

IMPACT OF DIFFERENT AMARANTHACEAE EXTRACTS ON THE BIOFILM-FORMING CAPACITY OF SEVERAL BACTERIAL PATHOGENS

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ABSTRACT

Amaranthaceae Juss. family encompasses many edible plants with prominent biological activity. This investigation tested the bioactive properties of ethanolic and methanolic extract of three well-known species: spinach (*Spinacia oleracea* L.), chard (*Beta vulgaris* L. subsp. *vulgaris*), and orache (*Atriplex hortensis* L.) through the determination of total phenolic and flavonoid content, antioxidant activity, and antibacterial properties. The particular goal was to evaluate the antibiofilm potential of extracts and to demarcate concentration-dependent changes in the biofilm-forming category of included bacterial strains. The mass of the chard and orache methanolic extracts gained by maceration are lower in comparison to the mass of ethanolic extracts obtained by the Soxhlet method. In the case of spinach, the results are the opposite. All extracts have an antiradical activity that can be attributed to the established amounts of phenols and flavonoids. Total phenolics in dry leaves ranged from 0.09 to 0.44 mg GAE/g dw, and total flavonoids from 0.42 to 1.9 mg RTE/g dw. All investigated extracts performed inhibitory potential in terms of bacterial growth, while there was no bactericidal effect observed. Values of the minimum inhibitory concentration ranged from 125 µg/ml to 500 µg/ml. Overall results suggested orache extracts as the strongest inhibitory agents. Antibiofilm assays showed that examined extracts of spinach, chard, and orache caused changes in the biofilm-forming capacity of investigated bacterial pathogens. Fluctuations in observed biofilm-forming categories after application of extracts were concentration-dependent.

Keywords: Amaranthaceae extracts, phenolic composition, antioxidant activity, minimum inhibitory concentration, antibiofilm activity

INTRODUCTION

One of the major global health challenges of the 21st century is antimicrobial resistance (Hernando-Amado *et al.*, 2019). In the search for novel antimicrobial agents, plants and their secondary metabolites play a very important role. Specific phytochemicals are capable of inhibiting the quorum-sensing process of bacteria and therefore could be used for fighting infections caused by biofilms (Bouyahya *et al.*, 2017). Since bacterial biofilms are involved in about 80% of infections (Paluch *et al.*, 2020) the need for new antibacterial agents of natural origin is emphasized. Family Amaranthaceae Juss. comprise around 165 genera and 2040 plant species of flowering plants (Christenhusz & Byng, 2016) that are widespread from tropical areas to cool temperate regions. Amaranthaceae species are characterized by the occurrence of different phytochemical compounds with bioactive properties (Müller & Borsch, 2005), but antibacterial activity, in particular, could be attributed to the presence of different flavonol sulfates (Chassagne *et al.*, 2021). Spinach (*Spinacia oleracea* L.) is rich in different bioactive molecules and therefore known as an antioxidant, anti-inflammatory, antitumor, antimutagenic, antineoplastic, chemopreventive, anti-obesity, and lipid-lowering agent (Adapa *et al.*, 2018; Olasupo *et al.*, 2018; Ribera *et al.*, 2020). Chard (*Beta vulgaris* L. subsp. *vulgaris*) is a popular edible plant that contains significant concentrations of valuable nutrients (Sacan & Yanardag, 2010). Phytochemical screening revealed many other compounds that can be associated with biological activities like antioxidant, anti-acetylcholinesterase, anti-diabetic, anti-inflammatory, antitumor, and hepatoprotective effects (Mzoughi *et al.*, 2019). *Atriplex hortensis* L., orache is a halophytic plant used as a vegetable as well as a curative agent in folk medicine (Bylka *et al.*, 2001). *Atriplex* species typically inhabit dry environments with extreme saline concentrations in the soil, and hence they are documented for different pharmacological activities (Zohra *et al.*, 2019). Considering that all investigated species are well known in the human diet and recognized as beneficial plants in terms of traditional medicine, this multidisciplinary investigation intends to reveal new insights into the bioactive properties of examined plants. The study aims to

determine the impact of ethanolic and methanolic extract of the three selected Amaranthaceae species on the biofilm-forming capacity of different bacterial pathogens, as well as to demarcate concentration-dependent changes in the biofilm-forming category of investigated bacteria. Since there is a strong connection between the antimicrobial properties of plant preparations and their chemical properties regarding the secondary metabolites, an evaluation of the total phenolic and flavonoid content and antioxidant activity of extracts was performed. Considering all the aspects of this research, as well as the different methods involved in the experiment, the scope of the study encompasses fundamental microbiology, biotechnology, food chemistry, and phytotherapy.

MATERIAL AND METHODS

Samples

Spinach, chard, and orache samples were gathered in the Tuzla region (Bosnia and Herzegovina) from field parts exposed to sunlight, in September 2020. After washing, samples were cut into thin strips and dried in the shade (30 °C) for a week. Dried samples were crushed, and homogenized.

Determination of antioxidative activity and total phenolic composition

Reagents

The materials used for this analysis are: ethanol and methanol; DPPH; Sodium carbonate; Aluminium chloride (Sigma Aldrich); Acetic acid, 99-100%; Formic acid; Sodium Hydroxide; Sodium Nitrite; Rutin; Gallic acid; Folin-Ciocalteu reagent, FCR (Merck, Germany). All other used reagents were of high analytical grade.

Extraction

Maceration and Soxhlet methods were used to extract each (4 g) sample of spinach, chard, and orache. Methanol was used for maceration (80 mL). After soaking and shaking, the mixture was at room temperature for 24 hours with continual stirring. The mixture was filtered through qualitative filter paper (Whatman, Grade 1) to eliminate coarse particles after soaking and stirring. Ethanol was utilized for the Soxhlet method. Samples were crushed in a pestle and mortar. A thimble (Cellulose, 43 x 123 mm), was placed in the center of the Soxhlet device with the crushed sample. The extraction with ethanol was appropriated 24 hours. The methanolic and ethanolic extracts were concentrated using a rotating vacuum evaporator after filtering. The obtained extracts were stored at 4-6 °C in the dark.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assays

According to Brand-Williams et al. (1995) and Sánchez-Moreno et al. (1998) the stable DPPH radical scavenging effect of spinach, chard, and orache extracts was measured. An aliquot of an ethanol solution containing different sample concentrations was added to 1.90 mL 130 mM solution DPPH in ethanol. The absorbance at 517 nm was measured at different time intervals until the reaction reached the steady state. The RSC value (Radical Scavenging Capacity) is calculated using Eq 1 to describe the antioxidant activity obtained by the DPPH method:

$$RSC(\%) = 100 - \left[(A_S - A_B) \cdot \frac{100}{A_C} \right]$$

A_S - absorbance of the sample at 517 nm

A_B - absorbance "blank" at 517 nm

A_C - absorbance "control" at 517 nm

Determination of total phenolic content (TPC)

The total phenolic content of the samples was determined using a modified Folin-Ciocalteu method (Singleton et al., 1990). Samples (10 ml) were introduced into test tubes, and then 1.3 ml 0.2 M Folin-Ciocalteu's reagent and 3.75 ml sodium carbonate (20%) were added. The absorbance of all samples was measured at 740 nm after incubating at 23 °C for 2 h. For three replications, all results were presented as mean (microgram gallic acid equivalents per gram of dry samples) ± STDEV.

Determination of total flavonoid content (TFC)

The total flavonoid content was determined using aluminum chloride ($AlCl_3$) by a well-known method (Ordonez et al., 2006; Lin & Tang, 2007) and rutin as standard. 0.2 mL of the plant extracts, 0.3 mL of a 5% $NaNO_2$ solution, 3.4 mL and 0.15 mL of 0.5 mL of a 10% $AlCl_3$ solution were added to 25 mL flask and was properly mixed. After 6 minutes, 2 mL of 1 M NaOH was added. To make the final volume of 25 mL, water was added. Absorption was measured immediately on the blank at 514 nm with a UV-1280 spectrophotometer (Shimadzu, Japan) in comparison to standards prepared similarly with known rutin concentrations. For three replications, the results are expressed as mean (micrograms of rutin equivalents per gram of dry samples) ± STDEV.

Antimicrobial activity

Bacterial species

Antibacterial and antibiofilm activity of tested extracts was evaluated using a total of 11 bacteria including the clinically important multidrug-resistant strains, as follows: *Staphylococcus aureus* ATCC 6538 (SA1); *S. aureus* ATCC 25923 (SA2); methicillin-resistant *S. aureus* (MRSA): *S. aureus* ATCC 33591 (SA3), and *S. aureus* NCTC 12493 (SA4); *Enterococcus faecalis* ATCC 29212 (EF); *Bacillus subtilis* ATCC 6633 (BS); *Escherichia coli* ATCC 14169 (EC1); *E. coli* ATCC 25922 (EC2); extended-spectrum beta-lactamase-producing (ESBL) *E. coli* ATCC 35218 (EC3); *Salmonella enterica* NCTC 6017 (SE); *Pseudomonas aeruginosa* ATCC 27853 (PA). Investigated bacteria were obtained from the American Type Culture Collection (ATCC) and National Collection of Type Cultures (NCTC) (MicroBioLogics, St. Cloud, Minnesota, USA). Inoculums were prepared according to EUCAST (2017). Overnight cultures were dissolved in sterile saline solution to the final turbidity of 0.5 McFarland standard, that is bacterial cell concentration of 1.5×10^8 CFU/ml.

Determination of minimum inhibitory and minimum bactericidal concentration

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). The minimum inhibitory concentration (MIC) of the extracts was determined using the broth microdilution method (CLSI, 2018). Pure extracts were dissolved in Dimethyl Sulfoxide, DMSO (Sigma-Aldrich) to the final concentration of 1000 µg/ml. The final volume of 100 µl of two-fold dilutions of extracts ranging from 1000 to 1.95 µg/ml, was applied in a

96 well microtiter plate containing the Mueller Hinton broth (Sigma-Aldrich). Then, 10 µl of bacterial inoculum was added to each well. As the positive control, pure bacterial culture was taken, while uninoculated media was used as the negative control. Results were read after overnight incubation, on a microplate reader (Biochrom EZ Read 400) at 595 nm. Experiments were performed in quadruplets. Minimum bactericidal concentration (MBC) was evaluated by replating the bacteria on sterile Mueller Hinton medium and observing the presence of growth after overnight incubation.

Evaluation of antibiofilm activity

The tissue culture plate method (TCP) in 96 well plates (Merritt et al., 2005) was used for the determination of biofilm formation in the presence of investigated extracts. Tryptic Soy Broth, TSB (Sigma-Aldrich) was used as the diluting medium. The initial concentration of tested extracts of 1000 µg/ml was two-fold diluted in TSB to an end concentration of 1.95 µg/ml, with the final volume of 100 µl per well. After that, each well was inoculated by 10 µl of the investigated bacterial strain. Inoculums were prepared as described above. As the negative control, uninoculated media was used.

The biofilm formation was determined through the adherence of tested bacterial strains only in the presence of TSB. The content of the plates was decanted after overnight incubation, washed in Phosphate Buffered Saline, PBS (Sigma-Aldrich, USA), and stained with 0.1% crystal violet solution for 10 minutes. Upon washing, 96% ethanol was added to each well. Results were read on the microplate reader (Biochrom EZ Read 400) at 595 nm. The experiment was performed in quadruplet, and results are given as mean value ± STDEV. Determination of the biofilm-forming category (Stepanović et al., 2007) was completed using the Biofilm Classifier Software ver 1.1.

The optical density cut-off value (ODc) was calculated as three standard deviations above the mean OD of the negative control, while the biofilm categories were determined as follows: $OD \leq ODc$: non-adherent (NA), $ODc < OD \leq 2 \times ODc$: weakly adherent (W), $2 \times ODc < OD \leq 4 \times ODc$: moderately adherent (M), and $4 \times ODc < OD$: strongly adherent (S). According to Jadhav et al. (2013) the percentage of biofilm inhibition was calculated.

RESULTS AND DISCUSSION

Yield of extracts

To get insight into the extraction capability and the effect of varied conditions under which the extraction was carried out, Soxhlet and maceration extraction methods were utilized. The mass fractions of dry leaves extract of selected Amaranthaceae plants are shown in the table 1.

Table 1 Mass fractions of dry leaves extract in selected plants of the Amaranthaceae family

Samples	Type of solvent	Extract mass (mg/g dry sample)
Spinach	EtOH	208.38±2.43
	MeOH	223.11±1.76
Chard	EtOH	158.57±2.93
	MeOH	157.25±1.99
Orache	EtOH	168.07±2.39
	MeOH	143.72±1.97

Data are shown as means with ± STDEV of three replicates. EtOH: ethanol. MeOH: methanol.

The mass fractions of chard and orache methanolic extracts obtained by maceration are lower than the mass fractions of ethanolic extracts obtained by the Soxhlet method. While with spinach the opposite is true (Tab 1). In terms of obtained yield of investigated extracts, the maceration and Soxhlet methods differ in that the mass fractions of chard and orache methanolic extracts obtained by maceration are lower than the mass fractions of ethanolic extracts obtained by the Soxhlet method. In the case of spinach, however, the reverse is true. Previous investigations showed that the utilization of different extraction techniques and solvents might result in changes in mass (Handa et al., 2008).

Antioxidant Activity

The DPPH method was used to assess the antioxidant activity of obtained extracts from dry leaves of Amaranthaceae plants. The resultant redox interaction between the extracts of the sample and the free DPPH radical was followed by a shift in absorption to 517 nm, i.e., the total free radical scavenger capacity (RSC) was calculated spectrophotometrically by detecting the radical DPPH• disappearance. Table 2 shows the RSC findings for examined samples. The IC_{50} value is another way to express a sample's antioxidant capability (half maximal inhibitory concentration). The extract concentration necessary to react with 50% DPPH radicals ($RSC=50\%$) under previously described experimental conditions has been determined as the IC_{50} value (mg/mL). The equation from curves that explain the relationship between RSC percent and concentration was used to compute IC_{50} .

Table 2 Antioxidant activity values of tested samples

Samples	Spinach		Chard		Orache		Ascorbic acid	
	Conc.(mg/mL)	EtOH	MeOH	EtOH	MeOH	EtOH		MeOH
RSC/%	0.05	2.21	6.02	3.81	2.70	8.23	6.02	23.71
	0.10	2.82	7.61	6.39	3.19	8.84	7.25	31.94
	0.20	6.52	8.48	7.74	6.64	10.82	9.10	44.35
	0.40	7.74	10.32	10.32	7.99	12.77	12.53	56.14
	0.60	10.44	11.91	11.55	9.95	13.26	13.14	66.09
	0.80	12.04	12.54	13.03	10.57	16.59	16.83	70.76
	1.00	13.02	13.14	14.62	14.13	16.70	16.95	83.17
R² value on a graph		0.9289	0.9451	0.9371	0.9448	0.9525	0.9499	0.9521
IC₅₀		6.03	5.23	4.40	4.30	4.57	3.71	0.39

Table 2 shows the R-squared value from the graph and the IC₅₀ concentrations for investigated samples. Based on the IC₅₀ values, it is clear that all tested samples exhibited anti-radical activity, with orache ethanolic extract having the highest antioxidant capacity and spinach methanolic extract having the lowest. All samples showed concentration dependency with respect to the expressed activity. Methanolic extracts of spinach, chard, and orache have higher antioxidant activity than ethanolic extracts, according to IC₅₀ values (Tab 2). As a result of the previous, we may deduce that the type solvent and extraction procedure have an impact on antioxidant capacity. The ability of tested samples to neutralize DPPH radicals was compared with the ability of ascorbic acid as a standard, i.e., the obtained IC₅₀ values of samples were compared to the obtained IC₅₀ value of ascorbic acid, which is used as a commercial antioxidant (Fig 1).

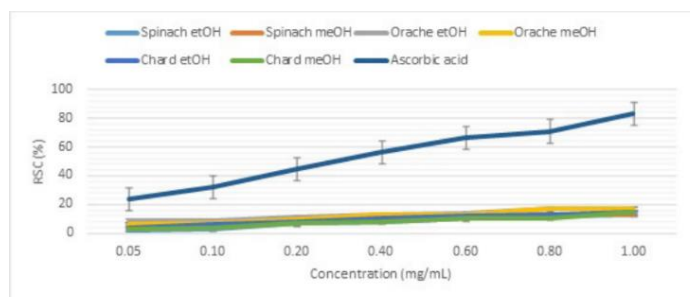


Figure 1 Graphic representation of the dependence of DPPH radical inhibition on the concentration of Amaranthaceae extracts compared to ascorbic acid

The concentrations of extracts which are required to neutralize 50% of DPPH radicals are much higher than the required concentration of ascorbic acid. For the evaluation of the antioxidant activity of investigated extracts, DPPH method was used, and this approach is useful because of its simplicity and quickness, as well as the ease with which it may be carried out (Huang et al., 2005). Results regarding the antioxidant activity of tested Amaranthaceae species revealed that all extracts possess anti-radical activity. Earlier study (Hatamjafari & Tazarv, 2013) found that the capacity to eliminate free DPPH radicals from dried spinach leaves ranged from 6.62 to 12.65%, which supports our results. However, there is found that antioxidant activity varies depending on the storage method (Galani et al., 2017). Fresh leaves spinach had a DPPH value of around 100 mg TE/100 g fw before storage, and about 80 mg TE/100 g fw after storage (15 days, at 4°C). According to recent investigation (Uslu et al., 2020) the DPPH values of dried orache leaves ranged from 18-95-65.64%, which is consistent with our reports. While for leaves of chard there is not enough information on DPPH values.

Table 4 The minimum inhibitory concentration of tested Amaranthaceae extracts (µg/ml)

Bacterial strain	Spinach		Chard		Orache	
	EtOH	MeOH	EtOH	MeOH	EtOH	MeOH
<i>Staphylococcus aureus</i> ATCC 6538	250	250	250	250	250	250
<i>S. aureus</i> ATCC 25923	500	500	250	250	250	250
<i>S. aureus</i> ATCC 33591 (MRSA)	250	250	250	250	250	250
<i>S. aureus</i> NCTC 12493 (MRSA)	250	250	250	250	250	250
<i>Enterococcus faecalis</i> ATCC 29212	500	500	250	250	250	250
<i>Bacillus subtilis</i> ATCC 6633	125	125	125	125	125	125
<i>Escherichia coli</i> ATCC 14169	250	125	125	250	125	125
<i>E. coli</i> ATCC 25922	250	250	125	125	125	125
<i>E. coli</i> ATCC 35218 (ESBL)	125	125	125	125	125	125
<i>Salmonella enterica</i> NCTC 6017	250	250	250	250	125	125
<i>Pseudomonas aeruginosa</i> ATCC 27853	125	125	125	125	125	125

Determined values of the minimum inhibitory concentration of the tested extracts ranged from 125 to 500 µg/ml. Spinach extracts showed the highest MIC value, which can be noticed in the case of SA2 and EF. MIC values of chard extracts

Total phenolic (TP) and flavonoid content (TFC)

The extraction yield of phenolics (mg GAE/g dw) and flavonoids (mg RTE/g dw) contents in dry leaves in the investigated Amaranthaceae plants are presented in the table 3. In dry leaf samples, total phenolics ranged from 0.09 to 0.44 mg GAE/g dw, while total flavonoids ranged from 0.42 to 1.9 mg RTE/g dw. The methanolic extracts of spinach and orache prepared by maceration contained more phenol and flavonoids than the ethanolic extracts, however, the methanolic extract of chard contained less phenol than the ethanolic extract (Tab 3).

Examination of total phenolic and flavonoid content showed that methanolic extracts mainly contain higher amounts of these chemical constituents. Total phenol and flavonoids values of spinach leaves varied between 29 and 915 mg GAE/100g and 838 and 1696 mg RTE/g depending on genotypes at three maturity stages (Pandjaitan et al., 2005). While certain researchers (Fan et al., 2011; Galani et al., 2017) published data for phenols and flavonoids in spinach leaves that are comparable to ours, there is a found of an average of 500 mg GAE/100g polyphenols in field-grown orache leaves and an average of 200 mg GAE/100g in greenhouse-grown (Uslu et al., 2020). While flavonoids were 2.52-4.35 mg Rutin/100g in field-grown orache leaves, and 0.75-2.04 mg Rutin/100g in greenhouse-grown. A previous study (Pyo et al., 2004) noted that chard had total phenols in the range of 290-1320 mg/100g fw and flavonoids in the range of 0.9-25.6 mg/100g fw. This is the first study to show the presence of both phenolic acids and flavonoids as antioxidant compounds in the chard methanolic extract.

Table 3 Total phenolics and flavonoids contents of the samples examined

Samples		Total phenolics	Total flavonoids
		(mg GAE/g dw)	(mg RTE/g dw)
Spinach	EtOH	0.09	0.96
	MeOH	0.15	1.90
Chard	EtOH	0.44	0.99
	MeOH	0.15	1.36
Orache	EtOH	0.27	0.42
	MeOH	0.33	0.61

Broth microdilution method

Obtained values of the minimum inhibitory concentration of ethanolic and methanolic extract of Amaranthaceae species against the tested Gram-positive and Gram-negative bacterial strains are presented in the table 4.

ranged from 125 to 250 µg/ml. Orache extracts showed balanced antibacterial activity, with the same MIC values for all bacterial strains. Overall results suggested orache extracts as the strongest inhibitory agents (Tab 4). After replating

the bacteria, and exposure to all tested concentrations of the examined extracts, there was no minimum bactericidal concentration.

Determination of biofilm-forming categories of investigated bacteria

Results regarding the biofilm-forming capacity alteration in presence of different concentrations of extract compared to the positive control, where no extract was added, were tested at the MIC value and at least five consecutive 50% dilutions of the extract below the MIC (subinhibitory concentrations) for the tested bacterial strain. The positive controls which were classified as strong biofilm formers included: SA1, SA4, EC3, and PA according to the previously mentioned method. SA2, SA3, BS, and EC1 expressed moderate biofilm-forming capacity, while EF and SE exhibited weak biofilm formation. EC2 used in this study did not show any biofilm-forming potential, since all performed replications suggested the non-adherent biofilm category for this strain. Results are presented in the figure 2.

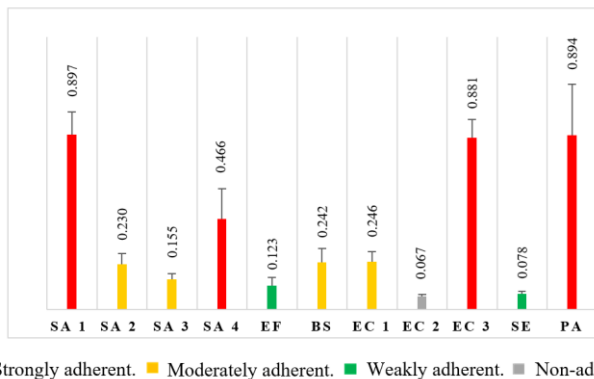


Figure 2 Observed biofilm-forming categories for tested bacterial strains according to the mean absorbance values (± STDEV). SA1: *Staphylococcus aureus* ATCC 6538; SA2: *S. aureus* ATCC 25923; SA3: *S. aureus* ATCC 33591 (MRSA); SA4: *S. aureus* NCTC 12493 (MRSA); EF: *Enterococcus faecalis* ATCC 29212; BS: *Bacillus subtilis* ATCC 6633; EC1: *Escherichia coli* ATCC 14169; EC2: *E. coli* ATCC 25922; EC3: *E. coli* ATCC 35218 (ESBL); SE: *Salmonella enterica* NCTC 6017; PA: *Pseudomonas aeruginosa* ATCC 27853.

Antibiofilm activity of tested Amaranthaceae extracts

Ethanollic and methanolic extracts of spinach, chard, and orache performed the ability to modify the biofilm-forming capacity of tested bacteria, through the change of biofilm-forming category in comparison to the positive control.

Spinach

The ethanolic extract at the concentration of 125 µg/ml caused 56.73% inhibition of SA1 biofilm, which is noted through the reduction of the biofilm-forming category from strongly- to moderately adherent. Biofilm formation of MRSA strain SA3 remained strong when treated with ethanolic extract, but methanolic extract decrease its biofilm-forming capacity in all subinhibitory concentrations at the amount of 3.18-17.00%. SE was sensitive to both extracts, and moderately-adherent biofilm was decreased to weakly adherent by all subinhibitory concentrations of ethanolic extract (7.55-30.94% inhibition), as well as destroyed by all dilution of methanolic extract (6.63-65.28% inhibition). PA biofilm-forming capacity was decreased to moderately adherent at 62.50 µg/ml of both extracts, while with lower dilutions biofilm remained strong. Inhibition of PA biofilm was 79.04% and 80.41% with ethanolic and methanolic extract respectfully (Fig 3). Strong biofilm formers including ESBL strain (EC3) and SA4 (MRSA) were completely resistant to spinach extracts in terms of antibiofilm activity, as well as the SA2, EF, BS, EC1, and EC2 strains.

Chard

Chard extracts led to the decrease in the biofilm-forming capacity of SA2, starting from 3.90 µg/ml of ethanolic (31.75% inhibition), and 15.63 µg/ml of methanolic extract (41.07% inhibition). Moderately adherent BS biofilm was reduced to weakly adherent by ethanolic extract in the range of 62.50-15.63 µg/ml, and the maximum inhibition was 14.53%. A concentration of 62.50 µg/ml of both extracts inhibited EC1 biofilm formation in the amount of 53.44% and 46.98% of ethanolic and methanolic extract respectfully. The same dilution led to a change of strongly adherent EC3 (ESBL) biofilm to weakly- and moderately adherent with inhibition of 72.61% (ethanolic extract) and 62.54% (methanolic extract), but the difference was observed with lower concentrations where the ethanolic extract was performed activity up to the last dilution, while methanolic extract acts as inhibitory agent only at 62.50 µg/ml. At that threshold, both extracts decreased strong PA biofilm to weakly- or moderately adherent, with inhibition of 23.72% (ethanolic extract) and 78.08% (methanolic extract). Although there was certain inhibition observed after application of the extracts, SE biofilm remained weakly adherent, except in

the case of 125 µg/ml of ethanolic extract where it was destroyed (22.07% inhibition). There was no antibiofilm activity of chard extracts against SA1, SA3 (MRSA), SA4 (MRSA), EF, and EC2 strains (Fig 4).

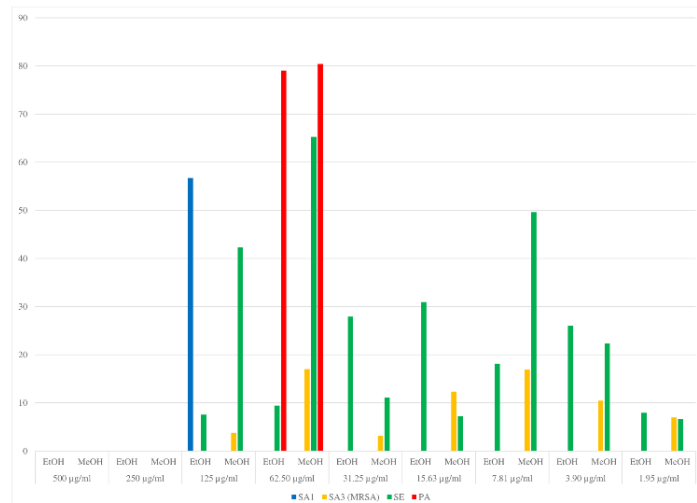


Figure 3 Bacterial strains where spinach extracts performed antibiofilm activity sufficient to change the biofilm-forming category. SA1: *Staphylococcus aureus* ATCC 6538; SA3: *S. aureus* ATCC 33591 (MRSA); SE: *Salmonella enterica* NCTC 6017; PA: *Pseudomonas aeruginosa* ATCC 27853. (MIC values of spinach extracts: SA1, SA3, and SE=250 µg/ml; PA=125 µg/ml).

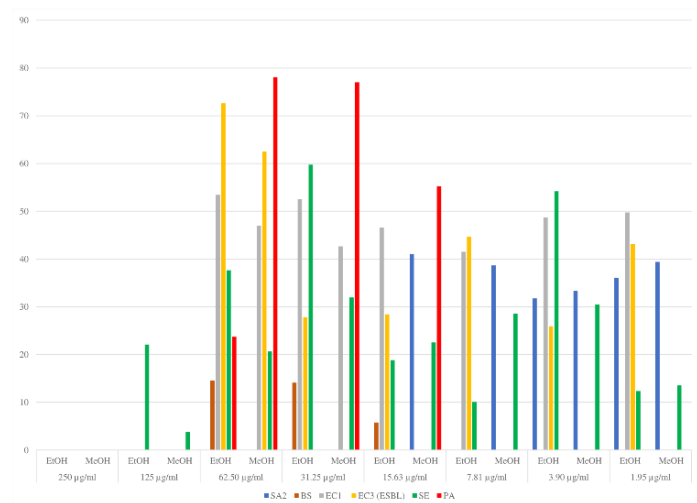


Figure 4 Antibiofilm activity of chard extracts on bacteria with changed biofilm-forming category. SA2: *S. aureus* ATCC 25923; BS: *Bacillus subtilis* ATCC 6633; EC1: *Escherichia coli* ATCC 14169; EC3: *E. coli* ATCC 35218 (ESBL); SE: *Salmonella enterica* NCTC 6017; PA: *Pseudomonas aeruginosa* ATCC 27853. (MIC values of chard extract: SA2, SE=250 µg/ml; BS, EC3, PA=125 µg/ml; EC1: EtOH=125, MeOH=250 µg/ml).

Orache

Ethanolic extract weakened the SA3 (MRSA) biofilm at 125 µg/ml (13.40% inhibition), and at 62.50 µg/ml (10.60% inhibition). Moderately adherent BS biofilm became weakly adherent in the range of 15.63-1.95 µg/ml of ethanolic extract, with inhibition of 5.33-24.19%. Furthermore, ethanolic extract decreased EC1 biofilm in all subinhibitory concentrations, with inhibition of 39.05-57.45%, while methanolic extract acted like this only at 62.50-31.25 µg/ml and achieved maximum inhibition of 57.08%.

EC3 (ESBL) biofilm became weakly- and moderately adherent at 62.50 µg/ml of both extracts with 75.36% inhibition achieved by ethanolic and 86.94% by the methanolic extract. PA biofilm was reduced to moderately adherent at 62.50 µg/ml and 31.25 µg/ml of ethanolic extract, with the highest inhibition of 73.24% (Fig 5). Chard extracts did not perform antibiofilm activity against SA1, SA2, SA4 (MRSA), EF, EC2, and SE biofilms.

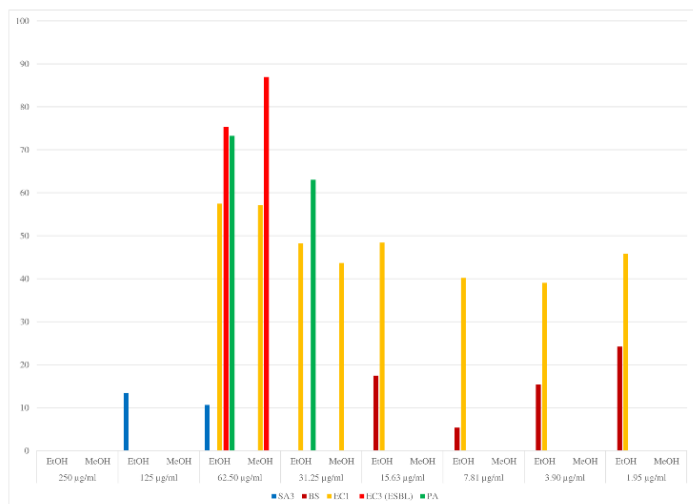


Figure 5 Bacterial strains with reduced biofilm-forming category due to the activity of orache extracts. SA3: *S. aureus* ATCC 33591 (MRSA); BS: *Bacillus subtilis* ATCC 6633; EC1: *Escherichia coli* ATCC 14169; EC3: *E. coli* ATCC 35218 (ESBL); PA: *Pseudomonas aeruginosa* ATCC 27853. (MIC values of orache extracts: SA3=250 µg/ml; BS, EC1, EC3, PA=125 µg/ml).

The antimicrobial activity of various spinach preparations was investigated in previous studies (Nasim et al., 2012; Akhtar et al., 2016; Alnashi et al., 2016; Altemimi et al., 2017; Issazadeh et al., 2017; Adapa et al., 2018; Olasupo et al., 2018; Shafique et al., 2021) and proven against many bacteria. The presence of flavonoids and terpenes, unsaturated fatty acids, specific cys-rich peptides known as defensins, and inorganic materials in spinach extracts can be related to the antimicrobial effects. In this study, spinach extracts performed antibacterial activity against all the tested bacterial strains. The antibacterial potential is illustrated through the inhibitory action, while bactericidal activity was not recorded. Previously reported findings regarding the chemical investigation and bioactive potential of chard (Sacan & Yanardag, 2010; Mzoughi et al., 2019) can be related to the antibacterial properties of its extracts registered in this study. According to the available literature, results considering the antimicrobial properties of orache extracts are scarce. Our results suggest the inhibitory potential of orache extracts towards bacterial growth. All investigated bacteria were susceptible to orache extracts, but there were no bactericidal effects. Earlier chemical investigations revealed flavonoids, saponins, and alkaloids as the main secondary metabolites of *Atriplex* species (Stanković et al., 2019), which can also be involved in antibacterial activity. Examined Amaranthaceae extracts have shown the ability to change the biofilm-forming capacity of investigated bacteria, including the multidrug-resistant strains through the concentration-dependant activity.

CONCLUSION

This investigation showed that tested extracts of Amaranthaceae plants possess bioactive properties. Detected antibacterial and antibiofilm activity can be attributed to the secondary metabolites proven in examined extracts. Evaluation of total phenolic and flavonoid content of extracts, in general, suggested higher amounts of these constituents in methanolic extracts. Furthermore, according to available data, this represents the first report of both phenolic acids and flavonoids as the antioxidant compounds in the chard methanolic extracts. All investigated extracts possess anti-radical activity whose expression was concentration-dependent. The study encompassed several bacterial pathogens, including the multidrug-resistant strains that are increasingly difficult to treat with synthetic antibiotics. Results suggest that Amaranthaceae extracts possess inhibitory potential against bacteria, and they as well act as the antibiofilm agents in particular concentrations. Obtained MIC values were between 125 and 500 µg/ml, and orache extracts were the strongest inhibitory agents. Nevertheless, the bactericidal properties of tested extracts were not detected, but at the same time, these samples caused alterations in the biofilm-forming capacity of tested bacteria. These findings can be important in light of growing antibiotic resistance and particularly in terms of biofilm formation that is associated with the majority of infections. Considering the fact that specific dilutions of the extracts caused increasing in the biofilm-forming capacity of some bacterial species, further investigations are needed to demarcate the impact of concentration and nature of the solvent on QS-signalling pathways of bacterial pathogens.

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