Composition and Antibacterial Activity of Aronia melanocarpa (Michx.) Elliot, Cornus mas L. and Chaenomeles superba Lindl. Leaf Extracts

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Abstract: The purpose of this study was to investigate the composition of leaf extracts from Aronia melanocarpa, Chaenomeles superba, and Cornus mas, and their antimicrobial activity against typical spoilage-causing and pathogenic bacteria found in meat and meat products. The highest total phenolic content (TPC) was detected in C. superba extract, followed by C. mas and A. melanocarpa extracts. The antioxidant capacity of the extracts was measured by DPPH and ABTS assays. The lowest IC50 values were found for C. superba extract, followed by C. mas and A. melanocarpa extracts. LC-MS and HPLC analysis revealed that A. melanocarpa and C. superba extracts contained hydroxycinnamic acid derivatives and flavonoids (mainly flavonols). Hydroxycinnamic acid derivatives were detected in the C. mas extract, as well as flavonols, ellagitannins, and iridoids. The antibacterial activity of the plant extracts was tested against Gram-negative bacteria (Moraxella osloensis, Pseudomonas fragi, Acinetobacter baumannii, Escherichia coli, Enterobacter aerogenes, Salmonella enterica) and Gram-positive bacteria (Enterococcus faecium, Staphylococcus aureus, Brochothrix thermosphacta, Lactobacillus sakei, Listeria monocytogenes) using the microculture method. The extracts acted as bacteriostatic agents, decreasing the growth rate (µmax) and extending the lag phase (tlag). C. mas showed most potent antibacterial activity, as confirmed by principal component analysis (PCA).

Keywords: polyphenols; iridoids; ellagitannins; Aronia melanocarpa; Chaenomeles superba; Cornus mas; antibacterial activity

1. Introduction

Due to its high water activity and content of nutrients, meat is a favorable environment for the growth of microorganisms. The main microbial populations associated with the spoilage of meat and meat products belong to the genera Bacillus, Pseudomonas, Carnobacterium, Enterococcus, Staphylococcus, Acinetobacter, Brochothrix, Lactococcus, Lactobacillus, Leuconostoc, Moraxella, Aeromonas, Acinetobacter, Arthrobacter, Flavobacterium, and Escherichia [1–3]. The pathogenic bacteria L. monocytogenes, Salmonella sp., Yersinia enterocolitica, and Staphylococcus aureus, pathogenic strains of E. coli and Pseudomonas aeruginosa, can also be detected in meat [4].

To inhibit the growth of microorganisms and spoilage processes, manufacturers add chemical food preservatives, including nitrates. Increasing consumer awareness of the use of chemical preservatives
in food is driving a demand for healthier alternatives. Researchers are considering natural methods of food preservation, including substances of animal origin (the peptides pleurocidin, defensins, and lactoferrin, as well as chitosan, lysozyme, and lipids), mushroom origin (fatty acids, polyphenols, lycopene, and polysaccharides), algae origin (fatty acids, steroids, and polyphenols), and microbial origin (the bacteriocins nisin and pediocin). However, plant substances, such as essential oils and extracts rich in bioactive compounds (polyphenols, saponins, and iridoids), have attracted the greatest attention [5–7]. Essential oils, due to their antioxidant and antibacterial properties, have been applied in meat products [8,9], but their organoleptic properties are a contentious issue.

Recently, polyphenolic extracts have been the subject of particular interest, due to their strong antimicrobial and antioxidant activity as well as health benefits. Polyphenols can be extracted from any part of a plant, including the fruits, buds, flowers, and leaves. However, the largest quantities are found in the leaves [10–12]. Therefore, leaf extracts have the potential to effectively inhibit the growth of microorganisms. So far, most studies have focused on tea leaf extracts [13], as well as some herbs and spices [14,15]. The antimicrobial activity of leaf extracts from fruit trees and shrubs has not been studied extensively. Extracts from olive leaves [16,17], walnut leaves [18], grape leaves [19], and eucalyptus leaves [20] have been investigated as potential antimicrobial agents.

Natural plant extracts have been applied in meat products to protect them from spoilage processes, extend their freshness, and inhibit the growth of pathogenic bacteria. Polyphenolic extracts have been found to prevent microbial growth, discoloration, lipid oxidation, and organoleptic changes, thereby improving meat quality. Leaf extracts have been suggested as an alternative to synthetic substances [21,22]. Cherry and blackcurrant [23], olive [24,25], mint [26], Phyllanthus acidus [27], and myrtle [28] leaf extracts have been used as natural preservatives in meat products.

The aim of this study was to investigate the antimicrobial activity of Aronia melanocarpa, Cornus mas, and Chaenomeles superba leaf extracts against typical spoilage and pathogenic bacteria found in meat and meat products. The precise compositions of the analyzed extracts were also studied, including the content of polyphenols and iridoids.

2. Results and Discussion

2.1. Composition and Antioxidant Activity of the Leaf Extracts

The highest total phenolic content (TPC) was detected in C. superba extract (3110.6 µg/mL), followed by C. mas (1867.7 µg/mL) and A. melanocarpa extracts (861.6 µg/mL). The extracts also showed strong antioxidant properties. The C. superba extract had the lowest IC50 values, according to both ABTS and DPPH assays (ABTS: IC50 = 6.2%; DPPH: IC50 = 4.8%), followed by C. mas (ABTS: IC50 = 6.8%; DPPH: IC50 = 8.7%) and A. melanocarpa (ABTS: IC50 = 44.6%; DPPH: IC50 = 18.2%) extracts (Table 1).

Table 1. Total phenolic content (TPC) and antioxidant activity in leaf extracts. The results are expressed as mean ± SD.

| Extract             | TPC [µg/mL] | Antioxidant Activity |   |
|---------------------|-------------|----------------------|---|
|                     |             | DPPH IC50 [%]        | ABTS IC50 [%] |
| Aronia melanocarpa  | 861.6 ± 23.0 c | 18.2 ± 0.50 a        | 44.6 ± 1.23 a |
| Chaenomeles superba | 3110.6 ± 86.1 a | 4.8 ± 0.71 c         | 6.2 ± 0.13 c  |
| Cornus mas          | 1867.7 ± 38.4 b | 8.7 ± 0.86 b         | 6.8 ± 0.18 b  |

a,b,c—statistically significant differences (p < 0.05).

We identified 15 phenolic compounds in the A. melanocarpa leaf extract (Table 2), including 4 phenolic acids and 11 flavonoids (flavonols). Among the phenolic acids we identified neochnorogenic, chlorogenic, caffeoyldeoxyhexose and p-coumaroylquinic acids. Unidentified hydroxycinnamic acid derivatives 1 ([M – H]− = 391), 2 ([M – H]− = 461) and 3 ([M – H]− = 705) were also detected. The A. melanocarpa...
extract contained flavonoids from the flavonols group: quercetin derivatives (quercetin-3-O-rhamnoside, quercetin-pentoside-deoxydihexoside, quercetin-3-O-dihexoside, quercetin-3-O-dirhamnosylhexoside, quercetin-3-O-vicianoside, quercetin-3-O-robinobioside, quercetin-3-O-rutinoside, quercetin-3-O-glucoside), isorhamnetin derivatives (isorhamnetin-3-O-rutinoside, isorhamnetin-hexoside-pentoside), and kaempferol-3-O-rutinoside. Interestingly, we identified hydroxytyrosol (a non-flavonoid compound typically found in olive extracts [29]) for the first time in \textit{A. melanocarpa}. In the \textit{A. melanocarpa} leaf extract, the total concentration of phenolic acids (114.66 µg/mL) was higher than that of flavonoids (93.40 µg/mL) (Table 3).

### Table 2. Identification of polyphenols and iridoids in leaf extracts.

| RT [min] | [M – H]⁺ | MS² | Compound Extract |
|----------|----------|-----|------------------|
|          |          |     | **Phenolic Acids *** |               |
| 3.19     | 337      | 179 | Caffeic acid derivative 1 | CM             |
| 3.25     | 309      | 179; 161 | Caffeoyl-deoxyhexose | AM             |
| 3.74     | 169      | -   | Gallic acid | CM             |
| 4.42     | 311      | 149; 179 | Caftaric acid isomer 1 | CM             |
| 6.01     | 391      | 183; 207; 211; 167; 323 | Hydroxycinnamic acid derivative 1 | AM             |
| 6.69     | 353      | 179; 191 | Neochlorogenic acid | AM, Ch         |
| 7.27     | 461      | 163; 177; 207; 297; 315 | Hydroxycinnamic acid derivative 2 | AM             |
| 7.59     | 705      | 513 | Hydroxycinnamic acid derivative 3 | AM             |
| 7.90     | 341      | 179; 161 | Caffeic acid dimer/caffeoyl hexoside | Ch             |
| 7.95     | 311      | 149; 179 | Caftaric acid isomer 2 | CM             |
| 8.17     | 337      | 163 | p-Coumaroylquinic acid isomer 1 | AM             |
| 9.24     | 353      | 191; 171 | Chlorogenic acid | AM, Ch         |
| 9.71     | 325      | 145; 163; 187 | p-Coumaroylhexoside isomer 1 | Ch             |
| 10.93    | 325      | 193 | p-Coumaroylhexoside isomer 2 | CM             |
| 11.25    | 337      | 191; 163 | p-Coumaroylquinic acid isomer 2 | Ch             |
| 11.88    | 431      | 341; 205; 367 | Hydroxycinnamic acid derivative 4 | Ch             |
| 12.46    | 337      | 191; 163 | p-Coumaroylquinic acid isomer 3 | Ch             |
|          |          |     | **Flavonols *** |               |
| 8.76     | 465      | 303; 285 | Dihydroquercetin hexoside | Ch             |
| 10.07    | 447      | 269; 401 | Quercetin-3-O-rhamnoside | AM             |
| 13.27    | 741      | 300/301; 489; 577; 409 | Quercetin-pentoside-deoxydihexoside | AM             |
| 13.37    | 625      | 300/301 | Quercetin-3-O-dihexoside | AM             |
| 13.82    | 755      | 300/301 | Quercetin-3-O-dirhamnosylhexoside | AM             |
| 14.42    | 609      | 301 | Quercetin-3-O-glucuronylpentoside | CM             |
| 14.72    | 595      | 301 | Quercetin-3-O-vicianoside | AM             |
| 14.99    | 593      | 447; 285 | Kaempferol-hexoside-deoxyhexoside | Ch             |
| 15.81    | 609      | 301 | Quercetin-3-O-robinobioside | AM             |
| 16.10    | 609      | 301 | Quercetin-3-O-rutinoside | AM, Ch, CM     |
| 16.16    | 477      | 301 | Quercetin-3-O-glucuronide | CM             |
| 16.42    | 463      | 301 | Quercetin-3-O-galactoside | Ch             |
| 16.80    | 463      | 301 | Quercetin-3-O-glucoside | AM, Ch, CM     |
| 17.58    | 609      | 315 | Isorhamnetin-hexoside-pentoside | AM             |
| 18.56    | 593      | 285 | Kaempferol-3-O-rutinoside | AM, Ch         |
| 19.19    | 461      | 285 | Kaempferol-3-O-glucuronide | CM             |
| 19.33    | 623      | 300; 315 | Isorhamnetin-3-O-rutinoside | AM             |
| 19.31    | 447      | 285; 327 | Kaempferol-3-O-hexoside | Ch             |
|          |          |     | **Flavones *** |               |
| 13.63    | 609      | 285; 447; | Luteolin-dihexoside | Ch             |
| 15.76    | 593      | 447; 431; 285 | Luteolin-3-O-rutinoside | Ch             |
|          |          |     | **Flavanones *** |               |
| 18.77    | 433      | 271 | Naringenin-7-O-glucoside | Ch             |
### Table 2. Cont.

| R_t [min] | [M – H]^+ | MS|^2 | Compound | Extract |
|----------|------------|------|----------|--------|
| Ellagittannins ** | | | | |
| 1.325 | 708−2 | 633; 301; 169 | Camptothin A isomer 1 | CM |
| 1.675 | 708−2 | 633; 301; 169 | Camptothin A isomer 2 | CM |
| 1.925 | 708−2 | 633; 301; 169 | Camptothin A isomer 3 | CM |
| 2.233 | 1100−2 | 633; 301; 169 | Cornusin F isomer 1 | CM |
| 2.45 | 783−2 | 301; 169 | Cornusin A isomer 1 | CM |
| 2.683 | 1100−2 | 633; 301; 169 | Cornusin F isomer 2 | CM |
| 2.817 | 708−2 | 633; 301; 169 | Camptothin A isomer 4 | CM |
| 3.200 | 783−2 | 301; 169 | Cornusin A isomer 2 | CM |
| Ellagic Acid ** | | | | |
| 11.85 | 301 | - | Ellagic acid | CM |
| Substituted Phenols * | | | | |
| 3.79 | 315 | 153 | Hydroxytyrosol | AM, Ch |
| Iridoids ** | | | | |
| 5.017 | 375 | 213 | Loganic acid isomer 1 | CM |
| 6.867 | 375 | 213 | Loganic acid isomer 2 | CM |
| 7.708 | 375 | 213 | Loganic acid isomer 3 | CM |
| 10.533 | 403 | 223 | Secoxyloganin | CM |
| 16.625 | 541 | 169 | Cornuside | CM |
| Unidentified Compounds * | | | | |
| 8.94 | 451 | 405 | Unidentified 1 | AM |
| 12.47 | 433 | 387 | Unidentified 2 | AM |
| 13.31 | 611 | 431; 251 | Unidentified 3 | AM |
| 14.04 | 649 | 605 | Unidentified 4 | Ch |
| 17.66 | 503 | 293; 457 | Unidentified 5 | AM |

*Compounds identified with LC-MS^n method according to 3.6.1. ** Compounds identified with UPLC–qTOF–MS/MS method according to 3.6.2. AM–Aronia melanocarpa. Ch–Chaenomeles superba. CM–Cornus mas.

### Table 3. Quantification of polyphenols and iridoids in leaf extracts (µg/mL).

| Compound | Aronia melanocarpa | Chaenomeles superba | Cornus mas |
|----------|-------------------|---------------------|----------|
| Phenolic Acids * | | | |
| Caffeic acid derivative 1 | - | - | 82.52 ± 6.60 |
| Caffeoyl-deoxyhexose | 3.65 ± 0.18 | - | - |
| Gallic acid | - | 1.61 ± 0.08 | - |
| Caftaric acid isomer 1 | - | - | 29.28 ± 1.46 |
| Hydroxycinnamic acid derivative 1 | 1.16 ± 0.06 | - | - |
| Neochlorogenic acid | 26.88 ± 1.88 | 97.23 ± 5.83 | - |
| Hydroxycinnamic acid derivative 2 | 11.44 ± 0.57 | - | - |
| Hydroxycinnamic acid derivative 3 | 13.41 ± 0.67 | - | - |
| Caffeic acid dimer/cafeoyl hexoside | - | 68.04 ± 5.44 | - |
| Caftaric acid isomer 2 | - | - | 27.54 ± 1.65 |
| p-Coumaroylquinic acid isomer 1 | 11.59 ± 0.81 | - | - |
| Chlorogenic acid | 46.53 ± 2.33 | 299.23 ± 14.96 | - |
| p-Coumaroylhexoside isomer 1 | - | 16.66 ± 0.83 | - |
| p-Coumaroylhexoside isomer 2 | - | - | 4.15 ± 0.21 |
| p-Coumaroylquinic acid isomer 2 | - | 143.62 ± 7.18 | - |
| Hydroxycinnamic acid derivative 4 | - | 13.72 ± 0.69 | - |
| p-Coumaroylquinic acid isomer 3 | - | 5.66 ± 0.40 | - |
| Total | 114.66 ± 6.50 | 644.16 ± 35.33 | 145.1 ± 10.01 |
| Compound                                      | *Aronia melanocarpa* | *Chaenomeles superba* | *Cornus mas* |
|----------------------------------------------|----------------------|-----------------------|-------------|
| **Flavonols *                              |                      |                       |             |
| Dihydroquercetin-hexoside                   | -                    | 84.50 ± 4.23          | -           |
| Quercetin-3-O-rhamnoside                    | 5.42 ± 0.27          | -                     | -           |
| Quercetin-pentoside-deoxydihexoside         | 2.00 ± 0.12          | -                     | -           |
| Quercetin-3-O-dihexoside                    | 10.63 ± 0.74         | -                     | -           |
| Quercetin-3-O-dihamnosylhexoside            | 17.36 ± 1.22         | -                     | -           |
| Quercetin-3-O-glucuronylpentoside           | -                    | 19.47 ± 1.36          | -           |
| Quercetin-3-O-vicianoside                   | 23.91 ± 1.20         | -                     | -           |
| Kaempferol-hexoside-deoxyhexoside           | 6.02 ± 0.36          | 8.25 ± 0.41           | -           |
| Quercetin-3-O-robinobioside                 | -                    | -                     | -           |
| Quercetin-3-O-galactoside                   | 15.28 ± 1.07         | 29.02 ± 1.74          | 7.68 ± 0.38 |
| Quercetin-3-O-glucuronide                   | -                    | -                     | 60.88 ± 4.26|
| Quercetin-3-O-galactoside                   | -                    | 96.78 ± 4.84          | 5.67 ± 0.28 |
| Isotheramatine-hexoside-pentoside           | 3.37 ± 0.17          | -                     | -           |
| Kaempferol-3-O-rutinoside                   | 2.92 ± 0.15          | 15.23 ± 0.76          | -           |
| Kaempferol-3-O-glucuronide                  | -                    | -                     | 17.96 ± 0.90|
| Isotheramatine-3-O-rutinoside               | 3.37 ± 0.24          | -                     | -           |
| Kaempferol-3-O-hexoside                     | -                    | 18.39 ± 1.29          | -           |
| **Total**                                   | 93.40 ± 5.66         | 252.17 ± 13.27        | 111.66 ± 7.19|
| **Flavones *                               |                      |                       |             |
| Luteolin-dihexoside                         | -                    | nd                    | -           |
| Luteolin-3-O-rutinoside                     | -                    | 17.28 ± 0.86          | -           |
| **Total**                                   | -                    | 17.28 ± 0.86          | -           |
| **Flavanones *                             |                      |                       |             |
| Naringenin-7-O-hexoside                     | -                    | 227.30 ± 11.37        | -           |
| **Total**                                   | -                    | 227.30 ± 11.37        | -           |
| **Ellagitannins **                          |                      |                       |             |
| Camptothin A isomer 1                       | -                    | -                     | 13.87 ± 1.54|
| Camptothin A isomer 2                       | -                    | -                     | 39.38 ± 1.45|
| Camptothin A isomer 3                       | -                    | -                     | 12.88 ± 0.10|
| Cornusin F isomer 1                         | -                    | -                     | 22.36 ± 1.17|
| Cornusin A isomer 1                         | -                    | -                     | 19.64 ± 0.25|
| Cornusin F isomer 2                         | -                    | -                     | 14.47 ± 1.02|
| Cornusin A isomer 3                         | -                    | -                     | 14.01 ± 1.47|
| Cornusin A isomer 4                         | -                    | -                     | 15.02 ± 1.77|
| **Total**                                   | -                    | -                     | 151.63 ± 8.77|
| **Ellagic Acid **                           |                      |                       |             |
| Ellagic acid                                 | -                    | -                     | 2.56 ± 0.27 |
| **Total**                                   | -                    | -                     | 2.56 ± 0.27 |
| **Substituted Phenols *                     |                      |                       |             |
| Hydroxytyrosol                               | 7.91 ± 0.40          | 75.00 ± 5.25          | -           |
| **Total**                                   | 7.92 ± 0.40          | 75.00 ± 5.25          | -           |
| **Iridoids **                               |                      |                       |             |
| Loganic acid isomer 1                        | -                    | -                     | 8.52 ± 0.58 |
| Loganic acid isomer 2                        | -                    | -                     | 12.76 ± 0.12|
| Loganic acid isomer 3                        | -                    | -                     | 2.96 ± 0.23 |
| Secoxyloganin                                | -                    | -                     | 6.21 ± 0.54 |
| Cornuside                                    | -                    | -                     | 3.45 ± 0.38 |
| **Total**                                   | -                    | -                     | 33.9 ± 1.85 |
| **Unidentified Compounds **                 |                      |                       |             |
| Unidentified 1                               | 4.02 ± 0.20          | -                     | -           |
| Unidentified 2                               | 6.67 ± 0.33          | -                     | -           |
| Unidentified 3                               | 6.67 ± 0.33          | -                     | -           |
| Unidentified 4                               | -                    | 63.49 ± 3.17          | -           |
| Unidentified 5                               | 3.29 ± 0.16          | -                     | -           |
| **Total**                                   | 20.65 ± 1.03         | 63.49 ± 3.17          | -           |

The results are expressed as mean ± SD. * compounds quantified with HPLC. PDA according to 3.7.1. ** compounds quantified with HPLC-PDA according to 3.7.2. nd–not detected with HPLC.
Teleszko and Wojdylo [12] identified only one additional phenolic compound, belonging to the flavan-3-ols (epicatechin), in *A. melanocarpa* leaves. Lee et al. [30], Tian et al. [31], Skupień et al. [33], and Skupień et al. [33] also found the following phenolic compounds from the group of phenolic acids: dicafeoylquinic, 3,4-dihydroxyphenylacetic, protocatechuic, caffeic, and *p*-coumaric acids. In addition, they detected the following flavonols: apigenin-7,4-di-*O*-rhamnoside, quercetin-3-*O*-galactoside, quercetin, quercetin-deoxyhexoside-deoxyhexoside, quercetin-deoxyhexoside, quercetin-rhamnosylhexoside, quercetin-arabinogalactoside kaempferol-coumaroylglucoside, kaempferol-hexoside-pentoside, isorhamnetin-rhamnosylhexoside isomers, and the non-flavonoid phenolic compound 4-(2-hydroxyethyl) phenolhexoside. Chlorogenic acid isomers were predominant in *A. melanocarpa* leaves, which is in agreement with our results. We found chlorogenic acid to be predominant, constituting 19.66% of all the compound present, followed by neochlorogenic acid at 11.35% (Table 3). Tian et al. [31] found similar percentages of chlorogenic acid isomers in *A. melanocarpa* leaves. In their study, chlorogenic acid constituted 17.75% and neochlorogenic acid 10.07% of all identified phenolic compounds. In our study, a quercetin derivative (quercetin-3-*O*-vicianoside) appeared in the highest concentration among the flavonoids and constituted 10.10% of all the presented compounds (Table 3). This is in agreement with the literature, where the highest concentrations of quercetin derivatives, such as quercetin-3-*O*-rutinoside [30], quercetin-3-*O*-arabinoglucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside [31], and quercetin [33] were detected in *A. melanocarpa* leaves.

In *C. superba* leaf extract (Table 2), we identified 6 phenolic acids: neochlorogenic, caffeic acid dimer/cafeoyl hexoside, chlorogenic, *p*-coumaroylhexoside isomer 1, *p*-coumaroylquinic acid isomers 2 and 3, and one unidentified hydroxycinnamic acid derivative 4 ([M − H] = 431). Among the flavonoids, flavonols (dihydroquercetin-hexoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-hexoside, kaempferol-3-*O*-rutinoside, kaempferol-hexoside-deoxyhexoside), flavanones (naringenin-7-*O*-hexoside), and flavones (luteolin-3-*O*-rutinoside, luteolin-dihexoside) were found, as well as hydroxytyrosol, a non-flavonoid phenolic compound also detected in *A. melanocarpa* extract.

To our knowledge, this is the first study of the composition of *C. superba* leaf extract. *C. superba* is a mixture of *Chaenomeles japonica* (Japanese quince) and *Chaenomeles speciose* (flowering quince). Therefore, some similarities in terms of phenolic composition may exist. *Chaenomeles japonica* leaves have been studied by Teleszko and Wojdylo [12] and Urbanaviciute et al. [34]. Similarly to our results for *C. superba*, the *C. japonica* leaves were found to contain chlorogenic acid and quercetin derivatives (galactoside, rutinoside, glucoside). In addition, *p*-coumaric acids were detected, as well as the flavan-3-ols catechin and epicatechin, and procyanidins B1, B2, B3, and C1. Ponder and Hallmann [35] and Turkiewicz et al. [36] characterized the fruits of *C. superba* and detected phenolic acids (gallic, chlorogenic, cryptochlorogenic, caffeic, *p*-coumaric acids), flavonols (quercetin-3-*O*-rutinoside, myricetin, quercetin, luteolin), and flavan-3-ols (catechin, epicatechin, procyanidins B2, B3, and C1, and unidentified procyanidin dimers, trimers, and tetramers). However, in our study no flavan-3-ols were detected, including procyanidins, which may be due to the different extraction methods used. Turkiewicz et al. [36] and Teleszko and Wojdylo [12] used methanol/water/acetic acid/ascorbic acid as a solvent, whereas we extracted the polyphenols with water. Water is not commonly used to extract flavan-3-ols, although Dhanani et al. [37] indicate that catechin, procyanidin B2, epicatechin, epigallocatechin gallate, and epicatechin gallate can be successfully extracted with water. Therefore, these compounds may not appear in *C. superba* leaves. Moreover, many other factors, such as plant variety/cultivar, the part of the plant, and the growing season, can influence the compositions of plants and their extracts [7].

In the *C. superba* extract, the total amount of phenolic acids (644.16 µg/mL of leaves) was higher than the total amount of flavonoids (496.75 µg/mL). Chlorogenic acid was a major component (23.39% of all the presented compounds). Naringenin-7-*O*-hexoside was the predominant flavonoid (17.76% of all the presented compounds) (Table 3). According to Urbanaviciute et al. [34], chlorogenic acid was also the predominant compound in *C. japonica* leaves, constituting 78.7–86.5% of the total polyphenols depending on the plant part and growing season.
on the cultivar. In a study by Ponder and Hallmann [35], C. superba, and C. japonica pear-shape fruits had the highest contents of total polyphenols among the C. superba species. In C. superba, the concentration of phenolic acids (10.69 mg/100 g fresh weight) was higher than the concentration of flavonoids (7.64 mg/100 g of fresh weight). Chlorogenic acid (4.57/100 g of fresh weight) was the predominant compound, which is in agreement with the results of our study. However, Turkiewicz et al. [36] found procyanidin B2 to be the predominant compound in all tested cultivars of C. superba fruit.

C. mas has attracted great interest in recent years, due to its beneficial properties such as antioxidative and anti-inflammatory effects and rich diversity of bioactive compounds [38,39]. However, the leaves of C. mas have not as yet been the subject of extensive research, and little is known about their composition. In our study, we identified 22 compounds in C. mas leaf extract (Table 2). Phenolic acids (gallic acid and caftaric acid isomers 1 and 2, p-coumaroylhexoside isomer 2) and flavonols (quercetin-3-O-glucuronylpentoside, quercetin-3-O-rutinoside, quercetin-3-O-glucuronide, quercetin-3-O-glucose, and kaempferol-3-O-glucuronide) were found. Unknown caffeic acid derivative 1 ([M – H]− = 337) was also detected.

Interestingly, caftaric acid isomers and quercetin-3-O-glucuronylpentoside have not been identified previously in C. mas leaves. However, recently Martinović and Cavoski [40] identified caftaric acid in C. mas fruits where it was the predominant compound (12.24 mg/100 g) among all phenolic acids. Badalica-Petrescu et al. [41] and Milenković-Andjelković et al. [42] analyzed the phenolic composition of C. mas leaf extract and found several different compounds belonging to the phenolic acids (p-coumaric acid derivative, chlorogenic acid, o-coumaroylhexoside, an unknown caffic acid derivative, unknown caffic acid hexosides), as well as flavan-3-ols (catechin, epicatechin) and flavonols (quercetin-3-O-galactoside, quercetin-3-O-galactoside-7-O-rhamnose, kaempferol-3-O-glucose, isorhamnetin-7-O-rhamnose, luteolin-3-O-glucoside). According to Milenković-Andjelković et al. [42], quercetin-3-O-glucoside was the predominant compound. In our study, the predominant compound was an unidentified caffeic acid derivative 1 ([M – H]− = 337), which constituted 18.55% of all the presented compounds, followed by quercetin-3-O-glucuronide (13.68% of all the presented compounds).

We also identified ellagic acid and ellagitannins in the C. mas extract. These phenolic compounds had not been detected previously in C. mas. According to Czerwińska and Melzig [39], tannins such as cornusiins and camptothis have been identified only in Cornus officinalis fruit. Gunduz et al. [43] measured the content of tannins in C. mas fruit at different stages of maturation, and found them to constitute 0.16–0.45% of fresh weight. In our study, we identified seven ellagitannins (camptothin A isomers 1–4, cornusiin F 1 and 2, cornusiin A 1 and 2). The ellagitannins constituted 34.08% of all the presented compounds. Camptothin A 2 appeared in the highest concentration, constituting 26.0% of all ellagitannins and 8.85% of all the compounds found in the C. mas extract. This suggests that ellagitannins are a significant group of compounds in C. mas leaves and their presence needs to be investigated further.

Apart from polyphenols, we also detected iridoids (loganic acid isomers, secoxyloganin, and cornuside), which are monoterpenoid compounds with strong antimicrobial, antioxidiant, anti-inflammatory, antitumor, and hepatoprotective activity [44]. Iridoids constituted 7.62% of all the presented compounds. Loganic acid isomer 2 was the predominant compound in this group, constituting 37.64% of all iridoids and 2.87% of all the presented compounds. To our knowledge, this is the first study to detect iridoids in C. mas leaves, although there have been previous reports of iridoids in C. mas fruit. Kucharska et al. [45] and Deng et al. [46] identified loganic acid, cornuside, sweroside, and loganin in C. mas fruit. Czyżowska et al. [47] also found loganic acid and cornuside in unripe C. mas fruit.

2.2. Antibacterial Activity of Leaf Extracts

We investigated the influence of A. melanocarpa, C. mas, and C. superba leaf extracts on the growth dynamics of meat spoilage bacteria. The maximum growth rate (µmax), time of lag phase (tLag), and maximum population density (log(Nmax)) were calculated. The Gompertz function used for data analysis appropriately describes the growth of bacteria, as confirmed by the high values for the
R² coefficient (0.9855 to 1.000). The Boltzman function was used to evaluate the effect of several concentrations of the extracts on $\mu_{\text{max}}$. The R² coefficient was in the range of 0.8902–1.0000 (Figure 1).

Our results indicate that *A. melanocarpa*, *C. superba*, and *C. mas* leaf extracts act as bacteriostatic agents. The extracts generally decreased $\mu_{\text{max}}$ and extended $t_{\text{Lag}}$, depending on the type of extract, concentration, and bacterial strain. We observed that $\mu_{\text{max}}$ changes according to the Boltzman function.
and is strictly related to the extract concentration. In some cases, the extracts slightly decreased log(N_{max}) and the differences were statistically significant. The greatest influence on the growth of almost all microorganisms was observed with an extract concentration of 5%. No difference was observed between using extract contents at 5% and 10% concentrations, especially regarding $\mu_{\text{max}}$ (Table 4, Figure 1, Table S1).

*C. mas* showed the highest antimicrobial activity. This suggests that antibacterial activity is not related to TPC, but rather to the composition of the extract. The *C. mas* extract contained iridoids and ellagitannins, which were not detected in the *A. melanocarpa* and *C. superba* extracts. Other studies confirm the strong antimicrobial activity of these compounds [48–50]. The *C. mas* extract showed the greatest inhibitory effect on *Moraxella osloensis* growth. Statistically significant changes in the growth parameters appeared already at a 1% concentration. At concentrations of 2% and higher, no bacterial growth was observed. We also noticed that the extract was more effective against Gram-negative bacteria than Gram-positive bacteria. The *C. mas* leaf extract reduced the $\mu_{\text{max}}$ of all Gram-negative bacteria at the lowest concentration (1%), except for *Pseudomonas fragi* (2%). The *C. mas* extract had a very strong influence on $t_{\text{lag}}$ of *P. fragi* and *Acinetobacter baumannii*, and at a concentration of 10% extended $t_{\text{lag}}$ for these bacteria by 23.02 h and 18.51 h, respectively. An effect was also observed on the log(N_{max}) of all Gram-negative strains. Of the extracts studied, *C. mas* had the strongest inhibitory effect on the pathogenic bacteria *L. monocytogenes*, reducing its $\mu_{\text{max}}$ 3.7-fold and its log(N_{max}) by 0.57 at a concentration of 10% (Table 4, Figure 1, Table S1).

Our studies are not in agreement with literature data, which suggest that Gram-positive bacteria are generally more sensitive to polyphenols than Gram-negative bacteria [14,51]. The cell walls of Gram-negative bacteria are covered by a lipophilic outer membrane, which results in less efficient permeability to hydrophilic substances. Moreover, enzymes in the periplasma space can damage molecules introduced from outside [52,53]. However, polyphenols can disintegrate the outer membrane of Gram-negative bacteria, leading to increased membrane permeability [54,55]. It should be emphasized that little is known about the antimicrobial activity of *C. mas* leaf extract. *C. mas* extract contains not only polyphenols, but also iridoids. Some interactions may occur between these compounds, explaining the stronger effect on Gram-negative bacteria in comparison to Gram-positive bacteria in our study. Moreover, the literature does not exclude the possibility of antibacterial activity against Gram-negative bacteria, which suggests that antibacterial activity may rather be related to the bacterial species or strain.

Only Milenković-Andjelković et al. [42] have discussed the antimicrobial activity of *C. mas* extract obtained from leaves, reporting that Gram-positive bacteria were more sensitive than Gram-negative bacteria. The most sensitive Gram-positive strains were *Sarcina lutea*, *Listeria monocytogenes*, and *Staphylococcus aureus*. The most sensitive Gram-negative strains were *Shigella sonnei* and *Salmonella enteritidis*. Dulger and Gonuz [56] analyzed the antibacterial activity of *C. mas* extract obtained from bark. The extract showed moderate antibacterial activity compared to other plant extracts, and inhibited the growth of *S. aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Micrococcus luteus*. No effect was observed for the bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Mycobacterium smegmatis*, or *L. monocytogenes*, nor for the yeasts *Candida albicans* and *Rhodotorula rubra*.

In our study, leaf extract from *A. melanocarpa* showed the weakest antibacterial activity. *L. monocytogenes* was most resistant bacteria to *A. melanocarpa*, which had no influence on its $\mu_{\text{max}}$ and $t_{\text{lag}}$ at any of studied concentrations. The most sensitive bacteria to *A. melanocarpa* were *S. aureus* and *Brochothrix thermosphaeta*. Of the extracts studied, *A. melanocarpa* had the strongest influence on the pathogenic bacteria *Salmonella enterica*, with statistically significant differences in $\mu_{\text{max}}$ and $t_{\text{lag}}$ observed even at the lowest concentration (1%). At a concentration of 10%, the extract reduced $\mu_{\text{max}}$ 2.2-fold and extended $t_{\text{lag}}$ by 1.06 h. No clear relationship was found between the influence of the plant extracts and whether the bacteria belonged to the Gram-positive or Gram-negative groups (Table 4, Figure 1, Table S1).
Table 4. Effect of leaf extracts on the lag time (tLag) and maximum population density (log10(Nmax)) of bacteria.

| Bacterial Strain | Extract Concentration [%] | Aromia melanocarpa | Chaenomeles superba | Cornus mas |
|------------------|----------------------------|--------------------|--------------------|------------|
|                  |                            | tLag [h]           | log10(Nmax)        | tLag [h]   | log10(Nmax) |
| Gram-Positive Bacteria |                        |                   |                   |            |            |
| Enterococcus faecium | [10.70 ± 0.17 A]       | 10.70 ± 0.14 A    | 11.58 ± 0.06 A    | 10.75 ± 0.38 B    | 10.75 ± 0.38 B    |
| Staphylococcus aureus | [11.97 ± 0.51 B]       | 11.97 ± 0.51 B    | 11.97 ± 0.51 B    | 11.97 ± 0.51 B    | 11.97 ± 0.51 B    |
| Brochothrix thermophosta | [18.45 ± 1.89 c]       | 18.45 ± 1.89 c    | 18.45 ± 1.89 c    | 18.45 ± 1.89 c    | 18.45 ± 1.89 c    |
| Lactobacillus sakei | [24.40 ± 2.07 a]       | 24.40 ± 2.07 a    | 24.40 ± 2.07 a    | 24.40 ± 2.07 a    | 24.40 ± 2.07 a    |
| Listeria monocytogenes | [10.67 ± 0.35 a]      | 10.67 ± 0.35 a    | 10.67 ± 0.35 a    | 10.67 ± 0.35 a    | 10.67 ± 0.35 a    |
### Table 4. Cont.

| Bacterial Strain | Extract Concentration [%] | \( t_{	ext{lag}} \) [h] | \( \log_{10}(N_{\text{max}}) \) | \( t_{	ext{lag}} \) [h] | \( \log_{10}(N_{\text{max}}) \) | \( t_{	ext{lag}} \) [h] | \( \log_{10}(N_{\text{max}}) \) |
|------------------|---------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| **Aronia melanocarpa** | | | | | | | |
| *Mesorhizobium phymatum* | 0 | 7.64 ± 0.16_ab | 8.49 ± 0.04_ab | 7.64 ± 0.16_c | 8.49 ± 0.04_a | 7.64 ± 0.16_b | 8.49 ± 0.04_a |
| | 1 | 5.00 ± 0.03_ab | 8.00 ± 0.01_ab | 5.00 ± 0.03_a | 8.00 ± 0.01_a | 5.00 ± 0.03_a | 8.00 ± 0.01_a |
| | 2 | 5.76 ± 0.05_ab | 8.76 ± 0.03_ab | 5.76 ± 0.05_a | 8.76 ± 0.03_a | 5.76 ± 0.05_a | 8.76 ± 0.03_a |
| | 3 | 6.00 ± 0.05_ab | 9.00 ± 0.03_ab | 6.00 ± 0.05_a | 9.00 ± 0.03_a | 6.00 ± 0.05_a | 9.00 ± 0.03_a |
| | 4 | 7.00 ± 0.05_ab | 10.00 ± 0.03_ab | 7.00 ± 0.05_a | 10.00 ± 0.03_a | 7.00 ± 0.05_a | 10.00 ± 0.03_a |
| **Chaenomeles superba** | | | | | | | |
| *Pseudomonas aeruginosa* | 0 | 7.64 ± 0.16_ab | 8.49 ± 0.04_ab | 7.64 ± 0.16_c | 8.49 ± 0.04_a | 7.64 ± 0.16_b | 8.49 ± 0.04_a |
| | 1 | 8.76 ± 0.05_ab | 9.00 ± 0.03_ab | 8.76 ± 0.05_a | 9.00 ± 0.03_a | 8.76 ± 0.05_a | 9.00 ± 0.03_a |
| | 2 | 7.64 ± 0.05_ab | 8.76 ± 0.03_ab | 7.64 ± 0.05_a | 8.76 ± 0.03_a | 7.64 ± 0.05_a | 8.76 ± 0.03_a |
| | 3 | 6.00 ± 0.05_ab | 9.00 ± 0.03_ab | 6.00 ± 0.05_a | 9.00 ± 0.03_a | 6.00 ± 0.05_a | 9.00 ± 0.03_a |
| | 4 | 7.00 ± 0.05_ab | 10.00 ± 0.03_ab | 7.00 ± 0.05_a | 10.00 ± 0.03_a | 7.00 ± 0.05_a | 10.00 ± 0.03_a |
| **Cornus mas** | | | | | | | |
| *Aronia melanocarpa* | 0 | 8.00 ± 0.05_ab | 8.76 ± 0.03_ab | 8.00 ± 0.05_a | 8.76 ± 0.03_a | 8.00 ± 0.05_a | 8.76 ± 0.03_a |
| | 1 | 8.76 ± 0.05_ab | 9.00 ± 0.03_ab | 8.76 ± 0.05_a | 9.00 ± 0.03_a | 8.76 ± 0.05_a | 9.00 ± 0.03_a |
| | 2 | 7.64 ± 0.05_ab | 8.76 ± 0.03_ab | 7.64 ± 0.05_a | 8.76 ± 0.03_a | 7.64 ± 0.05_a | 8.76 ± 0.03_a |
| | 3 | 6.00 ± 0.05_ab | 9.00 ± 0.03_ab | 6.00 ± 0.05_a | 9.00 ± 0.03_a | 6.00 ± 0.05_a | 9.00 ± 0.03_a |
| | 4 | 7.00 ± 0.05_ab | 10.00 ± 0.03_ab | 7.00 ± 0.05_a | 10.00 ± 0.03_a | 7.00 ± 0.05_a | 10.00 ± 0.03_a |

The results are expressed as mean ± SD, ab,ac—statistically significant differences between different concentrations of the extract (\( p < 0.05 \)), A,B,C—statistically significant differences between the same concentration of different extracts (\( p < 0.05 \)).
Tian et al. [57] investigated the antibacterial activity of A. melanocarpa leaf extract against E. coli, S. aureus, Bacillus cereus, L. monocytogenes, and S. enterica. Of the tested bacteria, E. coli showed the greatest resistance. No growth inhibition was observed when 10 µL of the extract was used. When 20 µL of extract was used, the cell suspension was reduced by 23%. Contrary to the results of our study, the most sensitive bacteria were L. monocytogenes and B. cereus, which were inhibited by 89% and 98%, respectively, by 20 µL of extract. The antibacterial activity of A. melanocarpa fruit extract was also studied. The extract revealed stronger activity against E. coli and L. monocytogenes compared to the leaf extract. Cvetanović et al. [58] analyzed the antimicrobial activity of A. melanocarpa extract from leaves, berries, and stems against two Gram-positive bacterial strains (S. aureus, Bacillus subtilis) and four Gram-negative bacterial strains (E. coli, K. pneumoniae, Proteus vulgaris, Proteus mirabilis), as well as against two fungal species (C. albicans and Aspergillus niger). The leaf extract showed the most potent activity against P. mirabilis (MIC = 19.53 µg/mL). The most resistant strains were K. pneumoniae, E. coli and A. niger (MIC = 312.5 µg/mL). Rauha et al. [59] studied the antimicrobial activity of A. melanocarpa berries. The A. melanocarpa extract inhibited the growth of M. luteus at a concentration of 1 mg/mL, and showed slight antimicrobial activity against S. aureus, B. subtilis, and E. coli, but was inactive against Staphylococcus epidermidis and fungi (A. niger and C. albicans).

In our study, C. superba extract showed slightly stronger antibacterial activity than the A. melanocarpa extract. The C. superba extract had the greatest effect on the t_{lag} of Lactobacillus sakei and P. fragi. The t_{lag} of L. sakei increased by 9.21 h and that of P. fragi by 9.68 h when C. superba was applied at a concentration of 10%. However, no effect was observed on the log(N_{max}) of any of the studied bacteria (Table 4, Figure 1, Table S1). To the best knowledge of the authors, there is no data in the previous literature regarding the antimicrobial activity of C. superba extract obtained from any parts of the plant. Previous studies have demonstrated the antibacterial activity of C. japonica extracts. Leaf extracts were found to be most active against Gram-positive S. aureus, but also showed activity against E. coli, P. aeruginosa, and C. albicans [60]. More research is needed into the composition and antimicrobial activity of C. superba. However, C. superba extract shows great biological potential, due to its high content and diverse range of phenolic compounds, as well as strong antioxidant activity.

Figure 2 presents a principal component analysis (PCA) of the growth parameters of all the investigated bacterial strains. PC1 and PC2 explain 70.91% of the total variance. All 34 variables had high significance for the separation of samples, with variable strengths of 0.838–0.996. Three main groups can be distinguished. All the samples with leaf extracts are separated from the control, indicating an inhibitory effect on bacterial growth. Samples with A. melanocarpa and C. superba belong to one cluster, suggesting similar antimicrobial activity. The samples with C. mas extracts are clearly separated from the others, confirming that C. mas extract had the strongest antibacterial activity.

The antibacterial activity of the extract is the result of the activity of all compounds and the interactions between them. Literature data show that flavonoids are the compounds of strong antibacterial properties. Their biological activity is well investigated. Flavonols most commonly identified in plants (quercetin, kaempferol, myricetin and its derivatives) as well as flavanones (naringenin found in citrus fruits), flavones (luteolin), chalcones and flavan-3-ols (catechins) were reported to inhibit bacteria growth [61]. In A. melanocarpa, C. mas and C. superba extracts we detected high amounts of quercetin and kaempferol derivatives. In C. superba extract naringenin and luteolin derivatives were also identified. However, no catechins of well documented antibacterial properties were found. Phenolic acids are poorly investigated in terms of their biological activity. The antibacterial activity of chlorogenic acid—most frequently found in plants—was demonstrated [62]. Studies show that ellagitannins [63] and iridoids [64,65] which we found in C. mas extract also reveal antibacterial properties, but they have not been widely examined.
The antibacterial activity of the extract is the result of the activity of all compounds and the interactions between them. Literature data show that flavonoids are the compounds of strong antibacterial properties. Their biological activity is well investigated. Flavonols most commonly found in plants are flavonol glycosides, flavone glycosides, flavanone glycosides, flavan-3-ols (catechins), and flavonols. In addition, flavonols are well-known for their antioxidant properties. Studies show that ellagitannins, iridoids, and polyphenols have been identified in leaves. The antibacterial activity of chlorogenic acid—most frequently found in plants—was demonstrated.

In summary, our studies show that *A. melanocarpa*, *C. superba*, and *C. mas* leaves constitute a rich source of bioactive compounds, mainly polyphenols from the phenolic acids and flavonoids groups. The *C. mas* extract was also found to contain iridoids. This is the first time these monoterpenoid compounds have been identified in *C. mas* leaves. Also for the first time, ellagitannins were detected in the *C. mas* leaves. The highest levels of polyphenolic compounds (TPC) were detected in *C. superba* leaf extract, which also showed the strongest antioxidant activity. We demonstrated the effect of *A. melanocarpa*, *C. superba*, and *C. mas* leaf extracts on the growth of spoilage and pathogenic bacteria found in meat and meat products. The extracts generally acted as bacteriostatic agents, influencing the bacterial growth parameters mainly by decreasing the growth rate (μ_max) and extending the lag phase (t_Lag). The strongest antibacterial activity was shown by *C. mas* leaf extract, followed by *C. superba* and *A. melanocarpa*. The *C. mas* leaf extract was more effective against Gram-negative bacteria than against Gram-positive bacteria. In terms of *A. melanocarpa* and *C. superba* extract, no clear relationship in Gram-staining was observed. It can be concluded that, due to the high content of bioactive compounds,
strong antioxidant capacity, and antibacterial properties, *C. mas*, *C. superba*, and *A. melanocarpa* leaf extracts have great potential for use as natural preservatives in meat products.

3. Materials and Methods

3.1. Plant Material

Plant material was collected in the region of Lodz (central Poland) on 15 July 2016. Leaves from *Aronia melanocarpa* (Michx.), Elliot (black chokeberry), and *Chaenomeles superba* Lindl. were collected in the village of Tymianka, and *Cornus mas* L. (*C. mas*) leaves from the village of Zadzim. Only fully developed, healthy leaves were selected. The plant material was transported to the laboratory, washed, and stored at −20 °C prior to extract preparation.

3.2. Bacterial Strains

The research material comprised typical spoilage and pathogenic bacterial strains found in meat and meat products (Table 5). The microorganisms were obtained from three culture collections, the American Type Culture Collection (ATCC, Manassas, VA, USA), the Polish Culture of Microorganisms (PCM, Wrocław, Poland), and the Collection of Industrial Microorganisms at the Institute of Fermentation Technology and Microbiology (LOCK, Łódź, Poland). Other strains were isolated from water, chicken meat, and meat packed in a modified atmosphere. These strains were genetically identified by 16S rRNA gene sequencing, and the sequences were deposited in the GenBank Database under appropriate accession numbers.

Table 5. Bacterial strains used in microbiological studies.

| Bacterial Strain | Source | Collection Number/Accession Number |
|------------------|--------|-----------------------------------|
| *Enterococcus faecium* WR1 | Water | MG911720 |
| *Staphylococcus aureus* | LOCK | MG911720 |
| *Brochothrix thermosphacta* MMAP4 | Meat packed in a modified atmosphere | HQ890943.1 |
| *Lactobacillus sakei* | ATCC | 15521 |
| *Listeria monocytogenes* | ATCC | 13992 |
| *Moraxella osloensis* | ATCC | 10973 |
| *Pseudomonas fragi* | ATCC | 4973 |
| *Acinetobacter baumanii* | ATCC | 19606 |
| *Escherichia coli* | ATCC | 10536 |
| *Enterobacter aerogenes* | PCM | 532 |
| *Salmonella enterica* MCH1 | Chicken meat | MG911721 |

3.3. Extraction of Polyphenols and Iridoids from Leaves

To extract polyphenols and iridoids, the leaves were crushed and approx. 200 g of each material was shaken for 1 h at 4 °C with 1000 mL of distilled water, then homogenized for 1 min at 20 °C. The samples were filtrated and centrifuged for 10 min at 10,000× g (5804R Centrifuge, Eppendorf, Hamburg, Germany). The supernatant was collected. The extracts were sterilized using microwaves (Microjet, EmbioTechnology, Oensingen, Switzerland), frozen at −20 °C and then stored prior to further analysis.

3.4. Total Phenolic Content

The total phenolic content (TPC) of the leaf extracts was determined using the Folin-Ciocalteu colorimetric method [68]. The reaction mixture contained the following components: 100 µL of polyphenolic extract; 200 µL of Folin-Ciocalteu reagent; 1 mL of 20% Na₂CO₃ solution, and 2 mL of distilled water. The blank sample contained 100 µL of distilled water instead of leaf extract. The solutions
were mixed and kept for 1 h in a dark place at room temperature. The absorbance was measured at 765 nm against the blank sample using a Cecil CE2041 spectrophotometer (Cecil Instruments Limited, Cambridge, UK). The TPC was quantified according to a calibration curve prepared for gallic acid and expressed as µg GAE/mL.

3.5. Antioxidant Activity

3.5.1. DPPH

Antioxidant activity was determined using the DPPH free radical scavenging assay, according to the methods described by Meda et al. [69] and Al et al. [70], with some modifications. The stock solution was prepared by dissolving 2.4 mg of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, St Louis, MO, USA) with 10 mL of 80% ethanol. To 1.95 mL of the DPPH free radical solution was added 50 µL of plant extract. The samples were left for 15 min in a dark place at room temperature. The absorbance was measured at 515 nm against 80% ethanol, using a Cecil CE2041 spectrophotometer (Cecil Instruments Limited, Cambridge, UK). The free radical scavenging activity determined by DPPH was expressed as the IC50 value (the concentration of extract required to inhibit 50% of the initial DPPH free radical).

3.5.2. ABTS

The ABTS radical scavenging assay was performed as described by Re et al. [71]. ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Sigma-Aldrich) was dissolved in water to a 7 mmol concentration. The ABTS radical cation (ABTS+) was obtained by reacting ABTS stock solution with 2.45 mmol K2S2O8 (final concentration), then kept in a dark place at room temperature for 12–16 h before use. The ABTS+ solution was next diluted to an absorbance of 0.700 at 734 nm. The reaction mixtures contained 3 mL of diluted ABTS+ solution and 30µL of plant extract. The samples were mixed and the absorbance measured after 10 min at 734 nm against distilled water. All analyses were performed in triplicate using a Cecil CE2041 spectrophotometer (Cecil Instruments Limited). The free radical scavenging activity determined by ABTS was expressed as the IC50 value (the concentration of extract required to inhibit 50% of the initial ABTS free radical).

3.6. Identification of Polyphenols and Iridoids

3.6.1. Identification of Phenolic Acids and Flavonoids with LC-MSn

Polyphenolic compounds were identified according to a previously published procedure [23]. The plant extracts were filtered through 0.45µm membrane filters prior to analysis. The HPLC was coupled on-line to an MS LTQ Velos mass spectrometer (ThermoScientific, Waltham, MA, USA). Chromatographic separation was achieved with a Hypersil Gold 150 × 2.1 column, particle size 1.9 µm (Thermo Scientific) operating at 45 °C. Detection wavelengths were set to 280, 320, and 360 nm. The mobile phase was a mixture of 0.01% formic acid (solvent A) and 95% acetonitrile (solvent B). The injection volume of the sample was 10 µL and the flow rate was 220 µL/min. The overall separation time was 55 min. The gradient elution applied was as follows: 8 min, 96–85% (A); 22 min, 85–70% (A); 10 min, 70–60% (A); 4 min, 60–50% (A); 3 min, 50% (A); 2 min, 50–96% (A); 6 min, 96%. The column was then washed and re-equilibrated.

Electrospray ionization mass spectrometry was performed using an LTQ Velos mass spectrometer (ThermoScientific) equipped with an ESI interface and controlled by Excalibur software. The analysis was performed in the negative mode across a range of 120–1000 m/z. The I spray voltage was 4 kV. The sheath gas flow rate was 25 and the aux gas flow rate 10. The desolvation temperature was 280 °C. The source temperature was 350 °C. Polyphenols were identified by comparison with the retention times and mass spectra of standards.
3.6.2. Identification of Ellagitannins and Iridoids by UPLC-qTOF-MS/MS

To identify ellagitannins and iridoids, the UPLC-qTOF-MS/MS method was applied, as described previously by Kucharska et al. [72]. An Acquity ultra-performance liquid chromatography (UPLC) system was used, coupled to a quadrupole-time of flight (Q-TOF) MS instrument (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) source. Separation was achieved on an Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm; Waters). The mobile phase was a mixture of 0.1% aq. formic acid v/v (A) and acetonitrile (B). The gradient program was as follows: initial conditions, 1% B in A; 12 min, 25% B in A; 12.5 min, 100% B; 13.5 min, 1% B in A. The flow rate was 0.45 mL/min and the injection volume 5 µL. The column was operated at 30 °C. UV-Vis absorption spectra were recorded on-line during UPLC analysis. Spectral measurements were made in the wavelength range of 200–600 nm, in steps of 2 nm. The major operating parameters for the Q-TOF MS were set as follows: capillary voltage, 2.0 kV; cone voltage, 40 V; cone gas flow, 11 L/h; collision energy, 28–30 eV; source temperature, 100 °C; desolvation temperature, 250 °C; collision gas, argon; desolvation gas (nitrogen) flow rate, 600 L/h; data acquisition range, m/z 100–2500 Da. The compounds were explored in the negative mode before and after fragmentation. The runs were monitored at the following wavelengths: iridoids at 245 nm; ellagic acid at 254 nm, ellagitanins at 280 nm.

3.7. Quantification of Polyphenols and Iridoids

3.7.1. Quantification of Phenolic Acids and Flavonoids Using HPLC-PDA

Polyphenolic compounds were quantified according to a previously published procedure [23]. Prior to analysis, the samples were filtered using 0.45 µm membrane filters. HPLC-PDA analyses were performed using a Finnigan Surveyor equipped with an autosampler and a diode array detector (Finnigan Surveyor PDA Plus Detector, Thermo Scientific), controlled with ChromQuest 5.0 chromatography software (Thermo Fisher Scientific Inc.). Separation was achieved using a Spherisorb ODS2 column (250 × 4.6 mm × 5 µm packing) (Waters), protected with a guard column of the same material. The volume of the injected sample was 50 µL, the flow rate was 0.8 mL/min, and the separation time was 60 min. The mobile phase consisted of 5% formic acid (solvent A) and 95% acetonitrile (solvent B). The samples were eluted with the following gradient: 2 min, 97% (A); 13 min, 97–85% (A); 9 min, 85–82% (A); 31 min, 82–75% (A); 5 min, 75–70% (A). Polyphenols were quantified according to calibration curves established for gallic acid (280 nm), caffeic acid (320 nm), and quercetin-glucoside (360 nm). The concentration of polyphenols was expressed as µg/g of leaves.

3.7.2. Quantification of Ellagitannins and Iridoids Using HPLC-PDA

To quantify the ellagitannins and iridoids, the HPLC-PDA method used, as described previously by Kucharska et al. [72]. The analysis was performed using a Dionex (Germering, Germany) system, equipped with an Ultimate 3000 diode array detector, an LPG-3400A quaternary pump, an EWPS-3000SI autosampler, and a TCC-3000SD thermostated column compartment. A C5-C18 Cadenza Imtakt column (75 × 4.6 mm, 5 µm) was used. The mobile phase was composed of solvent A (4.5% aq. formic acid, v/v) and solvent B (100% acetonitrile). The elution system was as follows: 0–1 min 5% B in C, 20 min 25% B in A, 21 min 100% B, 26 min 100% B, 27 min 5% B in A. The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 20 µL. The column was operated at 30 °C. Iridoids were detected at 245 nm, ellagic acid at 254 nm, ellagitanins at 280 nm. Isomers of loganic acid were expressed as loganic acid. Isomers of secoxyloganin and cornuside were expressed as loganin. Isomers of ellagic acid were expressed as the appropriate standards. Isomers of ellagitanins were expressed as gallic acid. All results were expressed as µg/mL.

3.8. Antibacterial Activity

The effect of the plant extracts on the growth of the bacteria was investigated using the microculture method (microliter plate assay). The bacterial strains were cultured for 24 h at 30 °C in a Tryptic Soy
Broth (TSB) medium. The extracts were added to the media at working concentrations of 1, 2, 3, 5, and 10%. The media were inoculated with bacterial strains. The control samples consisted of media containing only polyphenolic extracts and bacterial cultures without extracts. The wells of 96 microliter plates were filled with 200 µL samples and incubated at 30 °C until stationary phase was achieved. Bacterial growth was determined by measuring absorbance at 540 nm using a microliter plate reader (Biogenet, Józefów, Poland). Calibration curves were prepared for all bacterial strains and the OD$_{540}$ values were converted into CFU/mL.

3.9. Mathematical Models

Sigmoidal (S-shaped) curves produced by Gompertz and Boltzman functions are commonly used in biological studies to describe growth patterns. The cell numbers were fitted to the Gompertz equation using a Microsoft Excel add-in, DMFit 2.1 (Institute of Food Research, Norwich, UK): 

$$L(t) = A + C \exp\{−\exp[−B (t − M)]\}.$$  

The following growth parameters were calculated: maximum specific growth rate $\mu_{\text{max}} = BC/\text{e}$; lag time $t_{\text{lag}} = M − (1/B)$; maximum population density $\log_{10}(N_{\text{max}}) = A + C$.

Using the Origin 6.1. software (OriginLab, Northampton, MA, USA), a Boltzmann function producing a sigmoidal curve was fitted to describe the effect of different concentrations of plant extracts on $\mu_{\text{max}}$. The function was expressed by the following equation: 

$$y = A_2 + (A_1 - A_2)/(1 + \exp((x - x_0)/d_x)),$$ 

where $A_1$ = initial value, $A_2$ = final value, $x_0$ = center, $d_x$ = time constant.

3.10. Statistical Analysis

Mean values and standard deviations (SD) were calculated using Microsoft Excel 2013 (Microsoft, Waszyngton, WA, USA). Analysis of variance (one-way ANOVA) was performed using R 3.4.0 software (R Core Team, Vienna, Austria). Tukey’s Honestly Significant Differences (HSD) test was used to determine differences between variables ($p < 0.05$).

3.11. Chemometric Analysis

Principal component analysis (PCA) was performed using the Statistica 13 software (StatSoft, Poland, Kraków) to separate the leaf extracts, based on their inhibitory effects on the bacterial growth parameters.

Supplementary Materials: The following are available online, Table S1: Effect of leaf extracts on the maximum growth rate ($\mu_{\text{max}}$) of bacteria.

Author Contributions: M.E.-S., A.C. and A.N. conceived and designed the experiments; M.E.-S. and A.C. performed HPLC-PDA and LC-MSn analysis (polyphenols); A.Z.K. and I.F. performed HPLC-PDA and UPLC-qTOF-MS/MS analysis (ellagitannins and iridoids); M.E.-S. Prepared extracts and performed TPC, DPPH, ABTS and microbiological analysis. M.E.-S. Performed statistical and chemometric analysis; M.E.-S., A.C. and A.N. wrote the article. All authors have read and agreed to the published version of the manuscript.

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