Original research article

Antioxidant and antimicrobial activity of extracts from selected Mediterranean agro-food by-products, their mutual interaction and interaction with phenolic compounds

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Abstract

The by-products of Mediterranean agriculture that remain after the production of fruit wines, juices and extracts represent a large source of raw materials rich in phenolic compounds. As a contribution to the current state of knowledge, this study investigated the biological activity and phenolic composition of extracts obtained from blackberry, chokeberry and juniper by-products, as well as the interactions of the extracts in mixtures. The total phenolic content of the samples ranged from 44.42 to 250.28 mg gallic acid equivalents (GAE)/g dry extract, with the extracts from blackberry leaves and juniper needles being the highest. The highest concentration of phenolics detected by high performance liquid chromatography (HPLC) was also found in blackberry leaves (41.30 mg/g dry extract), with rutin dominating. The antioxidant activity of the samples, as measured by ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, ranged from 3.4 to 26.8 mM Trolox equivalents (TE), 15.8 to 58.4 mM TE and 80.1% to 91.8%, respectively. Among the extract mixtures, the highest synergistic interaction was observed between blackberry leaves and juniper by-products extracts at 1:2 and 2:1 ratios. The antimicrobial activity of the extracts was tested against Gram-positive and Gram-negative foodborne pathogens and spoilage bacteria, with the lowest minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values against *Staphylococcus aureus*, *Listeria innocua*, *Bacillus cereus*, *Campylobacter jejuni*, *Pseudomonas* spp. and *Shewanella* spp. On the other hand, the antimicrobial activity of blackberry leaf extract in combination with selected phenolic compounds (vanillic acid, catechin, rutin and apigenin) showed no synergy against the tested microorganisms. Overall, the results suggest that the tested by-products extracts could be utilized for the production of natural antioxidants and antimicrobials for further use in the food industry.

1. Introduction

Awareness of phenolics' beneficial effects and their potential applications in therapeutics, nutraceuticals, and food supplements is steadily increasing. The global market for phenolics is expected to reach USD 2.08 billion by 2025 (Grand View Research, 2022), reflecting their increasing use in the functional food and beverage industry.

Phenolics, naturally occurring secondary metabolites, play a crucial role in protecting plants from diseases, insects, and microorganisms. In foods, they contribute their bitterness, astringency, color, flavor, odor, and oxidative stability. Due to their diverse biological properties, phenolics are mainly being researched as effective antioxidants and antimicrobial agents for use in the food industry (Generalić Mekinić et al., 2019).

Berries, such as blackberries and chokeberries, are not only nutritious but also beneficial for human health, especially in preventing the negative effects of oxidative stress (Yamashita et al., 2017). In the Mediterranean region, berries such as blackberries, chokeberries, and juniper are used in the production of various food products, including fruit wine, pure juices, tea, and extracts. However, their production generates significant amounts of by-products in the form of seeds, pulp, peels, leaves, stems, and pomace. A transformative approach that aims to minimize waste generation and reduce the environmental footprint of industrial activities emphasizes their reuse, recycling and regeneration as a part of circular and green economy approach. Despite considerable progress, the generation of industrial waste remains a pressing problem. Large quantities of by-products are often disposed of in landfills or incinerated, contributing to pollution and greenhouse gas emissions. The environmental hazards posed by improper waste disposal are considerable. These include soil contamination, water pollution and harm to public health. The economic costs are also considerable and include expenditure on waste treatment, disposal and environmental remediation. The transition to a circular economy not only mitigates these threats, but also promotes sustainable development, resource efficiency and economic resilience, provides a holistic solution to the challenges posed by traditional waste

management practices (Dilucia et al., 2020; Gómez-García et al., 2021; Nakajima, 2000; Santeramo, 2022).

These by-products, which are rich in polyphenols, have attracted increasing interest in recent years as potential raw materials for bioactive compounds (de Aquino Souza Miskinis et al., 2023; Dueñas and García-Estévez, 2020; Ray et al., 2023; Sorrenti et al., 2023). In addition to phenolics, these solid wastes also contain dietary fiber, carotenoids, polysaccharides, oils, and vitamins, all of which are beneficial for human health. The extraction of these components can be utilized in the production of nutraceuticals, functional foods, and even for enhancing the quality of existing food products, including improving the physical, mechanical, antioxidant, and antimicrobial properties of foods and developing packaging solutions for food applications (Dilucia et al., 2020; Fehlberg et al., 2023; Fras Zemljič et al., 2022; Karimi Sani et al., 2023).

Despite the extensive studies of blackberries and chokeberries for their chemical composition and biological activity (Roda-Serrat et al., 2022; Rodríguez-Werner et al., 2019), information on juniper by-products from the Mediterranean region is still lacking. While juniper species have been studied primarily for their essential oils (Elshafie et al., 2020; Meng et al., 2016; Meringolo et al., 2022; Spengler et al., 2022), their extracts, especially in terms of their biological properties, are still less explored in the literature. The sole interest in the essential oils of juniper is not justified, as its extracts have also been studied for their antioxidant and antiproliferative potential (Meringolo et al., 2022). In recent literature, by-products from berries or junipers are considered as a potential source of antioxidants and antimicrobials (Barbieri et al., 2022; Gómez-García et al., 2021; Meringolo et al., 2022; Montanari et al., 2023; Paczkowska-Walendowska et al., 2021; Šimat et al., 2022). The antioxidant activity of extracts is not usually related to a specific compound, but to the synergy between them. Although the mechanism of their action is still unclear, the interaction effects between specific phenolic compounds and their biological activity are complex, and involve factors such as chemical conformation, hydroxyl group presence/position, and the ability to donate electrons and hydrogen

atoms (Palafox Carlos et al., 2012; Platzer et al., 2022). Existing evidence suggests that the interaction between specific phenolic compounds can be synergistic and enhance their antimicrobial and antioxidant potential (Skroza et al., 2019, 2022).

In this study, we investigated the antioxidant and antimicrobial activity of extracts from blackberry by-products (leaves and pomace), chokeberry by-products (pomace) and juniper by-products (residues from the production of extracts and needles) which remain after fruit/plant processing but are still valuable and rich sources of bioactive phytochemicals with potential health benefits. In addition to determining the biological potential of the extracts, we wanted to investigate the mutual interactions of the extract constituents and their joint effect in combination (in different ratios) to evaluate the possible synergistic/antagonistic activity leading to higher/lower antioxidant activity. Similarly, in the case of antimicrobial activity, the most potent extract was combined with the dominant pure phenolic compounds in binary mixtures, again with the aim of determining the potentially improved potency of the mixture compared to the individual samples.

2. Materials and Methods

2.1. Reagents

All standards, reagents and solvents used were of appropriate analytical or HPLC grade, and were purchased from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany), Merck (Darmstadt, Germany), Biolife Italiana Srl (Milan, Italy), Oxoid Ltd. (Hampshire, England), and Kemika (Zagreb, Croatia).

2.2. Sample collection and extraction procedure

The agro-food by-products were collected from traditional agro-production industries (production of berry wine, chokeberry juice, juniper essential oils) and wastes from these industries according to Table

1. The raw materials remained after production of juices (blackberry leaves - BL, blackberry pomace - BP), tea (choke pomace - CP), essential oil (juniper by-product - JB, collected after the hydro-distillation of juniper berries) and juniper needles separated during berry production (JN). All samples were shade-dried at ambient temperature (23 ± 2 °C) for about four to six days before being pulverized, and extracted in 50% ethanol (EtOH) using the advanced microwave assisted extraction (MAE) system (ETHOS X, Milestone Srl, Sorisole, Italy, 600 W, 5 minutes). The EtOH was evaporated at 50 °C using vacuum rotary evaporator (Laborota 4000, Heidolph, Schwabach, Germany) and the extracts were freeze-dried at -50 °C (FreeZone 2.5, Labconco, Kansas City, MO, USA) and stored in a cool dark place until analyses (Čagalj et al., 2021).

"Insert Table 1"

2.3. Total phenolic content (TPC) and HPLC determination of the individual phenolic compounds from extracts

The TPC of the extracts was determined by the Folin–Ciocalteu method (Amerine and Ough, 1980) using a spectrophotometer (SPECORD 200 Plus, Edition 2010, Analytik Jena AG, Jena, Germany). In brief, 25 µL of the extract was mixed with 1.5 mL of distilled water and 125 µL of Folin–Ciocalteu reagent. The solution was mixed and after one minute 375 µL of 20% sodium carbonate solution and 475 µL distilled water were added. After 2 h in the dark at room temperature (24 °C) the absorbance was read at 765 nm. The standard calibration curve was done using gallic acid (0–500 mg/L, $y=0.001x+0.0061$, $r^2=0.9997$) and the results were expressed as gallic acid equivalents (GAE) in mg/g of dried extract.

Phenolic compounds from the extracts were determined and quantified using an HPLC Ultimate 3000 (Termo Fisher Scientific, Whatman, MA, SAD) equipped with an ultraviolet-visible (UV-Vis) diode-array detector (DAD) according to the following protocol. Freeze-dried extracts (10 mg) were dissolved in 10 mL of 50% ethanol and filtered through 0.45-µm filter (LGG, Meckenheim, Germany).

Separation was performed using a Syncronis™ C18 column (250×4.6 mm, 5 µm particle size, Thermo Fisher Scientific, Waltham, MA, USA). The injected sample volume was 10 µL, the column temperature was set to 25 °C, and the flow rate to 0.8 mL/min. The total run time of the method was 80 min under the following conditions: a gradient consisting of solvent A (water/formic acid, 98:2, v/v), solvent B (acetonitrile), and solvent C (methanol) was applied as follows: from 96% A, 2% B, 2% C at 0 min to 50% A, 25% B, 25% C at 40 min, to 40% A, 30% B, 30% C at 45 min, to 0% A, 50% B, 50% C at 60 min, to 96% A, 2% B, 2% C at 70 min, maintaining 96% A, 2% B, 2% C for 10 min (80 min). The peaks of the individual phenolics were determined by comparing their retention times and absorption spectra (at two wavelengths 280 and 320 nm) with those of the corresponding standards (gallic acid, caffeic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, chlorogenic acid, *p*-coumaric acid, (-)-epicatechin, (+)-catechin, quercetin, rutin, astringnin, apigenin and (-)-epigallocatechin gallate). The phenolic compounds were quantified using external standard calibration curves for each of the above-mentioned compounds (injected at five different concentrations). Data were reported as means of the two injections of extracts from the same sample. Results were expressed in mg of compound per g of dry extract (mg/g).

2.4. Antioxidant activity

The antioxidant activity of the extracts was evaluated using methods based on two different mechanisms of action (hydrogen atom transfer - HAT (DPPH, ORAC) and electron transfer - ET (FRAP)). All analyses were done in triplicate and expressed as mean ± standard deviation.

The reducing activity was measured as FRAP (Benzie and Strain, 1996). Briefly, 300 µL of the FRAP reagent solution was pipetted into the microplate wells, and the absorbance was measured at 592 nm. Then 10 µL of the sample was added to the reagent and the change in absorbance was measured. The change in absorbance, calculated as the difference between the final absorbance value of the reaction mixture after a certain reaction time (4 min) and the absorbance of the FRAP reagent before sample

addition, was compared with the values obtained for the standard solution (Trolox, concentration range of 50-1000 µM). The results were expressed as millimoles of Trolox equivalents (mM TE).

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability of extracts was measured in 96-well microplates. The DPPH stock solution (4 mg/100 mL, in ethanol) was diluted to the reagent absorbance of 1.2 ± 0.02 (Katalinić et al., 2010). DPPH radical solution (290 µL) was pipetted into microplate wells, and absorbance was measured at 517 nm. Then, a 10 µL of sample was added to the wells and the decrease in absorbance was measured after 1 h using the plate reader. The antioxidant activity was expressed as inhibition percentages of DPPH radical (% inhibition).

The oxygen radical absorbance capacity (ORAC) method (Burčul et al., 2018) was performed to determine the antioxidant activity of the extracts by monitoring the inhibition of the action of free peroxyl radicals formed by the decomposition of 2,2-azobis (2-methylpropionamide)-dihydrochloride (AAPH) to the fluorescent compound fluorescein. Briefly, 150 µL of fluorescein and 25 µL of sample (or Trolox, or blank) were pipetted into microplate wells and thermostated for 30 minutes at 37 °C. After 30 minutes, 25 µL of AAPH was added and the change in fluorescence intensity was measured every minute for 80 min at 485 and 520 nm. The results were expressed as mM TE.

2.5. Antioxidant interaction of selected extracts detected by FRAP method

Potential synergistic (positive values) or antagonistic (negative values) effects were calculated according to the equation described in Skroza et al. (2022):

$$\text{Difference (\%)} = [\text{Combination AB} \times 100 / (\text{Individual A} + \text{Individual B})] - 100$$

where the combination AB represents the experimental FRAP value for the binary mixture of BL and JB or BL and JN extracts at a final concentration of 10 mg dry extract/mL, while the A/B value is the theoretical FRAP value calculated for each individual extract according to their molar ratio in the

198 mixtures (5 or 2.5 mg/mL). Positive values of the difference indicate a potential synergistic effect,
199 while negative values indicate an antagonistic effect.
200

201 **2.6.Antimicrobial testing against foodborne pathogens and spoilage bacteria**

202 The antimicrobial activity of the prepared extracts (at a concentration of 2 mg/mL, DMSO:water,
203 50:50, *v/v*) was evaluated by the broth microdilution method using 2-*p*-iodophenyl-3-p-nitrophenyl-5-
204 phenyl tetrazolium chloride (INT) and resazurin as indicators. The minimal inhibitory concentration
205 (MIC) was the lowest concentration at which no bacterial growth was detected as a reduction of
206 colorless INT to red formazan or blue resazurin to pink resorufin (Elez Garofulić et al., 2021; Klančnik
207 et al., 2010). The minimal bactericidal concentration (MBC) was the lowest concentration at which no
208 bacterial growth was observed after cultivation of the bacterial suspension where no change in color
209 occurred (Elez Garofulić et al., 2021). The analyses were performed with common foodborne bacteria
210 (*Staphylococcus aureus* ATCC 25923, *Listeria innocua* ŽM39, *Bacillus cereus* ŽMJ164, *Escherichia*
211 *coli* ATCC 11229, *Salmonella* Typhimurium ATCC 14028 and *Campylobacter jejuni* NCTC 11168)
212 and spoilage bacteria (*Shewanella baltica* NCTC 10735, *S. putrefaciens* ŽM654, *S. xiamenensis*
213 ŽM655, *Pseudomonas fragi* ATCC 4973, *P. fragi* ZIM B1064, *P. fragi* ZIM B1072, *P. fragi* ZIM
214 B1085, *P. fragi* ZIM B1092).
215
216 **2.7. Antimicrobial interaction activity of selected extract-phenolics mixtures**

217 Antimicrobial activity of BL extract in combination with selected phenolic compounds (vanillic acid,
218 catechin, rutin, and apigenin) against the most common foodborne pathogens: *S. aureus* ATCC 25923,
219 *B. cereus* ATCC 14579, *L. monocytogenes* ATCC 7644, *E. coli* ATCC 25922, *Enterococcus faecalis*
220 ATCC 29212, and *S. enteritidis* ATCC 13076 were analyzed according to the methodology described
221 in section 2.6. In addition, the extract was diluted in 40 mg/mL 4% dimethyl sulfoxide (DMSO) and
222 the mixtures were prepared by mixing 2 mL extract and 2 mL of pure phenolic solution: vanillic acid

(c= 10 mg/mL); catechin (c= 10 mg/mL); apigenin (c= 4.09 mg/mL); rutin (c= 10 mg/mL). The pure phenolics were diluted in DMSO:water (50:50, *v/v*). The interaction between the compounds in terms of antibacterial activity was determined by calculating the fractional inhibitory concentration index (FICI), which was calculated for each mixture according to the following formula (Skroza et al., 2019):

$$FIC_A + FIC_B = FICI$$

where FIC_A = MIC of BL in the extract-phenolic mixture/MIC of the BL alone and FIC_B = MIC of the phenolic compound in the extract-phenolic mixture/MIC of the phenolic compound alone. A synergistic interaction was defined when the FIC value was 0.5 or less, and an antagonistic interaction when the FICI was greater than 4. FICI values between 0.5 and 1 were interpreted as additive interactions and between 1 and 4 as indifferent interactions.

2.8. Statistical analysis

The statistical difference between the total phenolic content between the spectrophotometric data was determined using the analyses of variance (one-way ANOVA, followed by Fisher's least significant difference test). The significance level was set at $p < 0.05$. Analyses were performed using Statgraphics Centurion-Ver.16.1.11 software (StatPoint Technologies, Inc., Warrenton, VA, USA).

3. Results and Discussion

3.1. Phenolic content of extracts

The total phenolic content and phenolic profile of the investigated by-product extracts are shown in Figure 1 and Table 2. The highest total phenolic contents of 250 and 187 mg GAE/g were found in BL and JN extracts, respectively, with rutin dominating. Other phenolic components present in considerable amounts in the BL extract were caffeic acid, chlorogenic acid and astringinin. Gallic acid, protocatechuic and chlorogenic acids were found in CP extract, while vanillic acid was detected in the

247 highest concentration in JN extract. JB showed a similar phenolic profile to JN but with a lower
248 proportion of individual phenolic compounds.
249
250 *"Insert Figure 1"*
251
252 During the industrial processing of blackberries 20%-30% of the seeds, skins and part of the pulp
253 accumulate, especially in the production of fruit juice or wine (Struck et al., 2016). Several papers
254 report on the phenolic profile of berry fruits and their by-products after juice production. Struck et al.
255 (2016) found differences between the phenolic profiles of blackberry juice and pomace remaining after
256 juice production, indicating that most polyphenolic compounds (especially flavonols) are accumulated
257 in the fruit exocarp and are not extracted into the juice during processing. According to the authors, the
258 main phenolic compounds in blackberry pomace are flavonoids, especially catechin. Jazić et al. (2019)
259 also found a relatively high catechin content in blackberry pomace extracts from cultivated and wild
260 species. In addition, the pomace of wild blackberry had a high content of rutin, protocatechuic acid and
261 gallic acid. On the other hand, Subbiah et al. (2021) only reported the presence of protocatechuic acid
262 in blackberry extracts, while Čanadanović-Brunet et al. (2019) detected protocatechuic acid and gallic
263 acid in blackberry pomace extracts.
264 The blackberry leaf is a by-product that remains after plant cultivation and fruit processing. In general,
265 blackberry leaves are rich in tannins, flavonoids, phenolic acids, minerals and vitamin C (Ferlemi and
266 Lamari, 2016; Salevic et al., 2017). Particularly phenolic acids (caffeic acid, ellagic acid, gallic acid,
267 syringic acid), flavonols (quercetin, kaempferol, rutin, catechin) and their glycosides (rutin,
268 isoquercetin, hyperoside) and flavon-3-ols (catechin, epicatechin) were found to be responsible for
269 good antioxidant activity of blackberry leaf extracts (Paczkowska-Walendowska et al., 2021). Similar
270 to our results, Grabek-Lejko and Wojtowicz (2014) found a TPC of 115.92 ± 3.5 mg GAE/g (dry
271 weight) for the leaves of *R. plicatus*. According to a review conducted by Ferlemi and Lamari (2016),

the most abundant phenolic compounds in blackberry leaves are phenolic acids: caffeic acid, gallic acid, *p*-coumaric acid, and ellagic acid; followed by flavonoids: quercetin, kaempferol, myricetin, catechin, epicatechin, epicatechin gallate; and the anthocyanin cyanidin-3-*O*-glucoside (Zia-Ul-Haq et al., 2014). Relatively high content of ellagic acid, quercetin and rutin, and lower concentrations of polyphenolic acids have been reported for freeze-dried blackberry leaves (*R. ulmifolius*) (Martini et al., 2009). Leaves of wild and cultivated *Rubus* cultivars contained quercetin and kaempferol as dominant compounds in all samples and were a rich source of flavonoids, ellagic acid and tannins (Gudej and Tomczyk, 2004).

Chokeberries are considered to be one of the richest sources of polyphenols, mainly procyanidins, anthocyanidins and phenolic acids, while flavonols are usually found in low concentrations (Jurendić & Ščetar, 2021). It has been reported that chokeberries have significantly higher phenolic content and antioxidant activity than other berries (Oszmiański and Wojdylo, 2005), and this is also confirmed by this study. The TPC of chokeberry extracts was found in a wide range of 461-3436 mg GAE/L (Tolić et al., 2015), and protocatechuic acid, quercetin, (-)-epicatechin and chlorogenic acid were previously described in chokeberry pomace (Rodríguez-Werner et al., 2019).

"Insert Table 2"

Ben Mrid et al. (2019) investigated the chemical profile of *J. oxycedrus* subsp. *oxycedrus* needles and berries extracts. Dominant phenolic compounds found in needles extracts were salicylic acid and rutin, while in berries they were present in significantly lower concentration. *Juniperus oxycedrus* berries have a lower TPC compared to the results obtained for needles in this study. TPC of 17.89 ± 0.23 and 5.14 ± 0.06 mg GAE/g of extract and two phenolic acids, gallic acid and protocatechuic acid were detected in the berry extracts, with protocatechuic acid being the dominant one (3355 ± 0.88 µg/g extract). In a more recent study (−)-epicatechin, rutin, catechin, quercetin, quercetin-3-*O*-glucoside,

296 and luteolin was identified as the major constituents of the flavonoid class of *J. oxycedrus* (Meringolo
297 et al., 2022).
298
299 **3.2. Antioxidant activity of extracts**
300 The results of the antioxidant activity of the extracts are shown in Table 3. An exceptionally good
301 reducing power was observed for BL, with the highest FRAP values, but also for blackberry pomace
302 (BP) and JN.
303 *"Insert Table 3"*
304 Based on the results of antioxidant activity, BL, JB and JN were selected to determine their mutual
305 interactions when mixed in different ratios. The aim of combining the extracts was to test their potential
306 synergistic effect to evaluate their efficacy at lower concentrations of each extract in the mixture. This
307 was done using the FRAP assay (Table 4), as with the DPPH and ORAC method it is not possible to
308 determine the interaction effect using a mathematical equation as the relationship between
309 concentration and activity is not linear. The positive difference and synergy were observed for all
310 samples indicating a synergistic interaction, and the highest value was recorded for the binary mixture
311 of BL and JB in the 1:2 and 2:1 ratios. No analysis of the influence of the concentration or ratio of the
312 individual components in the mixture was considered. Our hypothesis was that combinations of
313 extracts would have improved efficacy in targeted biological assay and the data for the molar ratios of
314 the extracts in the 1:2 and 1:1 mixture confirmed this.
315 *"Insert Table 4"*
316 In the study by Salevic et al. (2017) blackberry leaf extracts showed higher antioxidant activity in terms
317 of the ferric reducing ability. The reported DPPH radical scavenging activity of the pomace from
318 different blackberry cultivars ranged from 0.2 to 10.9 μmol TE/g (Čanadanović-Brunet et al., 2019;
319 Cetojevic-Simin et al., 2017; Kalušević et al., 2016). Jazić et al. (2019) found that wild blackberry
320 pomace extracts had lower IC_{50} values for DPPH and ABTS radicals compared to the pomace of

cultivated species. The radical scavenging capacity of blackberry leaf extracts with high concentrations of ellagic acid in relation to Trolox (TEAC values) showed an antioxidant activity of 0.12. The study suggested that the antioxidant activity of blackberry leaf extract was also related to the content of gallic acid although it was present at low concentration in the sample (Martini et al., 2009). *Juniperus* is a typical genus of the Mediterranean marquis. The interest in this plant is mainly due to its aromatic berries and leaves, and the promising chemical profile of both the essential oils and the extracts (Barbieri et al., 2022). The literature has shown that the berries have been studied more for their antioxidant potential than the needles (Taviano et al., 2013; Živić et al., 2019), but in recent years there have been studies that have found both antioxidant potential and nutraceutical properties in the needles of *Juniperus* species. There are only few studies on *J. oxycedrus* needles. El Jemli et al. (2016) reported a TPC of 278.56 ± 9.67 mg GAE/g of *J. oxycedrus* needles, which is slightly higher than the value obtained in this study. The antioxidant activity of *J. oxycedrus* was previously investigated by Meringolo et al. (2022) using the FRAP and DPPH method and reported good activity. Similar to our results, Fieracsu et al. (2018) found that the DPPH inhibition ability of the *J. communis* extract was 81.6%.

3.3. Antimicrobial activity of extracts

The microbiological activity is associated with the strong antimicrobial properties of the phenolics. The results of the antimicrobial activity of the extracts against Gram-positive and Gram-negative foodborne pathogens and spoilage bacteria are shown in Tables 5 and 6. Against Gram-positive bacteria, *S. aureus*, *L. innocua* and *B. cereus*, BL, BP, JN and JB showed good antimicrobial activity. None of the extracts had any effect against Gram-negative bacteria, while they all showed antimicrobial activity against *C. jejuni*, with the lowest MIC values being obtained for CP extract. The antimicrobial activity against spoilage bacteria belonging to the genera *Pseudomonas* and *Shewanella* showed that

juniper species were active against *S. xiamenensis* and *P. fragi*. Among the extracts, BL and CP extracts were found to be the most effective, with MIC values of 0.5 mg/mL but higher MBC (2 mg/mL).

"Insert Table 5"

"Insert Table 6"

Blackberry leaf extracts could be a potential source of natural antimicrobial compounds and have antimicrobial activity against a wide range of microorganisms. Paczkowska-Walendowska et al. (2021) investigated the antimicrobial activity of water and hydroalcoholic leaf extracts of different blackberry varieties using the well-difussion method against *Lactobacillus* spp., *Bacillus* spp., *Gardnerella vaginalis*, *Streptococcus agalactiae*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. typhimurium* and *Candida* spp. Hydroalcoholic extracts showed better antimicrobial activity and inhibited the growth of all microorganisms tested. However, the strongest inhibition was recorded against *E. coli*, *S. aureus* and *Candida* spp. Milenković-Andjelković et al. (2016) determined MIC and MBC of blackberry leaf extracts against 12 bacteria and 1 yeast. The MIC values ranged from 8 to 63 μg/mL with the lowest inhibition concentration against *S. aureus*, *L. monocytogenes*, and *Sarcina lutea*, while the MBC values ranged from 8 to 125 μg/mL with the lowest bactericidal concentration against *S. lutea* (Riaz et al., 2011). Overall, the result suggest that the tested by-product extracts could be utilized for the production of products rich in natural antioxidants and antimicrobials for further use in the food industry..

Blackberry pomace extracts from two cultivars inhibited the growth of *E. coli*, *P. aeruginosa*, *L. monocytogenes*, *Salmonella* sp., *Staphylococcus* sp. and *Bacillus* sp. The values for MIC and MBC ranged from 0.39 to >25 mg/mL and, from 0.78 to >25 mg/mL, respectively (Cetojevic-Simin et al., 2017). Blackberry pomace extracts showed excellent inhibitory activity against *S. aureus* in the study by Struck et al. (2016), while Salaheen et al. (2016) found bactericidal activity of blackberry pomace extract against *S.* Typhimurium with MIC of 1.5 mg/mL and MBC of 1.7 mg/mL.

The potent antimicrobial activity of chokeberry pomace extracts was demonstrated in the study by Tamkutė et al. (2021). Extracts at a concentration of 6.6% and 3.3% inhibited the growth of *L.*

370 *monocytogenes, B. thermosphacta, C. jejuni* and *P. putida*. Furthermore, chokeberry pomace extracts
371 reduced the growth of *E. coli* by 4 logs at the concentration of 0.5 mg GAE/mL (Aditya et al., 2019).
372 Juniper species, *J. oxycedrus* and *J. communis*, are medicinal plants with various pharmacological
373 properties, including antimicrobial activity which has been mostly detected for their essential oils.
374 Recently, however, juniper extracts have been reported to be a great source of secondary metabolites,
375 including organic acids, terpenoids, and phenolic compounds (Mofikoya et al., 2023), with
376 antimicrobial activity against *L. monocytogenes* (Barbieri et al., 2022) and *Enterococcus faecium*
377 (Montanari et al., 2023). In addition, Taviano et al. (2011) investigated the antimicrobial activities of
378 five *Juniperus* branch extracts, all of which were only effective against Gram-positive bacteria. The
379 lowest MIC value was 4.88 μg/mL, which was found for *J. oxycedrus* against *S. aureus*.
380
381 **3.4. Antimicrobial interaction activity of extract-phenolic mixtures**
382 Information on the interaction of plant extracts and individual phenolic compounds is limited, but
383 crucial for the formulation of functional foods and the determination of interaction activity of different
384 matrices. Therefore, in this study, plant extracts with known antimicrobial activity, is combined with
385 pure substances, to understand their interactions. An extract of BL was chosen and retested for
386 antimicrobial interaction activity in combinations with pure phenolics, detected as dominant in the
387 extracts (refer to Table 1).
388 Table 7 presents the MIC and MBC values of the pure phenolic compounds and the BL extract,
389 assessed individually and in molar mixtures, against the most common foodborne pathogens. The pure
390 BL extract showed antimicrobial activity against *S. aureus*, with an MIC value of 0.63 mg/mL. The
391 MIC values for other bacterial species were either 5 mg/mL or above 10 mg/mL. The selected phenolic
392 compounds exhibited mostly similar antimicrobial activity against the tested bacteria, with MIC values
393 averaging 1.45 mg/mL. Notably, apigenin showed the best activity and had the lowest MIC values
394 (0.51 mg/mL) against all species. These findings are in line with numerous studies confirming the

commendable antibacterial activity of phenolic compounds against pathogenic bacteria (Araruna et al., 2012; Arima et al., 2002; Bhattacharya et al., 2016; Dong et al., 2013; Ibrahim et al., 2020; Kalogeropoulos et al., 2009; Kuete et al., 2010; Liu et al., 2013; Ma et al., 2019).

Comparing the results of the antimicrobial activity of the individual phenols and BL extract with those of the mixtures, the binary mixtures have almost the same effect (Table 7). All mixtures showed bactericidal activity against *S. aureus*. For the other binary mixtures, the MBC values were higher than the MIC or the MIC could not be confirmed.

The interaction activity between the components of the mixture was expressed by the FICI factor and the results are shown in Table 8. A range of 0.74 to 3.45 indicates that none of the tested mixtures had a synergistic effect against tested bacteria at given concentrations. To the best of our knowledge, there are no reports on the antimicrobial interaction of the plant extracts enriched with pure phenolics. However, it cannot be excluded that synergy is possible at higher concentrations (Sanhueza et al., 2017; Skroza et al., 2019).

"Insert Table 7"

"Insert Table 8"

4. Conclusion

In this study, blackberry, chokeberry, and juniper by-products were evaluated for their phenolic content, antioxidant, and antimicrobial activity. The highest TPC was found in BL extract which was especially rich in rutin. In addition, the BL extract showed the highest FRAP and ORAC values. The highest inhibition of the DPPH radical was found for JB extract. Based on these results, BL, JB and JN extracts were selected to determine the interactions between them when mixed in different ratios using FRAP assay. All samples showed synergistic interactions, with the best results obtained for the mixture of BL and JB at the 1:2 and 2:1 ratios. The antimicrobial analyses showed that Gram-positive bacteria were more susceptible to the extracts. BL, BP, JN and JB extracts showed good antimicrobial activity

against *S. aureus*, *L. innocua*, and *B. cereus*. None of the extracts had antimicrobial activity against Gram-negative bacteria *E. coli* and *S.* Typhimurium, but all showed good activity against *C. jejuni*. On the other hand, JN, JB, BL and CP extracts showed good antimicrobial activity against *Pseudomonas* and *Shewanella* spp. The combination of BL extract with pure phenolic compounds did not increase its activity, but it also did not reduce the antimicrobial effect. BL, JN and JB may be considered as a valuable raw material for the extraction of bioactive components and a sustainable source of antioxidants that have potential for the food industry. Considering that combining extracts may result in a lower concentration of their constituents in the mixture, further research should consider testing wider range of concentrations of extracts and detailed analyses of the compounds in the extracts as well as possibly demonstrate their health benefits.

5. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Author Contributions

Vida Šimat: Conceptualization, Resources, Data curation, Writing - review & editing, Supervision, Project administration, Funding acquisition. Martina Čagalj: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft preparation. Ivana Generalić Mekinić: Formal analysis, Investigation, Data curation, Writing - original draft preparation. Sonja Smole Možina: Methodology, Data curation, Supervision, Funding acquisition. Valentina Malin: Formal analysis. Giulia Tabanelli: Conceptualization, Resources, Writing - review & editing, Project administration. Fatih Özogul: Resources, Writing - review & editing, Supervision. Danijela Skroza: Formal analysis, Investigation, Methodology, Data curation, Writing - original draft preparation.

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7. Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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709 **10. Tables**

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711 Table 1. List of the agro-food by-products used in the study with their collection country and period,

712 and abbreviation.

By-product	Collecting country	Collection period	Mark
Blackberry (*Rubus fructicosus*) leaves	Croatia	August 2021	BL
Blackberry (*R. fructicosus*) pomace from juice production	Croatia	August 2021	BP
Chokeberry (*Aronia melanocarpa*) pomace from juice production	Slovenia	Summer 2020	CP
Juniper (*Juniperus oxycedrus*) needles	Croatia	Spring 2021	JN
Juniper *(J. communis)* by-product from essential oil production via hydro-distillation	Slovenia	November 2021	JB

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Table 2. Phenolic profile (mg/g of dry extract) of agro-food by-product extracts (n=2).

	BL[1]	BP	CP	JN	JB
Gallic acid	0.1±0.0	0.2±0.0	3.9±0.1	1.8±0.0	n.d.[2]
Caffeic acid	1.0±0.0	n.d.	0.7±0.1	n.d.	n.d.
Protocatechuic acid	n.d.	0.3±0.2	7.1±0.6	0.4±0.0	n.d.
p-hydroxybenzoic acid	n.d.	n.d.	n.d.	1.0±0.0	n.d.
Vanillic acid	n.d.	n.d.	n.d.	13.7±0.2	2.6±0.0
Chlorogenic acid	6.0±0.0	n.d.	4.8±0.3	n.d.	n.d.
p-coumaric acid	0.6±0.0	n.d.	n.d.	n.d.	n.d.
(-)-epicatechin	0.1 ± 0.0	n.d.	n.d.	0.7±0.0	0.1±0.0
(+)-catechin	n.d.	n.d.	n.d.	6.4±0.1	4.4±0.1
Quercetin	n.d.	n.d.	1.2±0.1	n.d.	n.d.
Rutin	29.0±0.5	1.3±0.2	1.1±0.3	9.1±0.1	7.2±0.1
Astringnin	2.3±0.0	n.d.	n.d.	n.d.	n.d.
Apigenin	n.d.	n.d.	n.d.	10.0±0.1	3.0±0.0
(-)-epigallocatechin gallate	n.d.	n.d.	n.d.	0.9±0.1	0.8±0.0
∑ of determined compounds	41.30	1.74	18.87	33.73	18.11

[1] Blackberry leaves (BL), blackberry pomace from juice production (BP), chokeberry pomace from juice production (CP), juniper needles (JN), juniper by-product from extract production (JB); [2] n.d. - not detected

Table 3. Antioxidant activity of the agro-food by-products' extracts (n=4)

Sample	FRAP (mM TE)	ORAC (mM TE)	DPPH (% inhibition)
BL	26.8 ± 1.4[a]	58.4 ± 1.6 [a]	87.7 ± 1.4 [a]
BP	12.9 ± 0.4 [b]	15.8 ± 0.5 [b]	86.1 ± 1.9 [a]
CP	10.1 ± 0.7 [c]	36.1 ± 1.1 [c]	80.1 ± 0.4 [b]
JN	13.2 ± 0.7 [b]	44.1 ± 0.6 [d]	89.1 ± 0.4 [c]
JB	3.4 ± 0.1 [d]	23.1 ± 3.3 [e]	91.8 ± 0.2 [d]

[*]Blackberry leaves (BL), blackberry pomace from juice production (BP), chokeberry pomace from juice production (CP), juniper needles (JN), juniper by-product from extract production (JB), Ferric Reducing/Antioxidant Power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) methods, Trolox equivalents (TE).

[a-e] mean value ± standard deviation in the same column followed by different superscript letter are significantly different (p<0.05)

Table 4. Antioxidant activity of the agro-food by-products' extract mixtures determined by FRAP (Ferric Reducing/Antioxidant Power) method.

Samples	Molar ratio	FRAP experimental (mM TE)	FRAP theoretical (mM TE)	Difference (%)
BL+ JB [1]	1:1	8.75 ± 0.15	8.23	5.9
BL+ JN	1:1	10.61 ± 0.29	9.69	8.7
BL+ JB	1:2	8.04 ± 0.15	7.11	11.7
BL + JN	1:2	9.66 ± 0.40	9.05	6.3

[1] Blackberry leaves (BL), juniper needles (JN), juniper by-product from extract production (JB)

773 Table 5. Antimicrobial activity of the agro-food by-products' extracts against selected common foodborne pathogens expressed as

774 MIC (minimal inhibitory concentration; mg/mL) and MBC (minimal bactericidal concentration; mg/mL).

Sample	*Staphylococcus aureus* ATCC 25923		*Listeria innocua* ŽM39		*Bacillus cereus* ŽMJ164		*Escherichia coli* ATCC 11229		*Salmonella* Typhimurium ATCC 14028		*Campylobacter jejuni* NCTC 11168	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
BL[1]	0.5	0.5	>2	>2	>2	>2	>2	>2	>2	>2	0.25	0.5
BP	>2	>2	0.25	0.25	>2	>2	>2	>2	>2	>2	0.125	0.25
CP	>2	>2	>2	>2	2	>2	>2	>2	>2	>2	0.0625	0.125
JN	1	1	0.5	2	>2	>2	>2	>2	>2	>2	2	>2
JB	0.25	0.5	0.25	0.5	>2	>2	>2	>2	>2	>2	2	>2

775 [1] Blackberry leaves (BL), blackberry pomace from juice production (BP), chokeberry pomace from juice production (CP), juniper needles

776 (JN), juniper by-product from extract production (JB)

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783 Table 6. Antimicrobial activity expressed as MIC (minimal inhibitory concentration; mg/mL) and MBC (minimal bactericidal concentration;
784 mg/mL) of agro-food by-products' extracts against spoilage bacteria.

Sample	*Shewanella baltica* NCTC 10735		*Shewanella putrefaciens* ŽM654		*Shewanella xiamenensis* ŽM655		*Pseudomonas fragi* ATCC 4973		*Pseudomonas fragi* ZIM B1064		*Pseudomonas fragi* ZIM B1072		*Pseudomonas fragi* ZIM B1085		*Pseudomonas fragi* ZIM B1092	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
BL[1]	2	2	/	/	/	/	0.5	>2	0.5	>2	0.5	>2	0.5	>2	0.5	>2
BP	1	2	1	2	2	>2	1	>2	1	>2	1	>2	0.5	>2	1	>2
CP	2	2	0.5	0.5	1	2	0.5	>2	0.5	>2	0.5	>2	0.5	>2	0.5	>2
JN	2	2	1	1	0.125	0.125	1	1	2	>2	2	>2	2	>2	2	>2
JB	>2	>2	1	>1	0.125	0.25	1	2	2	2	2	2	2	2	2	2

785 [1] Blackberry leaves (BL), blackberry pomace from juice production (BP), chokeberry pomace from juice production (CP), juniper needles
786 (JN), juniper by-product from extract production (JB)

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794 Table 7. Antimicrobial activity of blackberry leaf extract (BL) and its combinations with pure phenolics expressed as MIC (minimal
795 inhibitory concentration; mg/mL) and MBC (minimal bactericidal concentration; mg/mL).

Samples	Gram (+) bacteria						Gram (-) bacteria					
	Staphylococcus aureus (ATCC 25923)		*Bacillus cereus* (ATCC 14579)		*Listeria monocytogenes* (ATCC 7644)		*Escherichia coli* (ATCC 25922)		*Enterococcus faecalis* (ATCC 29212)		*Salmonella enteridis* (ATCC 13076)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
BL	0.63	1.25	5	>10	5	5	5	>10	5	>10	5	>10
Vanillic acid	>1.25	/	>1.25	/	>1.25	/	>1.25	/	>1.25	/	>1.25	/
Catechin	>1.25	/	1.25	>1.25	0.63	>1.25	1.25	>1.25	1.25	>1.25	1.25	>1.25
Rutin	>1.25	/	1.25	>1.25	1.25	>1.25	1.25	>1.25	1.25	>1.25	1.25	>1.25
Apigenin	>0.51	/	>0.51	/	>0.51	/	>0.51	/	0.51	>0.51	>0.51	/
BL + vanillic acid	1.25+0.31	2.5+0.63	5+1.25	>5+1.25	2.5+0.63	>5+1.25	2.5+0.63	≥5+1.25	5+1.25	>5+1.25	5+1.25	>5+1.25
BL+ catechin	0.63+0.16	1.25+0.31	5+1.25	>5+1.25	1.25+0.31	5+1.25	5+1.25	>5+1.25	5+1.25	5+1.25	5+1.25	>5+1.25
BL+ rutin	1.25+0.31	2.5+0.63	5+1.25	>5+1.25	5+1.25	5+1.25	5+1.25	>5+1.25	5+1.25	>5+1.25	5+1.25	>5+1.25
BL+ apigenin	1.25+0.13	2.5+0.26	5+1.25	>5+0.51	5+0.51	>5+0.51	>5+0.51	/	>5+0.51	/	>5+0.51	/

796 Blackberry leaf extract (BL) was diluted 40 mg/mL in 4% DMSO; Mixtures were prepared by mixing 2 mL pure extract and 2 ml of
797 phenolics solution: vanillic acid (c= 10 mg/mL); catechin (c= 10 mg/mL); apigenin (c= 4.09 mg/mL); rutin (c= 10 mg/mL).

798 Table 8. Interaction activity of blackberry leaf extract (BL) and phenolic compounds expressed as FICI (fractional inhibitory concentration
799 index).

Mixtures	*Staphylococcus aureus*	*Bacillus cereus*	*L isteria monocytogenes*	*Escherichia coli*	*Enterococcus faecalis*	*Salmonella enteridis*
BL + vanillic acid	1.25	2.00	1.00	1.00	2.00	2.00
BL + catechin	2.11	2.00	0.74	2.00	2.00	2.00
BL + rutin	2.23	2.00	2.00	2.00	2.00	2.00
BL + apigenin	2.24	3.45	2.00	2.00	2.00	2.00

800 The interactions between the compounds in the mixtures in relation to the antibacterial activity are expressed as FICI values. FICI ≤0.5
801 indicates a synergistic interaction, FICI = 0.5–1.0 additive, FICI = 1.0–4.0 indifferent interaction and FICI >4.0 indicates antagonism among
802 the tested phenolic compound.

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804 **11. Figure Legend**

805 **Figure 1.** Total phenolic content (mg gallic acid equivalents (GAE)/g dry extract) of extracts

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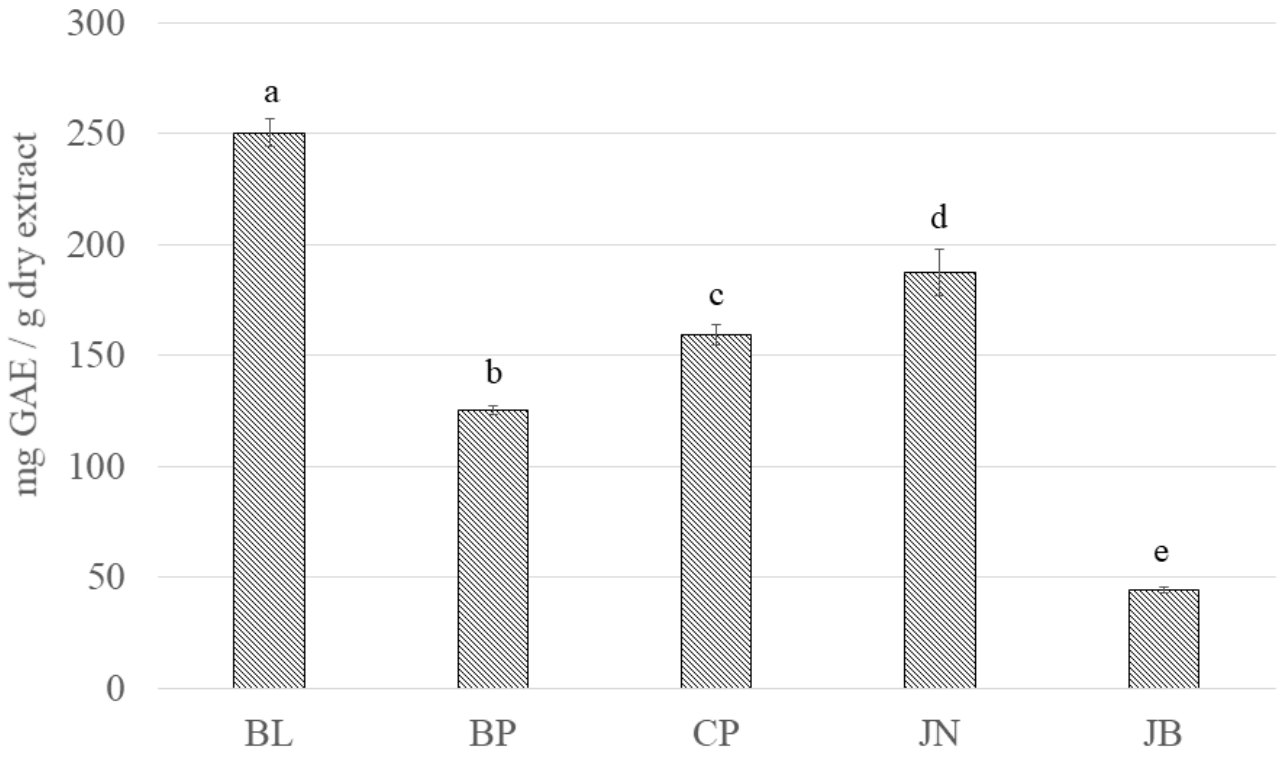
807 **12. Figures**



*Blackberry leaves (BL), blackberry pomace from juice production (BP), chokeberry pomace from juice production (CP), juniper needles (JN), juniper by-product from extract production (JB) a-e mean value ± standard deviation marked by different letter are significantly different ($p<0.05$)

Figure 1.