).

317 4. Discussion

318 The scientific interest into biological properties of Turkish wild plants has received much
319 attention in recent years and a lot of papers have confirmed their traditional uses (Güzel et al., 2015;
320 Polat et al., 2013; Turker and Usta, 2008). In this context, the study contributed to enrich the
321 literature data exploring the activity of five Turkish plant extracts. The findings pointed out that the
322 leaves of *P. granatum* L. and *R. coriaria* L. possess interesting antibacterial, antibiofilm and
323 antioxidant potential and are highlighted as bioactive promising plant-materials. The higher activity
324 of *P. granatum* L. and *R. coriaria* against Gram-positive bacteria than Gram-negative bacteria is
325 probably due to the differences in cell wall structure between these microrganisms. The Gram-
326 negative bacteria have an outer membrane that provides an additional selective permeation barrier
327 to prevent the entry of noxious compounds (Nikaido, 2003). Moreover, antigenotoxicity was
328 observed for the non-bacterial-toxic concentrations of both plant extracts.

329 *P. granatum* L. and *R. coriaria* L., commonly known as nar and sumac respectively, are
330 recognized for their well documented biological activities including antibacterial, antifungal,
331 antiviral, antiparasitic, antioxidant, anti-inflammatory and anticancer activities (Fawole et al., 2012;
332 Fazeli et al., 2007; Jurenka, 2008; Rayne and Mazza, 2007; Shabbir, 2012). However, while the
333 published studies are focused on the efficacy of gel, fruits and flowers only few data are performed
334 on the antibacterial activity of leaves.

335 The results of the study emphasize the higher polyphenols content and stronger antibacterial
336 and antioxidant activity of *P. granatum* L. and *R. coriaria* L. leaves extracts compared to the other
337 plant extracts. The bioautographic analysis of these plant extracts revealed bioactive bands in
338 correspondence of flavonoidic fractions, effectively identified by HPLC analysis as myricetrin and
339 quercitrin in *R. coriaria* L. and apigenin 4′-O-β-glucoside in *P. granatum* L.. These components
340 belonging to flavonols and flavones are a large group of naturally occurring plant compounds that
341 received a great deal of attention because of their antibacterial and antioxidant activity (Cushnie and
342 Lamb, 2011; Gould and Lister, 2006). In this context, Madikizela et al., (2013) demonstrated
343 antimicrobial activity of flavonol glycosides myricetin-3-O-arabinopyranoside, myricetrin-3-O-

344 rhamnoside and quercetin-3-O-arabinofuranoside isolated from *Searsia chirindensis* L..
345 Furthermore, Sato et al., (2000) suggested the potential use of apigenin and related flavonoids
346 against methicillin-resistant *Staphylococcus aureus* (MRSA) infections.

347 Regarding the effect on biofilm formation, the extracts of *P. granatum* L. and *R. coriaria* L.
348 leaves produced a significant ($p<0.05$) inhibition of *L. monocytogenes* and *S. aureus* biomass
349 respect to control. Biofilm formation is still a worldwide public health concern especially in terms
350 of nosocomial infections and foodborne illness. The research of novel molecules efficacy to prevent
351 the biofilm formation is, therefore, a priority. Once again, the antibiofilm activity of *P. granatum* L.
352 and *R. coriaria* L. has been rarely investigated and the few published papers are focused on the
353 activity of different plant-material (gel, fruit and flower). One of the previous studies confirmed the
354 potential of peel extract of *P. granatum* L. on inhibition of biofilm formation and disruption of
355 preformed biofilm (Bakkiyaraj et al., 2013), and later papers reported the effect of *P. granatum* L.
356 and *R. coriaria* L. on oral biofilm in patients using fixed orthodontic appliances or orthodontic wire
357 (Vahid Dastjerdi et al., 2014a; Vahid Dastjerdi et al., 2014b). In this study, the ability of the plant
358 extracts to prevent the bacterial adherence could be related to the inhibitory effect of its flavonoidic
359 components. Apigenin has been reported as a promising natural anti-biofilm compound against
360 *Streptococcus mutans* (Koo et al., 2003) while myricetin and quercetin have been studied for their
361 inhibition of biofilm formation of *S. aureus* strains, including clinically isolated MRSA strains
362 (Arita-Morioka et al., 2015; Lee et al., 2013). Interestingly, myricetin, quercetin and quercitrin
363 exhibited strong sortase inhibitory activity (Kang et al., 2006; Liu et al., 2015), an enzyme
364 modulating the ability of the bacteria to adhere to host tissue and responsible for anchoring surface
365 protein virulence factors to the peptidoglycan cell wall layer of *S. aureus* (Mazmanian et al., 1999).
366 Generally, the direct antibacterial activity of different flavonoids may be attributable to up to three
367 mechanisms: cytoplasmic membrane damage, inhibition of nucleic acid synthesis and inhibition of
368 energy metabolism (Cushnie and Lamb, 2011). Additional evidence has also been presented for two
369 new mechanisms: inhibition of cell membrane synthesis and inhibition of cell wall synthesis by D-

370 alanine–D-alanine ligase inhibition (Wu et al., 2008). Although the mechanism behind biofilm
371 inhibition is still unclear, the reason for the observed effects could be due to multiple factors acting
372 in concert rather than alone. The primary adhesion to surfaces is a crucial event for the biofilm
373 formation and it is affected by many factors such as physic-chemical properties of surfaces, bacteria
374 characteristics and environmental components (Simoes et al., 2007). Then it is conceivable that the
375 inhibition of biofilm may be related to the ability of plant extracts/flavonoidic components to
376 inactivate microbial adhesins and enzymes leading to an alteration of the bacterial surface thereby
377 interfering and compromising the cell-substratum interactions, attachment phase and normal biofilm
378 development. The aggregatory effect of flavonols on whole bacterial cells (Cushnie et al., 2007)
379 could also determine a preferred interaction of bacterial cells between themselves rather than with
380 the surface. Further studies are required to clarify the action mechanism(s).

381 In conclusion, having more effective antimicrobial agents, with no citoxicity or DNA
382 damage and also effectives to prevent or at least interfere with biofilm formation, is a considerable
383 achievement. To the best of our knowledge, this is the first study reporting the antibiofilm activity
384 of the leaf extract of *P. granatum* L. and *R. coriaria* L.. Therefore the present findings provide
385 scientific basis to promote the value-adding of *P. granatum* L. and *R. coriaria* L. leaves as natural
386 antimicrobial/antioxidant agents for control of food-related bacterial biofilms.

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551 **Fig. 1.** Radical scavenging activity. IC_{50} with spectrophotometric DPPH test. Trolox was used as
552 positive control. Inhibition percentage (Ip) of DPPH was calculated according to the following
553 formula Ip DPPH% = $(A_B - A_A) / A_B$ x100 where A_B and A_A are the absorbance values of the blank
554 sample and of the test sample respectively, after 30 min.
555
556
557 **Fig. 2.** Effect of different subMIC doses of plant extracts on planktonic bacterial growth on
558 polystyrene microtitre plates. The optical density (OD_{492nm}) was measured using a spectrophotometer
559 EIA reader. As an indicator of bacterial vitality, 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) was
560 added. Values are expressed as means ± standard deviations.
561
562
563 **Fig. 3.** Effect of different subMIC doses of plant extracts on biofilm formation on polystyrene
564 microtitre plates. The safranin stained biofilm was resuspended in 30% acetic acid and the optical
565 density (OD_{492nm}) was measured using a spectrophotometer EIA reader. Values are expressed as
566 means ± standard deviations.
567
568 **Fig. 4.** HPTLC bioautographic assay of (a) *P. granatum* L. and (b) *R. coriaria* L. extracts: for each
569 plant, chromatograms were respectively visualized at 254 nm, sprayed with DPPH solution and
570 treated with a thin layer of *S. aureus* (10^7 CFU/ml) proper agarized medium mixed with triphenyl
571 tetrazolium chloride (TTC) solution.
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Table

Table 1
Total polyphenols of Turkish plant extracts.

Plant extract	Parts used	Total yield (g)	Total polyphenols[a]
Ficus carica L.(Incir),	leaves	2.7	3.29±0.12
Juglans regia L. (Ceviz)	leaves	4	10.50±0.38
Juglans regia L. (Ceviz)	fruit peel	3.9	4.22±0.43
Olea europaea L. (Zeytin)	leaves	2.2	8.18±0.33
Punica granatum L. (Nar)	leaves	4.3	17.63±0.43
Rhus coriaria L. (Sumac)	leaves	6.6	25.38±1.03

[a] $g_{\text{gallic acid}}$ /100 g of plant extract.

Table 2

Table 2
Antibacterial activity of Turkish plant extracts.

Plant extract	Parts used	Test	*L. monocytogenes* ATCC 7544	*S. aureus* ATCC6538P	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 9027
Ficus carica L.	leaves	Ø[a]	6	6	<6	<6
		MIC[b]	5000	5000	>5000	>5000
		MBC[c]	>5000	>5000	>5000	>5000
Junglans regia L.	leaves	Ø	10	10	6	6
		MIC	2500	2500	5000	5000
		MBC	>5000	>5000	>5000	>5000
Junglans regia L.	fruit peel	Ø	7	6	<6	<6
		MIC	5000	5000	>5000	>5000
		MBC	>5000	>5000	>5000	>5000
Olea europaea L.	leaves	Ø	12	12	6	6
		MIC	625	625	1250	5000
		MBC	>5000	2500	>5000	>5000
Punica granatum L.	leaves	Ø	12	19	8	10
		MIC	625	156	625	1250
		MBC	>5000	2500	>5000	>5000
Rhus coriaria L.	leaves	Ø	21	20	9	18
		MIC	78	78	312	312
		MBC	>5000	625	>5000	>5000

[a]Ø, Disc diffusion test, inhibition diameters in mm.
[b]MIC Minimum inhibitory concentration expressed in μg/ml.
[c]MBC Minimum bactericidal concentration expressed in μg/ml.

Table

Table 3
Identification of phenolic compounds in *P. granatum* L. and *R. coriaria* L. active fractions.

	$[M-H]^-$ (m/z)	MS/MS (m/z)	λ_{max}	Compound
Punica granatum L. $R_f=0.7$	431	269	267	Apigenin 4'-O-β-glucoside
Rhus coriaria L. $R_f=0.5$	463	316	263	Myricitrin[a]
	447	301	263	Quercitrin[a]

[a]Verified against pure standard

Table

Table 4

SOS Chromotest results showing the lack of genotoxic activity (IF<1.5) of *P. granatum* L. and *R. coriaria* L. extracts compared to a known mutagen 4NQO; and the antigenotoxic activity of the same extracts expressed as percentage of inhibition of genotoxicity induced by 4NQO

		Genotoxic activity	Antigenotoxic activity
	Dose (μg/ml)	Induction Factor (IF)	Inhibition of genotoxicity (%)
4NQO[a]	2.5	2.6	0
NC[b]	0	1.0	0
Punica granatum L.	0.06	1.0	44.510
	0.3	0.7	58.097
	0.6	0.9	51.046
	3	1.1	52.460
	6	1.2	38.729
	30	/[c]	/[c]
	60	/[c]	/[c]
Rhus coriaria L.	0.06	0.9	73.224
	0.3	0.8	77.059
	0.6	0.9	77.007
	3	0.9	77.543
	6	1.4	76.268
	30	0.8	78.359
	60	1.1	83.096

[a] 4NQO, 4-nitroquinoline-1-oxide, positive control of genotoxicity.
[b] NC, negative control (non-treated cells).
[c] The extract exhibited citotoxicity at this concentration.

Figure 1

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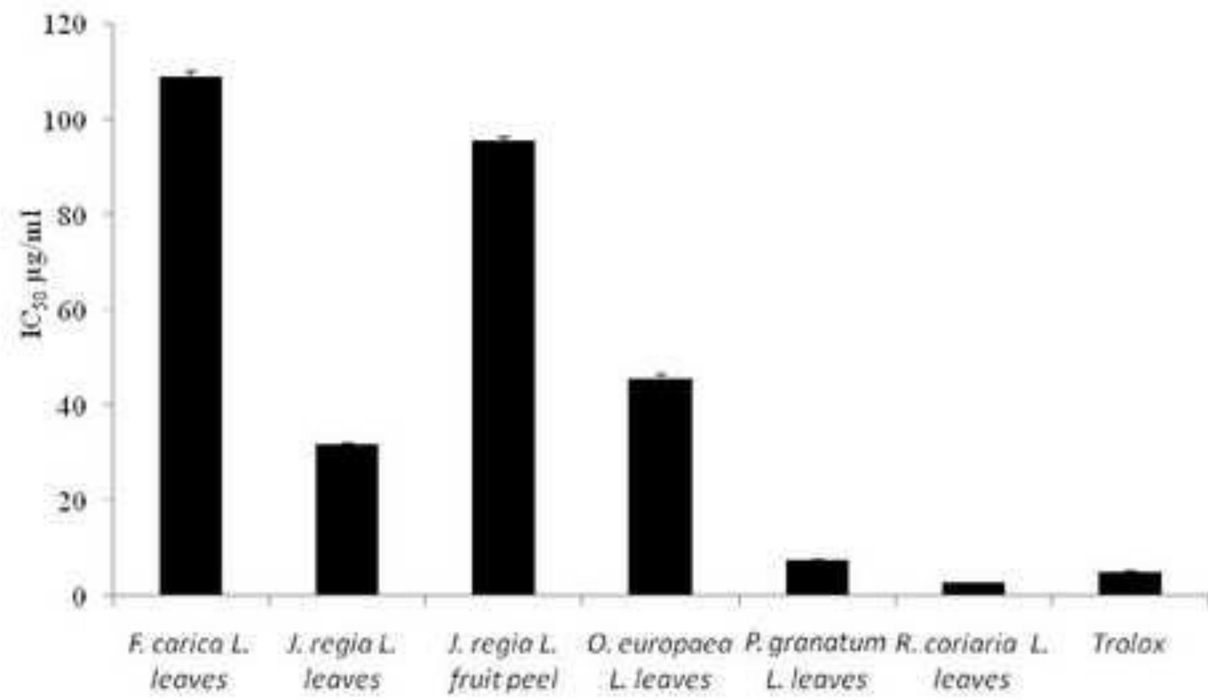

Figure 2

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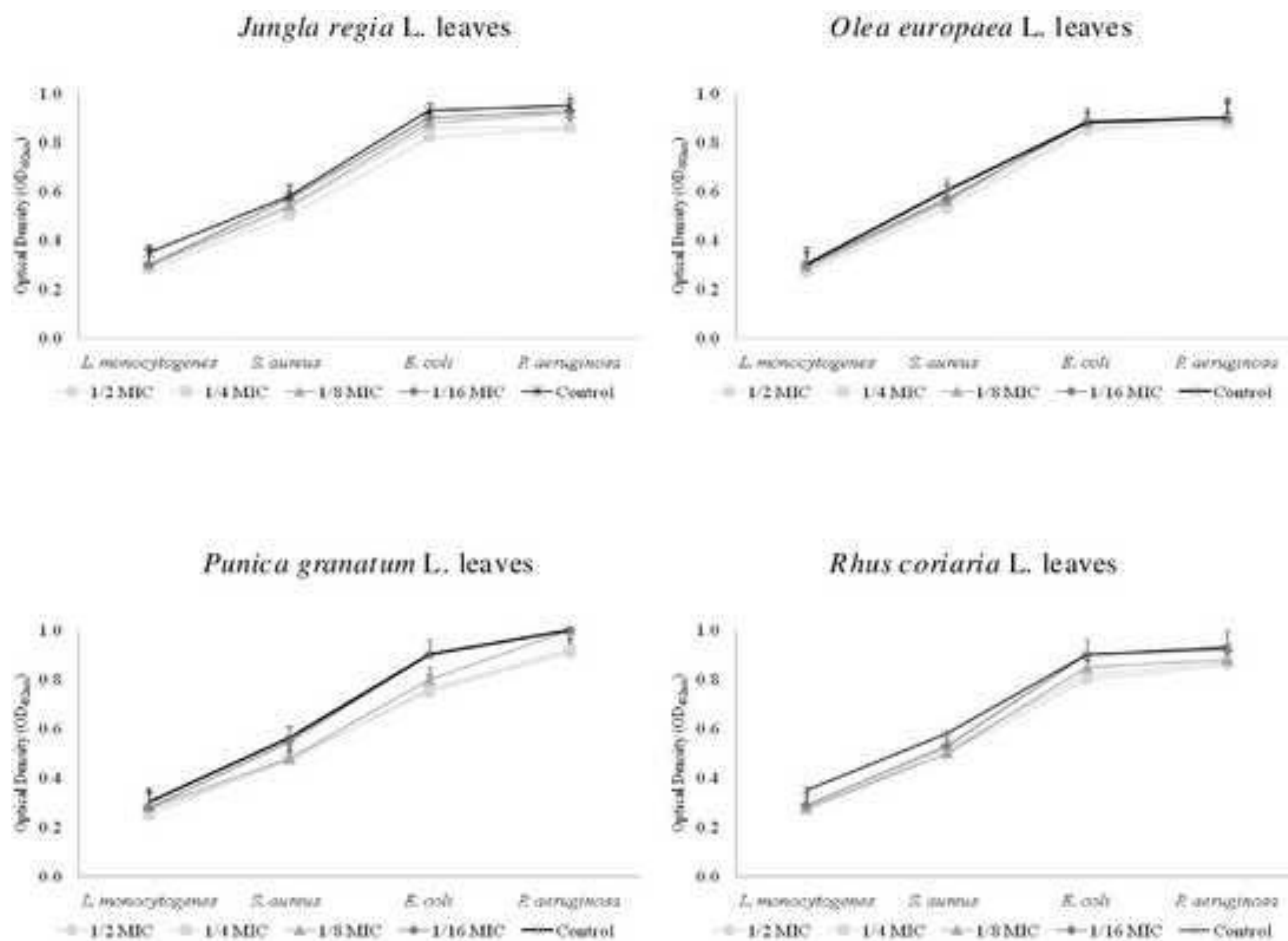

Figure 3
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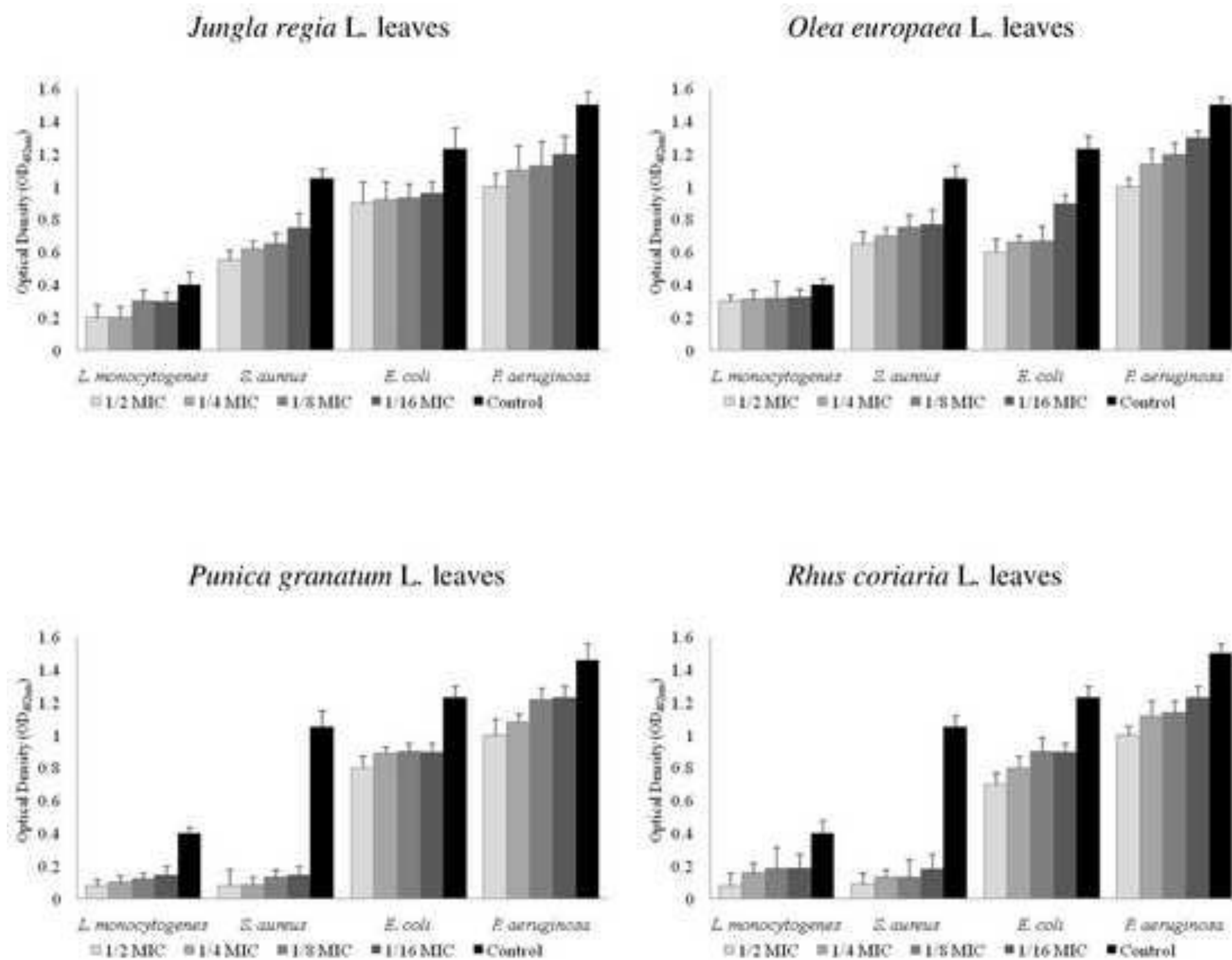

Figure 4

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