Antimicrobial potential of polyphenols extracted from almond skins

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Abstract

Aims: To evaluate the antimicrobial properties of flavonoid-rich fractions derived from natural and blanched almond skins, the latter being a by-product from the almond processing industry.

Methods and Results: Almond skin extracts were tested against Gram-negative bacteria (Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Serratia marcescens), Gram-positive bacteria (Listeria monocytogenes, Enterococcus hirae, Staphylococcus aureus, Enterococcus durans) and the yeast Candida albicans. Almond skin fractions were found to have antimicrobial activity against L. monocytogenes and Staph. aureus in the range 250–500 μg ml−1, natural skins showing antimicrobial potential against the Gram-negative Salm. enterica. The interactions between three almond skin flavonoids were also evaluated with isobolograms.

Conclusions: Pairwise combinations of protocatechuic acid, naringenin and epicatechin showed both synergistic and indifferent interactions against Salm. enterica and Staph. aureus. Antagonism was observed against L. monocytogenes with all combinations tested. Further studies need to be performed to understand the mechanisms responsible for these interactions.

Significance and Impact of the Study: Almond skins are a potential source of natural antimicrobials.

Introduction

Almonds production is around 1.7 million metric tons, with California currently producing 80% of the world’s almonds (http://www.almondboard.com). Almonds (Prunus dulcis Miller D.A. Webb) are a rich source of nutrients and phytochemicals such as vitamin E, monounsaturated fatty acids and polyunsaturated fatty acids (Mandalari et al. 2008). Positive effects of almond consumption have been demonstrated in reducing risk factors for coronary heart disease with markedly improved serum lipid profile (Sabate et al. 2003). Other health promoting compounds present in almond skins are polyphenols (Mandalari et al. 2010a), which have been shown to be protective agents against cancer and cardiovascular disease (Knekt et al. 2002; Liu 2004). Almond skins are industrially removed from the nut by hot water blanching and represent 6–8% of the total almond weight. Currently, they are under-utilized by-products of the almond processing industry, mainly used in cattle feed (Grasser et al. 1995) and in gasification plants to produce energy (González et al. 2006). However, almond skins are known to have a number of nutritional benefits, mainly based on the presence of polyphenols and the high dietary fibre content (Mandalari et al. 2010a). Among polyphenols, flavonoids are secondary metabolites well documented for their biological effects, including anticancer, antiviral, antimutagenic and anti-inflammatory activities (Benavente-García et al. 1997; Vuorela et al. 2005). There is also evidence suggesting...
that dietary flavonoids can influence gastrointestinal microbiota, and there is considerable in vitro data on the direct and indirect activity of polyphenols, including naringenin and hesperetin, against Helicobacter pylori (Bae et al. 1999; Mabe et al. 1999; Puuponen-Pimiä et al. 2001; Tombola et al. 2003; Funatogawa et al. 2004). Flavonoids are generally present in glycosylated forms in plants, the sugar moiety being an important factor, which determines their bioavailability. We have recently shown that polyphenols from almond skins are bioaccessible in the upper gastrointestinal (GI) tract and therefore potentially available for absorption during digestion (Mandalari et al. 2010a). The major flavonoids identified were (+)-catechin, (−)-epicatechin, kaempferol and isorhamnetin, the latter (as the 3-O-rutinoside or 3-O-glucoside) being predominant in almond skins. The main conjugated sugars were rhamnose (Rha) and glucose (Glc); mono-glycosides (mono-Glc) or rutinosides (−Glc [6 → 1]Rha).

There is an increased effort in trying to avoid foods with chemical preservatives and this is manifested by the food industries growing interest in finding natural compounds with antimicrobial activity (Beuchat and Golden 1989; Gould 1996). The enhanced demand for natural and minimally processed ingredients is also attributed to the legislations governing the use of current preservatives. A number of aromatic plant oils have found industrial applications as preservatives of raw and processed foods (Lis-Balchim and Deans 1997; Hammer et al. 1999). The aim of the present study was to evaluate the antimicrobial properties of almond skin fractions rich in flavonoids against Gram-negative bacteria, Gram-positive bacteria and yeast.

Materials and Methods

Materials

Natural almonds (Maisie Jane’s California) were kindly provided by the Almond Board of California and stored in the dark. Natural almond skins (NS) were prepared by a freeze-thaw method using liquid nitrogen and then milled with an analytical mill (Janke & Kunkel A 10) following the method previously reported (Mandalari et al. 2010a). Blanched almond skin powder (BS) prepared by hot water extraction was produced by ABCO Laboratories (Almond Skins powder 1912) and supplied by the Almond Board of California. All flavonoids were obtained from Extrasynthese (Genay, France).

Almond skin extracts

Almond skin extracts rich in flavonoids were prepared as previously reported (Mandalari et al. 2010a). Briefly, NS and BS (5 g) were extracted three times with n-hexane (10 ml) under constant agitation to remove the lipids. After filtration, the residue was mixed with 50 ml of methanol/HCl 0·1% (v/v), sonicated and centrifuged three times. All methanolic fractions were combined and evaporated, after which the residue was dissolved in 20 ml of distilled water and extracted four times with 20 ml of ethyl acetate. The organic phases were combined, dried with Na2SO4 for 20 min and dissolved in methanol. The polyphenolic composition of NS and BS has been previously reported (Mandalari et al. 2010a).

Microbial strains and culture conditions

The following strains were used as indicators for the antimicrobial testing and were obtained from the University of Messina’s in-house culture collection (Messina, Italy): Escherichia coli ATCC 25922, Salmonella enterica ssp. enterica serovar Typhimurium ATCC 14028, Listeria monocytogenes ATCC 7644, Staphylococcus aureus ATCC 6538P, Enterococcus hirae ATCC 10541, Enterococcus durans V3 (wild-type strain), Pseudomonas aeruginosa ATCC 27853, Serratia marcescens AM1 (wild-type strain) and Candida albicans ATCC 10231.

Cultures for antimicrobial activity tests were grown in Mueller–Hinton Broth (MHB; Oxoid, CM0405) at 37°C (24 h) for bacteria and Sabouraud Liquid Medium (SLM; Oxoid, CM0147) at 30°C (48 h) for the yeast. For solid media, 1·5% (w/v) agar (Difco) was added.

Antimicrobial testing

The minimum inhibitory concentrations (MICs) of NS and BS and the pure flavonoid compounds naringenin, catechin, protocatechuic acid, epicatechin and isorhamnetin-3-O-glucoside were determined by the broth microdilution method, according to CLSI (2008). The MICs were also performed in the Bioscreen C (Labystems Oy, Helsinki, Finland) for all strains as previously reported (D’Arrigo et al. 2010). Twofold serial dilutions of NS, BS and each flavonoid compound were added to broth and inoculated with a final inoculum of approximately 5 × 10⁵ CFU ml⁻¹. The tested concentrations ranged from 1000 to 1·95 μg ml⁻¹, and no significant changes of the pH of the growth medium were detected after addition of the almond extracts and the pure flavonoid compounds.

In the combination assays, the ‘checkerboard’ procedure described by White et al. (1996) was followed. This method allows varying the concentrations of each antimicrobial along the different axes, thus ensuring that each well of the assay plate contained a different combination. The combination assays were performed in Bioscreen C.
honeycomb 100-well plates containing appropriate media with test compounds represented by flavonoids (naringenin, epicatechin and protocatechuic acid) at different concentrations. Diluted cell cultures were then added, and the bacterial growth was monitored using Bioscreen C for 20 h. The OD$_{540}$ was measured at 10-min intervals. Controls grown with equivalent levels of media were included in all assays. The MIC of almond skins or flavonoid compound, alone or in combination, was considered as the lowest concentration, which completely inhibited bacterial growth (OD$_{540}$ cut-off point ≤0.1 = no growth) after 20 h.

MIC data of each flavonoid tested were converted into fractional inhibitory concentration (FIC), defined as the ratio of the concentration of the antimicrobial in an inhibitory concentration with a second compound to the concentration of the antimicrobial by itself (Olasupo et al. 2004).

$FIC_A = \text{MIC of A with B/MIC of A}$

The FIC index was then calculated as follows: FIC index = $FIC_A + FIC_B$.

The interaction of the antimicrobial combinations was determined with the isobologram as previously described (Davidson and Parish 1989; Hwang et al. 2004; Mandalari et al. 2007).

All experiments were performed in triplicate.

**Results**

**Antimicrobial activity of almond skin extracts**

The MIC values of almond skin extracts against all the bacteria tested and *C. albicans* are presented in Table 1. Results of negative controls indicate the complete absence of inhibition of all the strains tested (data not shown). Both NS and BS flavonoid-rich fractions were active against the Gram-positive food-borne pathogens *Staph. aureus* and *L. monocytogenes*, but were not active against any of the Gram-negative bacteria (with the exception of NS showing activity against *Salmonella enterica* ser. Typhimurium) or the yeast tested in this study. NS was more effective than BS, and this could be explained by the total amount of flavonoids present, being about 10-fold higher in the skin extracted from natural almonds (results not shown). On the other hand, the antimicrobial properties of BS could be attributed to the presence of catechin, epicatechin, kaempferol-3-O-rutinoside, isorhamnetin-3-O-rutinoside and naringenin, all being the most abundant polyphenols retained in this by-product.

*Staph. aureus* and *L. monocytogenes* were the most sensitive strains (complete inhibition achieved with a concentration of 250 μg ml$^{-1}$ NS), followed by *Salm. enterica*

### Table 1 Minimum inhibitory concentration of almond skin extracts against Gram-positive bacteria, Gram-negative bacteria and yeast. Values are expressed as μg ml$^{-1}$

<table>
<thead>
<tr>
<th>Strain</th>
<th>Natural almond skin flavonoid-rich extract</th>
<th>Blanched almond skin flavonoid-rich extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus hirae ATCC 10541 &gt;1000 &gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus durans V3 (wild type) &gt;1000 &gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Listeria monocytogenes ATCC 7644 250 500</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 6538P 250 500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella enterica ssp. enterica ser. Typhimurium ATCC 14028 500 &gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Serratia marcescens</em> AM1 (wild type) &gt;1000 &gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 27853 &gt;1000 &gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC 25922 &gt;1000 &gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em> ATCC 10231 &gt;1000 &gt;1000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(500 μg ml$^{-1}$ NS). The inhibitory effect of almond skin extracts against all the strains tested was bacteriostatic rather than bactericidal. This was indicated by colony formation on agar plates inoculated with cells from cultures exposed to MIC levels of the samples under investigation (data not shown).

**Antimicrobial activity of almond skin flavonoids**

The MICs of the five most abundant almond skin flavonoids against *Staph. aureus*, *L. monocytogenes* and *Salm. enterica* are reported in Table 2. The pure organic compounds showed similar activity against the three strains tested, *Staph. aureus* being the most sensitive strain to these pure compounds. Naringenin showed the greatest activity with MICs in the range of

### Table 2 Minimum inhibitory concentration of almond skin flavonoids against *Staphylococcus aureus* ATCC 6538P, *Listeria monocytogenes* ATCC 7644 and *Salmonella enterica* ser. Typhimurium ATCC 14028. Values are expressed as μg ml$^{-1}$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Staph. aureus</th>
<th>L. monocytogenes</th>
<th>Salm. enterica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>&gt;500</td>
<td>500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>250</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>250</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Isohamnetin-3-O-glucoside</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>500</td>
</tr>
<tr>
<td>Naringenin</td>
<td>250</td>
<td>500</td>
<td>250</td>
</tr>
</tbody>
</table>
250–500 μg ml⁻¹. Epicatechin and protocatechuic acid were the next most effective compounds, whereas catechin and isorhamnetin-3-O-glucoside were only active against *L. monocytogenes* and *Salm. enterica*, respectively.

The modes of interaction of naringenin, epicatechin and protocatechuic acid are presented as FIC isobolograms in Figs 1–3. As previously reported by Davidson and Parish (1989), the shape of the isobologram curve convex, linear or concave is indicative of the synergistic, additive and antagonistic interactions, respectively. In this study, we have interpreted as synergistic for values \(<0.5\), additive or indifferent if \(>0.5\) but \(<4\) and antagonistic if \(>4\) (Visalli et al. 1998). Indifference tending to synergism was observed between naringenin and protocatechuic acid against *Staph. aureus* (Fig. 1a) and *Salm. enterica* (Fig. 2a) but not against *L. monocytogenes*.

**Figure 1** FIC isobolograms for combinations of naringenin with protocatechuic acid (a) or epicatechin (b) and protocatechuic acid with epicatechin (c) against *Staphylococcus aureus*. The dotted line indicates the theoretical additive line. FIC, fractional inhibitory concentration.

**Figure 2** FIC isobolograms for combinations of naringenin with protocatechuic acid (a) or epicatechin (b) and protocatechuic acid with epicatechin (c) against *Salmonella enterica*. The dotted line indicates the theoretical additive line. FIC, fractional inhibitory concentration.
where an antagonistic effect between the two compounds was observed (Fig. 3a). The combination of naringenin and epicatechin showed an indifferent to synergistic effect against *Staph. aureus* (Fig. 1b) and *Salm. enterica* (Fig. 2b), whereas a mainly antagonistic interaction was observed against *L. monocytogenes* (Fig. 3b). Antagonism was evident in the combination of protocatechuic acid and epicatechin against *Salm. enterica* and *L. monocytogenes* but synergism was observed against *Staph. aureus* (Figs 2c, 3c and 1c, respectively).

**Discussion**

The present study has demonstrated that flavonoids present in almond skins are active against a range of food-borne pathogens. Blanched skins, a by-product of the almond processing industry, are a potential source of natural antimicrobials. Purified components from almond skins could be used as natural antibacterial agents in food systems, thus extending the shelf life of processed foods. Among plant antioxidants, several groups of polyphenols, including flavanones and isoflavones, have found industrial applications as nutraceuticals and/or functional foods as well as for dietary, pharmaceutical and cosmetic purposes (Espin *et al.* 2007). Furthermore, the *in vitro* antimicrobial activity of natural compounds could provide with potentially useful information for developing novel antibiotics. Medicinal plants could be used as a possible way to treat diseases caused by multidrug-resistant bacteria (D’Arrigo *et al.* 2010). The combination of the flavonol galangin with gentamicin has successfully been used for its synergistic effects against methicillin-resistant *Staph. aureus* (Lee *et al.* 2008).

The antimicrobial activity of crude plant extracts has been reported (Puupponen-Pimiä *et al.* 2006). Bergamot peel was effective against Gram-negative food-borne pathogen bacteria and the Gram-positive *Bacillus subtilis* (Mandalari *et al.* 2007). The antimicrobial activity of purified flavonoids may result in susceptibility differences against species with different origins and background (Taguri *et al.* 2004). This could explain the difference in sensitivity to naringenin between *Staph. aureus* ATCC 6538P used in this work and a previously tested *Staph. aureus* isolated from food (Mandalari *et al.* 2007). Protocatechuic acid has been shown to be active against both Gram-negative and Gram-positive strains, including *E. coli*, *Ps. aeruginosa*, *Staph. aureus* and *B. cereus*. Increased concentrations of catechin in a film used during storage of sausages resulted in a decrease in *E. coli* O157:H7 and *L. monocytogenes* populations, the latter well known for causing outbreaks in ready-to-eat meat products (Ku *et al.* 2008). Various polyphenols, including naringenin, rutin (quercetin-3-rutinoside), ellagic acid and kaempferol, have been shown to be active against *H. pylori* (Martini *et al.* 2009).

In the present study, almond skin fractions were inhibitory to Gram-positive bacteria only, NS being more active than BS. *Salm. enterica* var. Typhimurium was the only Gram-negative organism inhibited by NS at the concentration of 500 μg ml⁻¹. The highest antibacterial potential of NS could be explained by the amount of flavonoids.

**Figure 3** FIC isobolograms for combinations of naringenin with protocatechuic acid (a) or epicatechin (b) and protocatechuic acid with epicatechin (c) against *Listeria monocytogenes*. The dotted line indicates the theoretical additive line. FIC, fractional inhibitory concentration.
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present, being 10-fold higher compared to BS. However, the activity showed by BS may result from the interactions of different polyphenols retained by this by-product. Most studies on the antimicrobial potential of polyphenols have focused on the inhibitory activity of individual components, while information on the effects of these compounds in combination against food-borne microorganisms is limited. We have shown that the interactions between various compounds can alter the antimicrobial effectiveness of the almond skin flavonoids against food-borne bacteria. The inhibitory effect of phenolics could be explained by absorption to cell membranes, interactions with enzymes, substrate and metal ion deprivation (Scalbert 1991). The synergism observed between naringenin with enzymes, substrate and metal ion deprivation (Scalbert et al. 1994). The antagonistic interaction observed with all combinations against L. monocytogenes may be the result of a number of mechanisms such as competition for target sites or inhibition of uptake by the cells. Alternatively, direct interaction between the two compounds may result in changes of the structural conformation thus reducing the inhibitory activity.

It is important to consider the dietary intake of bioactive phenolics when applying these compounds as food preservatives. Chocolate consumption (439 mg proanthocyanidins and 147 mg catechin) resulted in increased excretion of flavonol-derived acids in urine (Rios et al. 2003). Most flavonoids are present in plasma in their conjugated forms, and the sugar moiety is the major determinant of their absorption in the GI tract (Manach and Donovan 2004). In the gut, phenolic conjugates, which are not absorbed, will reach the large bowel where they may be converted to aglycones by the colonic microbiota known to produce glycosidases, which convert flavonoid rutinosides, neohesperidoses, rhamnosides and glucosides into their aglycones, and further to simple phenolic acids (Rechner et al. 2004; Jenner et al. 2005; Simons et al. 2005).

In summary, the results of the present study showed that flavonoids from almond skins are effective against a range of food-borne pathogens. However, further studies need to be performed to understand the precise mechanisms responsible for these interactions.

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References

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