High Added-Value Compounds with Antibacterial Properties from Ginja Cherries By-products

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Abstract

Purpose To test the antimicrobial properties of the extracts of stems and leaves of Ginja cherry plant. Both stems and leaves are waste in the production of the cherry liquor and they could be valorised by extracting valuable compounds, making the process more environmentally sustainable.

Methods The ethanol extracts from both stems and leaves were analysed by LC-ESI/MS to determine the phenolic composition. They were tested against Gram positive and Gram negative bacteria (*Bacillus subtilis*, *Staphylococcus aureus* MSSA, *Staphylococcus aureus* MRSA, *Pseudomonas* sp., *Pseudomonas aeruginosa*, *Flavobacterium* sp., *Escherichia coli*, *Salmonella*), using the disk diffusion technique and the broth dilution technique.

Results The extracts showed good antibacterial properties towards Gram positive and Gram negative bacteria. The values of the Minimum Inhibitory Concentration (MIC) were lower for Gram positive bacteria (10–15 mg/ml) than for Gram negative ones (10–100 mg/ml). The values of Minimum Bactericidal Concentration (MBC) were between 2 and 4 times higher than the MICs.

Conclusions The waste from Ginja cherry plants can be successfully employed to extract valuable compounds such as polyphenols, with antibacterial properties.

Keywords Agricultural waste · Cherry by-products · Polyphenols · Antibacterial properties

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Introduction

The production of large quantities of waste is nowadays a cause of great concern all over the world; considering for instance the European Union area only, about 2 billions tonnes of wastes were produced in the year 2006. Furthermore, an increase in the waste production is estimated: in fact by the year 2020 the production of waste will be 45% higher than it was in 1995. Agricultural waste constitutes about 30% of the total generated waste, therefore the treatment or valorisation of these products is a very important sector [1].

Agricultural waste consists of both natural and non-natural waste; currently there are EU legislations for the disposal of both kind of waste, depending on the nature of the substances and on the potential hazard they can cause to the environment [2].

In recent years, there has been an increasing interest in the recovery and reuse of some of the materials from the agricultural waste, especially for natural ones. Natural wastes are often parts of plants/vegetables not used in the food production process or by-products, whose disposal is a cost for the manufacturer. In many cases these by-products may contain very high value species, showing bioactive properties; their recovery and reuse in other fields (i.e. pharmaceutical, food and cosmetic), has been subject of several studies.

Phenolic compounds are a class of molecules with many interesting properties; they are in fact reported to have an antioxidant and anti-inflammatory action [3]; furthermore, they show anti-allergic, anti-mutagenic, anti-ageing and antibacterial activity [4–6].

Several studies were reported in literature about the recovery of phenolic compounds from agricultural waste: in their review on this topic, Moure et al. [7 and reference

therein] report about many applications of different parts of plants, including fruit seeds, potato peel waste and fruit husk. Considering more recent publications, the extraction of polyphenols from Olive Mill Wastewater and Cake [8, 9], olive leaves [10], waste from the production of fruit juice [11], from the production of fruit and vegetable [12], walnut and cashew nut by products [13, 14] were reported.

Ginja cherry (variety *Prunus cerasus*, L. Rosaceae) is a kind of cherry native from Portugal; it is mainly used to produce Ginjinha, a traditional Portuguese liquor. Both the stems and the leaves of the plants are solid waste products; they can be considered for the extraction of valuable compounds such as polyphenols.

Previous work done before on these materials [15] showed that the extracts of both stems and leaves are rich in polyphenols; the composition—both qualitative and quantitative—of the extracts changed depending on the solvent used for the extraction. These extracts showed antioxidant properties; these preliminary data confirmed the importance of these by-products and the potential for their use.

In this work, we report about the antibacterial properties of these extracts; in fact, the presence of both antioxidant and antibacterial properties for the extracted compounds would make them even more valuable and with more varied applications, for instance for fields like food additive or food active packaging components. Ethanol extracts were chosen, because they were the ones with the highest antioxidant activity. The antimicrobial properties were tested on several microorganisms, isolated from different sources (soil, clinical and food products), both Gram positive and negative.

Materials and Methods

Samples

Ginja cherry (variety *Prunus Cerasus*) stems and leaves were obtained by a Ginjinha liquor producer (Frutobidos, Óbidos, Portugal). They were collected in July 2009; just after the collection, stems were manually separated from leaves and then they were both stored at −25°C, until the start of the extraction process.

Solvent Extraction of the Phenolic Compounds

Prior to extraction, either stems or leaves were dried at 45°C and grinded into fine powder, with a kitchen grinder (Ciatronic, Germany). The ethanol extracts were obtained by subjecting powdered samples to maceration in 70% aqueous ethanol solution (Merck, Darmstatd, Germany) at room temperature for 20 h under continuous stirring. The mixture was filtered through Whatman (no. 2) paper under vacuum and the clarified extract was collected. After filtration, clarified extracts were evaporated to dryness by rotary evaporator (Buchi, Switzerland) at 50°C. The dried extracts were kept under nitrogen and stored in freezer at −25°C for further analysis. The yields of the dried extracts of stems and leaves, referenced to 100 g of dry samples, were 17.09 and 17.63% respectively.

LC-ESI/MS Analysis

The chromatographic system consisted of a Prostar 210 LC pump (Varian, CA, USA) coupled with a Varian 1200 triple quadrupole mass spectrometer (Varian, CA, USA) with electrospray ionization in positive and negative modes. A 5 μm C18 column (4.6 mm × 100 mm, Merck) was used for the separation at a flow rate of 0.4 ml/min. For the analysis, a LC/MS/MS method has been developed. The separation was performed by gradient elution (eluent A, water with 0.1% formic acid; eluent B, 100% methanol) in 33 min. For MS/MS fragmentation, Argon atoms were used (pressure 1.20 mtorr; collision energy of 15 V). Data were acquired by Varian LC-MS 1200L Workstation.

Antibacterial Activity

The antibacterial activity of the natural extracts was tested against a total of eight different bacteria. Isolates used were obtained from environmental, clinical and food samples. The Gram-positive bacteria tested were: *Bacillus subtilis* (isolated from soil, Accession number GU930753), *Staphylococcus aureus* (Methicillin Sensitive *Staphylococcus aureus* NCTC 8532 (MSSA)), *Staphylococcus aureus* (Methicillin Resistent *Staphylococcus aureus* ATCC 29213 (MRSA)); for the Gram-negative species, *Pseudomonas* sp. (isolated from soil, Accession number GU930780), *Pseudomonas aeruginosa* (isolated from a food source, internal collection), *Flavobacterium* sp. (isolated from soil, Accession number GU930759), *Escherichia coli* (*Escherichia coli* NCTC 9001), *Salmonella* (*Salmonella* spp. ATCC 3076) were used.

These microorganisms were cultured aerobically in Agar Mueller Hinton (Sigma, Aldrich, UK), at 37°C, for 24 h. Fully grown cultures were used as inoculum source for each experiment.

To test the antibacterial activity, the dried ethanolic stems and leaves extracts were dissolved in 1% (v/v) solution of ethanol to a final concentration of 0.25 g/ml. A preliminary screening of the extracts antibacterial properties was performed using the disk diffusion technique [16]. Briefly, liquid culture of each microorganism was prepared in 1% KCl solution with an optical density adjusted to 0.2 at $\lambda = 610$ nm, corresponding to about 10^8 CFU/ml.

Bacterial cultures were then spread onto Mueller Hinton Agar and blank sterile disks (6 mm diameter, Oxoid, UK) were placed on the inoculated agar. Blank discs were impregnated with 40 or 80 µl of extracts solution. Discs were allowed to dry at room temperatures and then incubated at 37°C for 24 h. A maximum of two disks were placed on each plate. The antibacterial activity was determined considering the inhibition halo on the bacterial growth. A negative control was performed using a KCl solution containing no extract but 1% (v/v) ethanol.

Minimum antibacterial concentration (MIC) and minimum bactericidal concentration (MCB) were also assessed. Bacterial liquid culture was prepared in Mueller Hinton (MH) broth, with an approximate concentration of 10^8 CFU/ml. Solutions with 1% of inoculum were used on variable concentrations of the extracts prepared in MH broth, with a final volume of 500 µl.

To determine MIC, extract samples with concentration between 10 and 100 mg/ml were used. For concentration up to 40 mg/ml, the bacterial growth was followed during 24 h, at 37°C, using a Microplate Optima—BMG Labtech, measuring the absorbance at $\lambda = 610$ nm. Negative controls were performed, using a solution of 1% ethanol in MH broth, and a biotic control were also made, and both were inoculated with 1% inoculum. For concentration higher than 40 mg/ml, the bacterial growth was evaluated considering the cloudiness of the solution. The lowest extract concentration which inhibited completely the bacteria growth after 24 h was determined as the MIC value.

Similarly, for the evaluation of the MBC value, solutions with extract concentration equal or higher to the MIC values were considered, with a maximum concentration of 100 mg/ml. Bacterial cultures were incubated at 37°C for 24 h, then 50 µl for MIC and highest concentrations were inoculated on Agar Mueller Hinton plates by spread plate technique, and the plates were incubated again at 37°C, for 24 h. The lowest extracts concentration that showed no growth on the plate was the MCB value.

All tests were performed in triplicate, with the average values considered ± the standard deviation.

The growth curves recorded with the Microplate Optima instrument were used to calculate the bacterial lag time and growth rate; the latter was calculated considering the slope of the plot of the natural logarithm of the absorbance versus time.

Results

The composition of the extracts, determined by LC-ESI/MS, is reported in Table 1; it can be seen how the total phenolic composition of stems extracts is higher than the one of leaves. Table 2 shows the ratios between the concentrations of some polyphenols detected in the extracts; a difference between the stems and the leaves can be observed.

Table 1 Composition of the polyphenols extracts (µg/ml) determined by LC-MS

Compound	Stems	Leaves
Protocatechuic acid	33.82 ± 0.62	13.51 ± 2.14
p-Coumaric acid	20.20 ± 0.58	21.20 ± 1.50
Ferulic acid	43.27 ± 0.71	ND
Naringenin	28.36 ± 5.59	ND
(+)-Catechin	567.95 ± 7.50	73.37 ± 1.33
Chlorogenic acid	93.94 ± 8.41	23.11 ± 1.32
Quercetin	4.04 ± 0.61	ND
Total phenolic content	791.58	131.19

Figure 1 shows as an example the results of the disc diffusion technique for stems extracts against *Flavobacterium*: it can be seen the formation of a clear area of inhibition around both discs, and that the area is bigger for the discs impregnated with higher volume of extracts. Tests for other bacterial strains for both stems and leaves extracts gave unclear results; for instance stems extracts gave positive results for *Pseudomonas* sp. and *Bacillus subtilis*, but not for other strains. However, as previously reported [17], the absence of an inhibition area does not always mean that the tested compounds are inactive towards the bacteria, because the concentration tested in the disk may be insufficient to induce inhibition or non-polar compounds may not diffuse into the medium.

To confirm and refine results on the antimicrobial activity and to determine the MIC and MBC values, the broth dilution technique experiments was performed. Figures 2 and 3 report the growth curves for each bacteria as a function of time, for stems and leaves extracts respectively; the growth with no extract in solution ($c = 0$) is compared with the ones with extracts in variable concentration.

The curves corresponding to the solutions with extracts not inoculated (not reported in the figures) did not evidence any change in the absorbance with time; this indicate that polyphenols do not degrade and/or undergo any reaction during the experiment, and no contamination was observed. These curves were considered as baseline and subtracted from the growth curves corresponding to the inoculated extract solutions.

Considering stems extract (Fig. 2), it can be seen how they show antibacterial activity against the majority of the strains tested: in fact, they inhibit completely the growth of all tested Gram positive bacteria—*Bacillus subtilis*, *Staphylococcus aureus* MSSA, *Staphylococcus aureus* MRSA—and *Flavobacterium* for $c < 15$ mg/ml. For Gram negative bacteria, a complete inhibition is observed for

Table 2 Ratios between some polyphenols concentration for leaves and stem extracts

Compounds	Concentrations ratio for stems	Concentrations ratio for leaves
p-Coumaric acid/ protocatechuic acid	0.6	1.6
(+)-Catechin/ protocatechuic acid	16.8	5.4
(+)-Catechin/*p*-coumaric acid	28.1	3.5

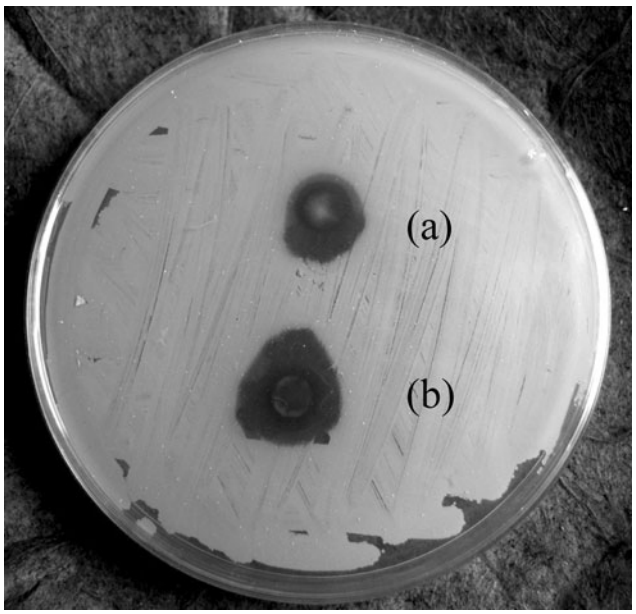

Fig. 1 Agar diffusion test for stems extracts against *Flavobacterium*; extract volume: (*a*) 40 µl, (*b*) 80 µl

Pseudomonas sp., even if at higher concentration (30 mg/ml); for *E. coli* and *Salmonella*, on the contrary, there is only a slight inhibition of the growth for $c > 30$ mg/ml, while no effect was observed towards *Pseudomonas aeruginosa*. For leaves extracts, the same pattern can be observed: in fact the same five bacteria strains are completely inhibited, *E. coli* and *Salmonella* are only partially inhibited and *Pseudomonas aeruginosa* is not affected at all.

Although the growth curves for leaves and stems extracts are similar for susceptible bacteria, comparing the values of MIC (Tables 3, 4), it can be seen how a higher concentration of leaves extracts is necessary to have a complete inhibition of the bacterial growth; the only exception is for *Pseudomonas* sp., where both stems have leaves extracts showed a MIC of 30 mg/ml.

Regarding the values of the MBC, a similar trend can be observed; in fact the extracts from the stems showed to be more active, since they present values 2 or 3 times lower than the ones corresponding to the leaves. Furthermore both extracts do not show any bactericidal activity towards *Pseudomonas aeruginosa*, *E. coli* and *Salmonella*.

Tables 5 and 6 report the values of the bacterial growth rate and the lag time for the stem and leaves extracts, respectively. It can be seen how they are both affected by the presence of the extracts, even if in a different way, depending on the nature of the microorganism and the concentration of the extracts. As expected, in most cases the values of the growth rate decrease while the ones of the lag time increase if the extracts are present; there are, however, some exceptions, for instance *Pseudomonas aeruginosa* has a more irregular behaviour with both types of extracts. Also, the effect on the lag time is more enhanced for the leaves extracts.

Discussion

Table 1 reports the composition of the ethanolic extracts from stems and leaves; the conditions and the procedure for the extraction were chosen considering the instability of polyphenolic compounds at higher temperatures. For this reason, different extraction techniques such as Soxhlet extraction could not be used, because of the degradation of thermolabile compounds associated with it [18].

Stems extracts show a higher total phenolic content compared to leaves (Table 1); therefore it is expected that the stems extracts show higher antimicrobial activity.

Most species detected in the extracts have been reported to have antibacterial activity, for example ferulic and *p*-coumaric acid is effective against *E. coli* and *Staphylococcus aureus* [19], protocatechuic acid against *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *E. coli* [6, 20], naringenin against several strains of *Staphylococcus aureus* [21]. However, the concentration in the extracts of some of the phenolic compounds is lower than the MIC value reported in literature. For instance MIC for protocatechuic acid against *E. coli* is 2667 µg/ml [6], while in stems extracts the concentration is only 33.8 µg/ml. (+)-Catechin is the polyphenol with the highest concentration in both extracts; however, its antimicrobial activity is not very high [22]. This can explain why the values of MICs for these extracts are slightly higher compared to some other extracts from natural sources [6, 17].

Our extracts, however, show antibacterial properties towards all studied Gram positive and Gram negative species; this is not always observed for natural extracts: extracts from walnut leaves, for instance, do not show any activity towards Gram negative bacteria [13]. Similar results are reported about extracts from *Cirsium* plants leaves [23].

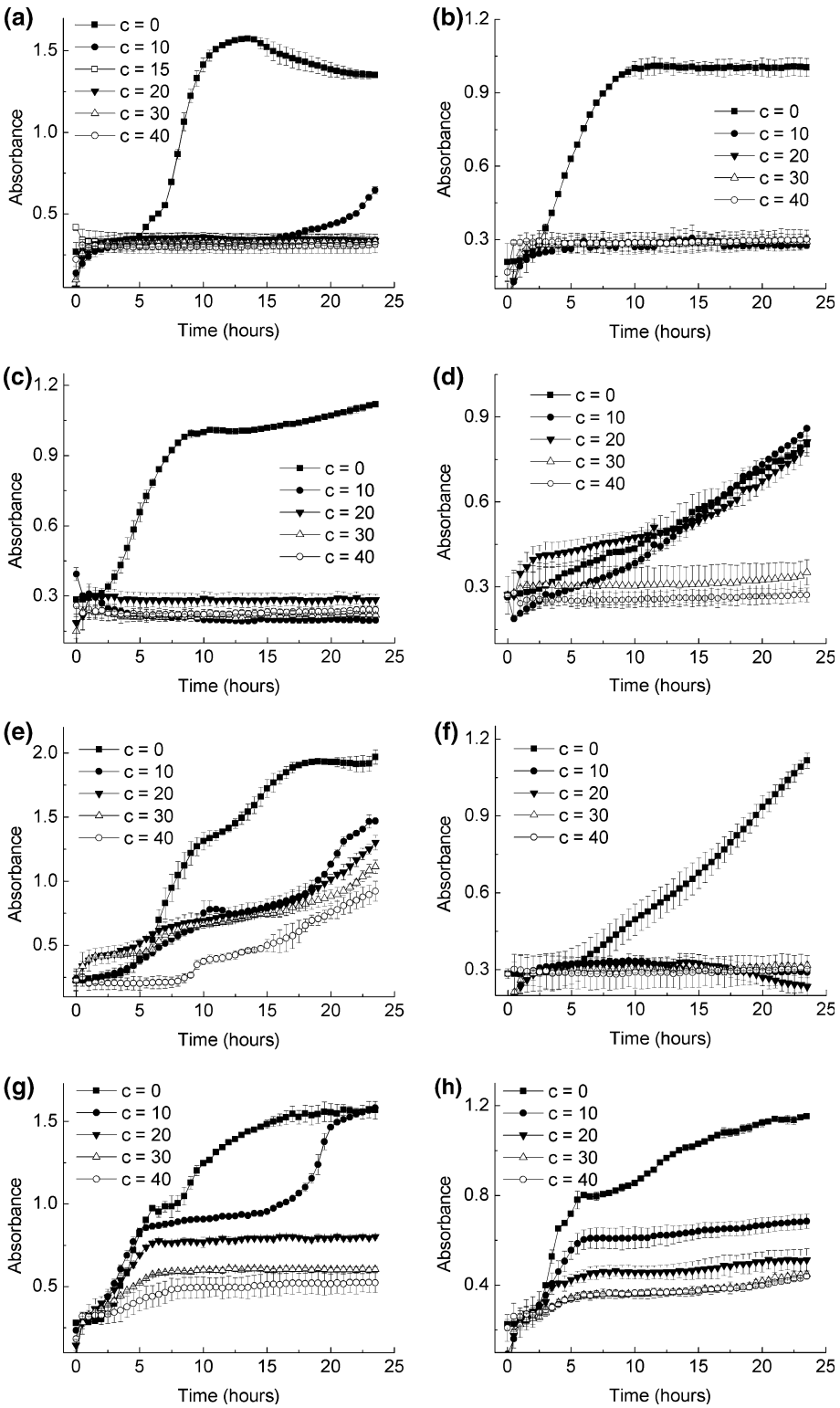

Fig. 2 Growth curves obtained by absorbance reading at 610 nm, for different bacterial strains in solutions containing different concentrations of stem extracts. **a** *Bacillus subtilis*, **b** *Staphylococcus aureus* MSSA, **c** *Staphylococcus aureus* MRSA, **d** *Pseudomonas* sp., **e** *Pseudomonas aeruginosa*, **f** *Flavobacterium*, **g** *E. coli*, **h** *Salmonella*. All concentrations are expressed in mg/ml

Compared with other strains, *Pseudomonas* sp. (isolated from tannin wastewater wetland) was resistant to higher concentrations of polyphenols in the extracts. This is in agreement with literature data: in fact previous study has demonstrated that *Pseudomonas* sp. are not only tolerant but can also degrade some type of polyphenol (as tannic acid) [24]. Chung suggested that polyphenol antimicrobial activity could be due to their strong binding capacity to iron complex [25]. However, the inhibition mechanism remains unclear and needs to be understood more clearly.

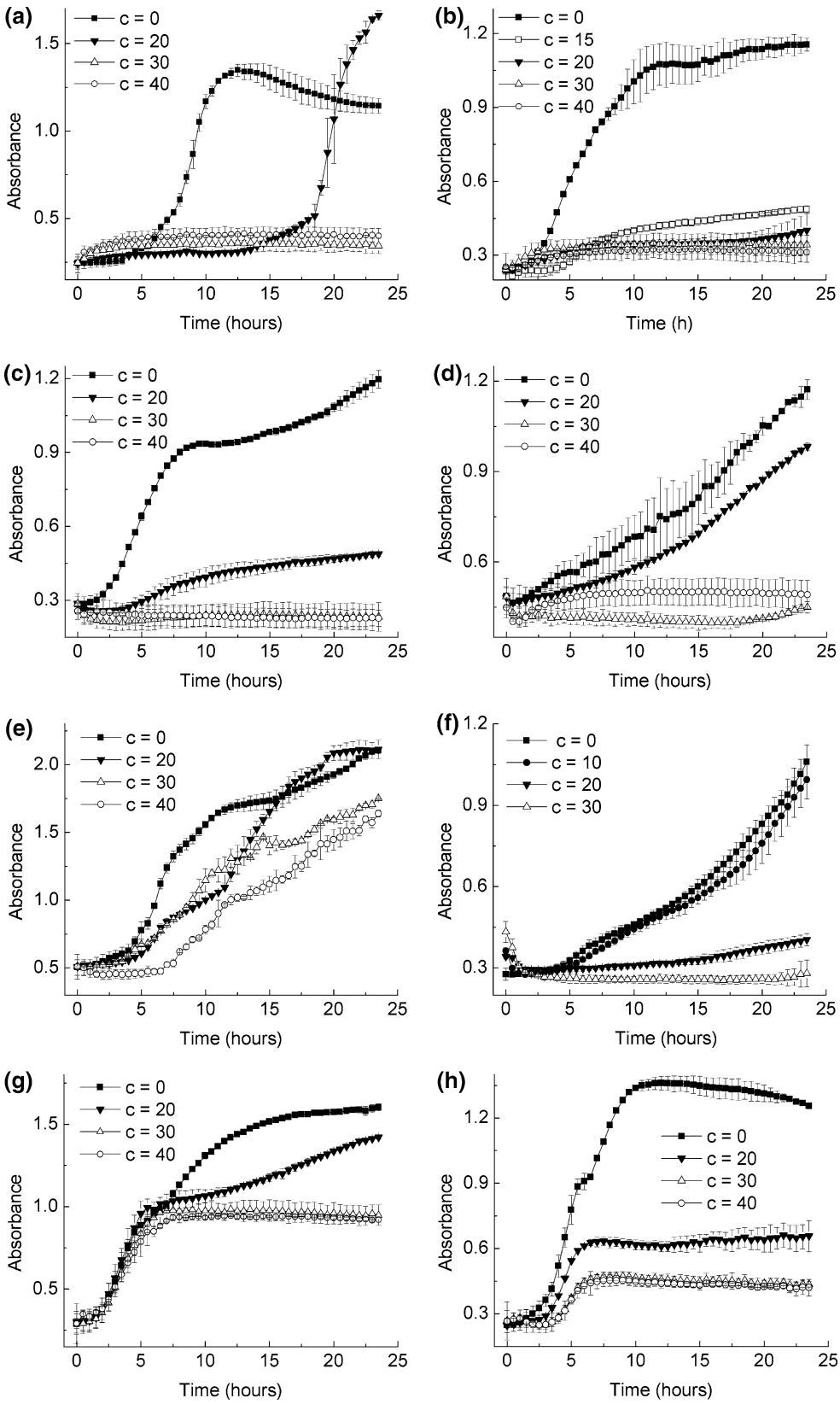

Fig. 3 Growth curves obtained by absorbance reading at 610 nm, for different bacterial strains in solutions containing different concentrations of leaves extracts. **a** *Bacillus subtilis*, **b** *Staphylococcus aureus* MSSA, **c** *Staphylococcus aureus* MRSA, **d** *Pseudomonas* sp., **e** *Pseudomonas aeruginosa*, **f** *Flavobacterium*, **g** *E. coli*, **h** *Salmonella*. All concentrations are expressed in mg/ml

The effect of natural compounds on the growth rate and lag time of microorganisms was previously reported before; for instance Munoz studied the behaviour of *Listeria monocytogenes* in the presence of natural essential oils [26], while Karanika analysed the effect of plant natural extracts on *Yarrovia lipolitica* [27]. Both studies

Table 3 Values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for stems extracts

Bacterial strain	MIC (mg/ml)	MBC (mg/ml)
Bacillus subtilis	15	60
Staphylococcus aureus MSSA	10	40
Staph. aureus MRSA	10	30
Pseudomonas sp.	30	40
Pseudomonas aeruginosa	100	>100
Flavobacterium	10	30
E. coli	100	>100
Salmonella	100	>100

Table 4 Values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for leaves extracts

Bacterial strain	MIC (mg/ml)	MBC (mg/ml)
Bacillus subtilis	30	100
Staphylococcus aureus MSSA	20	60
Staph. aureus MRSA	30	100
Pseudomonas sp.	30	60
Pseudomonas aeruginosa	100	>100
Flavobacterium	20	40
E. coli	100	>100
Salmonella	100	>100

showed a clear influence of the natural compounds on the two parameters, even if the effect was very different depending on the extracts considered. Karanika for instance reports that in some cases the growth rate of *Listeria monocytogeneses* was higher in the presence of the extracts; these data are in agreement with what observed here for *Pseudomonas aeruginosa*. In the study of Munoz, the natural compounds did not always have an effect on the lag time; a similar behaviour was observed here especially for the stem extracts. These examples show how the mechanisms of the interactions between microorganisms and antibacterial species are quite complex and not completely clear.

From Table 3, it can be seen how the values of MBC for the stem extracts are at maximum 4 times higher than the corresponding MIC; the same thing can be observed for the extracts from leaves. These data show the good bactericidal properties of the analysed samples. Comparing these values with literature data, other natural extracts show similar properties (i.e. not a big difference between MIC and MBC): Furiga in fact reports a ratio MBC/MIC = 2 for some pine bark extracts [28]. In other cases, however, this ratio is much higher, like for instance for rosemary extracts [17]. This indicates that the polyphenols present in stems and leaves extracts are more effective in targeting

Table 5 Growth rate (GR, h^{-1}) and lag time (LT, h) for the stem extracts

	Bacillus subtilis		MSSA		MRSA		*Pseudomonas* sp.		*Pseudomonas aeruginosa*		*Flavobacterium*		*E. coli*		*Salmonella*	
	GR	LT	GR	LT	GR	LT	GR	LT	GR	LT	GR	LT	GR	LT	GR	LT
C = 0	0.232 ± 0.017	5	0.213 ± 0.010	1	0.182 ± 0.008	1.5	0.048 ± 0.005	0	0.284 ± 0.019	4	0.068 ± 0.004	3.5	0.261 ± 0.020	1.5	0.314 ± 0.025	1
C = 10	0.070 ± 0.005	15.5	0	–	0	–	0.063 ± 0.006	0	0.084 ± 0.005	0	0	–	0.260 ± 0.008	0	0.286 ± 0.030	0
C = 15	0	–	0	–	0	–	–	–	–	–	–	–	–	–	–	–
C = 20	0	–	0	–	0	–	0.031 ± 0.005	0	0.044 ± 0.002	0	0	–	0.183 ± 0.006	0	0.124 ± 0.011	0
C = 30	0	–	0	–	0	–	0	–	0.043 ± 0.002	0	0	–	0.122 ± 0.005	0	0.119 ± 0.008	0
C = 40	0	–	0	–	0	–	0	–	0.081 ± 0.003	7.5	0	–	0.066 ± 0.003	0	0.061 ± 0.003	0

Table 6 Growth rate (GR, h^{-1}) and lag time (LT, h) for the leaves extracts

	Bacillus subtilis		MSSA		MRSA		*Pseudomonas* sp.		*Pseudomonas aeruginosa*		*Flavobacterium*		*E. coli*		*Salmonella*	
	GR	LT	GR	LT	GR	LT	GR	LT	GR	LT	GR	LT	GR	LT	GR	LT
C = 0	0.262 ± 0.014	3.5	0.142 ± 0.012	1	0.199 ± 0.010	1	0.040 ± 0.003	0	0.210 ± 0.01	1	0.060 ± 0.001	3	0.299 ± 0.025	0.5	0.301 ± 0.025	0.5
C = 10	–	–	–	–	–	–	–	–	–	–	0.059 ± 0.001	3	–	–	–	–
C = 15	–	–	0.069 ± 0.006	3.5	–	–	–	–	–	–	–	–	–	–	–	–
C = 20	0.353 ± 0.037	18	0	–	0.058 ± 0.003	3.5	0.034 ± 0.001	0	0.086 ± 0.007	3.5	0	–	0.307 ± 0.018	1	0.307 ± 0.022	2.5
C = 30	0	–	0	–	0	–	0	–	0.106 ± 0.002	3	0	–	0.293 ± 0.017	0.5	0.269 ± 0.011	3
C = 40	0	–	0	–	0	–	0	–	0.127 ± 0.005	6.5	0	–	0.231 ± 0.013	0	0.252 ± 0.010	3.5

the bacterial cell walls compared to the ones in rosemary extracts.

Considering the values of MIC and MBC for stems and leaves, it can be seen how they are maximum of 3 times lower for leaves extracts, despite the Total Phenolic Content (TPC) being about 6 times lower. This means that the difference in concentration cannot be the only factor affecting the antibacterial properties, but a synergic effect between the different molecules has also to be considered. Table 2 shows the ratios in concentration between the major compounds detected in stems and leaves extracts; it can be seen how the ratios between (+)-catechin and both protocatechuic and *p*-coumaric acids are much higher for stems extracts than for the leaves ones (16.8 and 28.1 vs. 5.4 and 3.5 respectively). As mentioned above, (+)-catechin has a weaker antibacterial activity compared to other polyphenols [22]; its presence in smaller proportion in the leaves extracts could explain their comparatively higher antibacterial properties.

Furthermore, the ratio between protocatechuic and *p*-coumaric acids is almost 3 times higher in leaves than in stems extracts; this difference in acid composition could also affect the activity towards the bacteria.

Sinergistic effect was already reported previously for affecting the antibacterial properties of polyphenols, especially those from natural sources. For instance, in Sasaki a synergistic effect of the monomeric polyphenols from tea leaves extracts is described [29]; moreover, Obied reports about the synergistic effect of the polyphenols in olive mill wastewaters, comparing the activity of the extract with the one of the individual components [9]. A synergistic effect of polyphenols with other antibiotics was also observed [21, 30]. Synergistic effect in polyphenols can influence the antioxidant properties too; this is an area of growing interest and study, but at present not fully understood.

Conclusions

In this paper we reported about an effective way of valorising agriculture waste, by obtaining valuable compounds from its treatment.

It was shown that the waste from traditional cherry liquor production can be used to extract antibacterial species: in fact the ethanolic extracts from both stems and leaves of Ginja cherry plants were rich in polyphenols. These extracts were tested against several bacterial strains (both for Gram positive and Gram negative species) and showed good antibacterial activity, with an inhibition of the bacterial growth, effect on the bacterial growth rate and a bactericidal effect.

These antibacterial properties, combined with the antioxidant activity previously studied [15], make these

extracts particularly valuable and suitable for several applications (i.e. cosmetic and/or food packaging).

This valorisation process makes the cherry liquor production more environmentally sustainable; a similar approach can be applied to other fields of agriculture.

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