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ORIGINAL PAPER

The antimicrobial effects of propolis collected in different regions in the Basque Country (Northern Spain)

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Abstract The antimicrobial activity of 19 propolis extracts prepared in different solvents (ethanol and propylene glycol) (EEP/PEP), was evaluated against some bacterial and fungal isolates using the agar-well diffusion method. It was verified that all the samples tested showed antimicrobial activity, although results varied considerably between samples. Results revealed that both types of propolis extracts showed highly sensitive antimicrobial action against Gram-positive bacteria and fungi at a concentration of 20% (*Staphylococcus aureus, Streptococcus mutans*, *Candida albicans* and *Saccharomyces cerevisae*) with a minimal inhibitory concentration (MIC) ranging from 0.5 to 1.5 mg/ml, with a moderate effect against *Streptococcus pyogenes* (MIC from 17 to 26 mg/ml). To our knowledge, this is the first study showing elevated antimicrobial activity against Gram-negative bacteria [*Salmonella enterica* (MIC from 0.6 to 1.4 mg/ml)] and lesser activity against *Helicobacter pylori* (MIC from 6 to 14 mg/ml), while *Escherichia coli* was resistant. This concluded that the Basque propolis had a strong and dose-dependent activity against most of the microbial strains tested, while database comparison revealed that phenolic substances were responsible for this inhibition, regardless of their geographical origin and the solvent employed for extraction. Statistical analysis showed no significant differences ($P \leq 0.05$) between EEP and PEP extracts.

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Introduction

Propolis is a resinous mixture that honeybees collect from tree buds, sap flows, and other botanical sources. This resin is masticated, salivary enzymes added, and the partially digested material is mixed with beeswax and used in the hive. Chemical analyses have showed the presence of beeswax at between 2 and 30%, and vegetable resins at between 40 and 80% in propolis powder, while the colour varies depending on its botanical source (Bonvehí and Coll 2000). Different studies have reported that European propolis (poplar-type) antibacterial activity is attributed to a number of phenolic compounds, mainly flavonoids, phenolic acids and their esters (Bankova et al. 2002). The Brazilian-type propolis is rich in terpenoids and prenylated derivatives of *p*-coumaric acid (Da Silva et al. 2006), and polyisoprenylated benzophenones in Cuban propolis (Cuesta-Rubio et al. 2002). The substance is sticky at and above room temperature and is used by bees as a glue and general purpose sealant. The antimicrobial property of propolis has been widely reported to be responsible for the low incidence of bacteria and moulds within hives (Marcucci 1995). Its antiseptic properties keep the hives healthy. Synergism between propolis and antibacterial agents has been observed, and the bacteriostatic effect is reflected in its constituent, which may differ from area to area and from season to season, according to different plant sources (Drago et al. 2000; Cuesta-Rubio et al. 2002; Boyanova et al. 2005). Most of the antifungal research into propolis has concentrated mainly on yeasts, such as different species of *Candida*, or dimorphic human and animal pathogenic

fungi, with satisfactory fungistatic and fungicidal results in both in vitro and in vivo experiments (Sawaya et al. 2002). Filamentous fungi are generally less sensitive to propolis activity than bacteria and yeasts, regardless of the origin or composition of the propolis. It is well established that the antibacterial, antifungal and anti-inflammatory properties of propolis are due to the existence of low-molecular-weight compounds of diverse polarity, such as phenolic compounds (flavonoids, phenolic acids and their esters), distributed in glycosides of anthocyanidines, flavones, flavonols, and flavanones in nature with aromatic acids (e.g., caffeic acid and *p*-coumaric acids) (Bonvehí and Jordà 1995; Hegazi et al. 2000; Kalogeropoulos et al. 2009).

Compounds which are identified in propolis are generally typical constituents of food/or food additives, and have gained a significant position in recent years as GRAS (Generally Recognized As Safe) products (Burdock 1998). In a previous paper, it was also reported that propolis exerted antimicrobial activity on Gram-positive bacteria, but had a limited response with Gram-negative bacteria (Mohammadzadeh et al. 2007; Tosi et al. 2007). However, its biological properties may vary according to different plant sources (Bankova et al. 2000). The chemical composition and biological activity of propolis has been studied extensively in many European countries (Kosalec et al. 2003; Uzel et al. 2005; Popova et al. 2009), but only a few reports can be found on Spanish propolis (Bonvehi and Gutiérrez 2011). Based on these observations, this study aimed to measure the antibacterial activity of propolis extracts (prepared with ethanol and propylene glycol) against Gram-positive bacteria (*S. mutans, S. pyogenes* and *S. aureus*), Gram-negative bacteria (*H. pylori, S. enterica* and *E. coli)* and fungi strains (*C. albicans* and *S. cerevisae*).

Materials and methods

Propolis samples

In this study, 19 samples of raw propolis produced by *Apis mellifera* honeybees were collected from apiaries located in naturally preserved areas of the Basque Country (Bizkaia, Gipuzkoa and Araba) (Fig. 1). Propolis samples were collected from the same apiary from spring to winter (2005 and 2008) employing the frame-scraping technique described in the Basque Apicultural Programme protocol.

Pollen grains may be introduced into propolis by worker bees during manufacture, and may stem from bee loads from anemophilous plant species. The palynological processing of samples was determined according to Barth (1998). The main plant species visited that contributed to the propolis were: poplar [*Populus* sp. (Aigeros section],

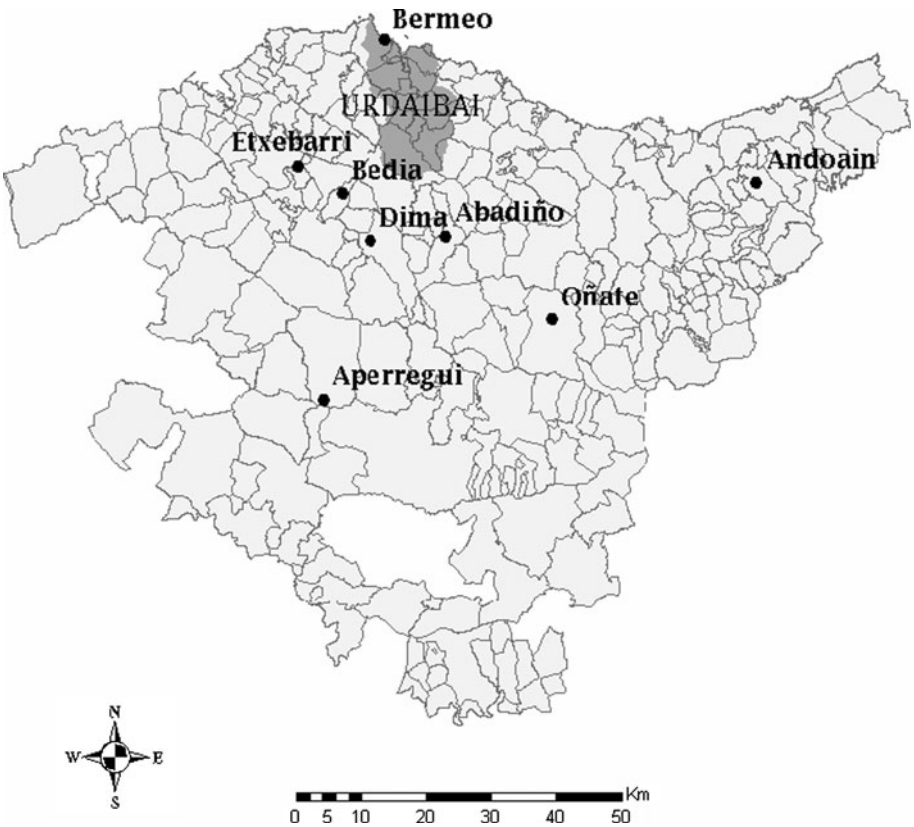

Fig. 1 Map of Basque Country, with the distribution of sampling areas

ash (*Fraxinus* sp.), elm (*Ulmus* sp.), willow (*Salix* sp.), chestnut (*Castanea sativa*), blackberry (*Rubus ulmifolius*), oak (*Quercus* sp.), and birch (*Betula* sp.)]. Representative samples were collected (500 g) and sent to the laboratory with the corresponding collection and location data (Table 1). After harvesting and prior to analysis, macroscopic impurities were removed. Two to three sub-samples from different parts of each lot were then taken to create the 50 g samples. The samples, previously cooled at −20°C for 24 h, were milled in an IKA A 10 analytical mill (LabSource, UK). The resulting product was packaged in foil, with 25 g being used as a control sample and 25 g being stored in darkness at 4°C, to carry out different assays. Samples were either homogenized or pulverized if necessary and analysed in triplicate.

Preparation of ethanolic and propylene glycol extracts of propolis

For the evaluation of the antimicrobial activity, the active compounds were extracted from 20 g of finely ground propolis with 70% ethanol or 100% propylene glycol (100 ml), with intermittent manual shaking, at room temperature in the dark for a week. The insoluble fraction was separated by filtration. The filtrate was then taken up to 100 ml with the corresponding solvent, and stored in sealed bottles at 4°C for 1 week. All extracts were kept in the dark, at room temperature prior to antibacterial testing. Their composition is shown in Table 2.

Reagents and standards

Ethanol and propylene glycol were analytical grade and supplied by Panreac (Barcelona, Spain), and phenol was obtained from Sigma-Aldrich (Steinheim, Germany).

Strain and culture conditions

Bioassays of the antimicrobial activities of the Basque propolis were performed using (a) three species of Gram-positive bacteria (*Streptococcus mutans* CECT 479T, *Staphylococcus aureus* CECT 435 and *Streptococcus pyogenes* CECT 191); (b) three species Gram-negative bacteria (*H. pylori* CIP 103995, *Salmonella enterica* CIP 6062 and *Escherichia coli* CECT 101); and (c) two yeasts species (*Candida albicans* CECT 1394 and *Saccharomyces cerevisae* CECT 1383). The indicator organisms were procured from the Spanish collection of microorganisms and cell cultures [The Spanish Type Culture Collection (CECT) is a general service of the University of Valencia, Spain], and the Institute Pasteur Collection (CIP). Microbiological media were purchased from Oxoid Ltd (Basingstoke, UK) and Biolife (Milan, Italy).

Table 1 Propolis sample collect from different locations throughout Basque Country

Sample no	Collecting area	Year of sampling	Apiary no
1	Bermeo	2005	1
2	Urdaibai	2005	2
3	Etxebarri	2005	3
4	Bedia	2005	4
5	Dima	2005	5
6	Abadiño	2005	6
7	Oñate	2005	7
8	Andoain	2005	8
9	Aperregi	2005	9
10	Bermeo	2008	1
11	Urdaibai	2008	2
12	Etxebarri	2008	3
13	Bedia	2008	4
14	Dima	2008	5
15	Dima	2008	5
16	Abadiño	2008	6
17	Oñate	2008	7
18	Andoain	2008	8
19	Aperregi	2008	9

Determination of antimicrobial activities

Antimicrobial activity evaluated by the agar diffusion method

The agar well diffusion method was used to determine the antimicrobial activities of EEP and PEP (modified Kirby-Bauer method). The inoculum was prepared from pure cultures, incubated in selective media [*C. albicans* (Sabouraud 4% glucose agar), *E. coli* (tryptone soy agar), *S. mutans* (brain–heart infusion), *S. aureus* (trypticase soy broth), *S. cerevisae* (yeast extract-peptone-trehalose), *S. enterica* (tryptone soy agar), *S. pyogenes* (brain–heart infusion) and *H. pylori* (Columbia agar with defibrinated horse blood)] for 24 h. All types of media were sterilized to autoclaving at 121°C and 1.03 bars for 15 min. The inoculum size was adjusted so as to deliver final inoculums of approximately 10^8 colony forming units (c.f.u.)/ml, equivalent to McFarland 0.5 standard. One millilitre of the cellular suspension obtained was added to 10 ml of previously melted Mueller–Hinton agar, mixed, poured into Petri dishes and left to solidify for 1 h. A sterile punch was used to make 8 mm-diameter staggered wells in the solidified agar. Forty microliter EEP or PEP were added in peripheral holes and 40 µl 70% ethanol of 100% propylene glycol was added in the central hole for negative control. Incubation was performed at 37°C for 24 h. Antimicrobial activity was based on measurement of the diameter of the

Table 2 Chemical composition of ethanolic and propylene glycol extracts of Basque propolis (g/100 g)

Sample no.	Total phenolic content	Flavone and flavonol content	Flavanone and dihydroflavonol content	Total flavonoids content
1	21/23	3.1/2.8	5.5/5.7	8.6/8.5
2	30/28	5.2/5.7	7.3/6.3	12.5/12.0
3	27/24	4.5/4.3	5.9/6.2	10.4/10.5
4	26/24	4.8/4.7	8.9/8.4	13.7/13.1
5	29/27	6.2/7.8	9.9/8.0	16.1/15.8
6	30/28	6.1/7.4	10/8.5	16.1/15.9
7	23/21	3.7/2.6	6.1/6.6	9.8/9.2
8	28/26	4.9/4.6	8.2/8.3	13.1/12.9
9	32/28	6.0/5.5	8.2/6.5	14.2/12.0
10	31/27	5.9/5.2	8.2/6.7	14.1/11.9
11	28/25	4.4/3.1	7.6/8.2	12.0/11.3
12	21/20.3	2.6/1.2	6.1/6.0	8.7/7.2
13	27/26	5.9/2.0	9.6/10.8	15.5/12.8
14	29/26.5	4.5/1.6	8.0/8.6	12.5/10.2
15	29/24.7	5.1/2.6	7.0/7.5	12.1/10.1
16	31/30.3	4.7/2.5	7.3/7.7	12/10.2
17	33/29.6	5.1/4.7	8.6/7.4	13.7/12.1
18	21/20	3.9/1.9	7.9/6.7	11.8/8.6
19	34/30	4.9/3.3	8.6/7.8	13.5/11.1
X	27.9/25.7	4.82/3.87	7.88/7.47	12.7/11.3
SD	3.97/3.10	1.00/1.89	1.30/1.23	2.19/2.27
Range	21–34	2.6–6.2	5.5–10	8.6–16.1
	20–30.3	1.2–7.8	5.7–10.8	7.2–15.9

According Bonvehi and Gutiérrez (2011)

Calibration curves: Total phenolic content [mg gallic acid equivalents/g ($y = 232.66 x + 2.7333$; $r^2 = 0.999$)]; Flavone and flavonol content [mg quercetin/g ($y = 163.08 x + 0.4298$; $r^2 = 0.9999$)]; Flavanone and dihydroflavonol content [mg naringenin/g ($y = 2754.7x$; $r^2 = 0.9975$)]; Results for different extracts are separated by (/); first value in ethanol extract propolis (EEP); second value in propylene glycol extract propolis (PEP); *SD* standard deviation

inhibition zone formed around the well, and the effect was calculated as the mean of the triplicate determinations. The measurement was compared to the criteria set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2011). Based on the criteria, the organism can be classified as being Resistant (R), Intermediate (I) or Susceptible (S).

Determination of the minimum inhibitory concentration (MIC)

The MIC was defined as the lowest solution concentration that inhibited at least 90% of the microorganism growth after incubation. The antibacterial activity was assayed by the agar-well diffusion method proposed by Allen et al. (1991), with minor modifications. Solid media containing different concentrations of EEP or PEP were used (0.025–2%, v/v). In addition, concentrations of 1, 5 and 10% were incorporated into the media for bacterial strains that were insensitive to the lower concentrations. Phenol aqueous standard solutions from 1 to 10% (w/v) were prepared to use as comparative standards. All samples were prepared aseptically, assayed immediately after dilution, and were handled away from direct sunlight.

The cellular suspension (1.5×10^8 c.f.u./ml) was diluted in sterile physiological saline solution to a concentration of 10^6 c.f.u./ml. Large square plates ($125 \times 125 \times 15$ mm) seeded with the different microorganisms were prepared by adding 100 µl aliquots of each prepared microbial suspension to 150 ml of sterilized Mueller–Hinton agar cooled to 45°C. *Candida albicans* required an additional glucose solution of 10% to the nutrient agar, with a final concentration of 1/‰. The plates were poured on a level surface immediately after mixing, and stored at 4°C overnight before use the next day. Twenty-five wells were cut in the agar using a cooled flamed 8 mm cork borer, and using a quasi-Latin square as a template. The wells were numbered, and the samples were tested in triplicate, by adding 80 µl of the solution of propolis to each of three cells with the same number. The plates containing microorganisms were incubated at 37°C for 24 h, under aerobic conditions. After incubation, confluent microbial growth was observed. The diameter of the clear zones was measured using a ruler and compared with those obtained with different phenol concentrations. A standard graph was plotted representing the percentage of phenol against the square of the mean diameter of the clear zone. The MICs of each strain were expressed as the lowest

propolis concentration of the sample that caused a clear (1–3 mm) zone of inhibition. All MICs were determined in triplicate at all dilutions.

Statistical analysis

Analysis of variance (ANOVA) was applied to determine significant differences among the geographical origins of the samples analysed. ANOVA was performed according to the fixed factor model, considering locality, year, and antimicrobial activity as sources of variation, using F distribution and unpaired Student's test at a level of $P \leq 0.05$. Pearson correlation was used in order to verify a possible correlation between flavonoid content and antimicrobial activity of EEP and PEP extracts. The results were analysed by means of Statistica software for Windows 5.5 (StatSoft Inc., Tulsa, OK, USA). The mean of three replicates was taken as the variation limit for each parameter.

Results and discussion

Antimicrobial activity

As a general rule, an extract was considered as active against both bacteria and fungi if the zone of inhibition was greater than 6 mm. The inhibition diameters obtained in the different samples are shown in Table 3. The largest inhibition zones were noticed against *H. pylori* (from 13 to 20 mm), *S. enterica* (from 10 to 18 mm), and *S. aureus* (from 10 to 16 mm). The results obtained agreed with those of Boyanova et al. (2005), who observed inhibition zones ≥15 mm for *H. pylori*. Similarly, the antimicrobial activity of Brazilian propolis at 30% of concentration against *H. pylori* was also determined by using the agar-well diffusion method and the inhibition zone diameter was established at 21.4 mm (Kimoto et al. 1998).

On the other hand, medium-size inhibition zones were recorded against *S. pyogenes* and *S. mutans* (from 4 to 12 mm), and less fungicidal activity was shown against *C. albicans* and *S. cerevisae* (from 4 to 10 mm). With most of the samples tested, the diameter of the inhibition zones ranged between 8 and 14 mm against bacterial species and the extracts considered more active presented an inhibition zone diameter from 15 to 20 mm (Table 3). This variation may be correlated to the chemical composition of the propolis. This explanation was supported by Bonvehi and Gutiérrez (2011), when they found that the antioxidant activity of Basque propolis differed according to differences in phenolic compositions. European researchers support different flavonoid aglycones as the antibacterial agents in propolis (Bonvehí et al. 1994; Bankova et al. 2000). Tetracycline and ampicillin showed an antibacterial activity with inhibition diameters between 22 and 32 mm against *S. aureus* and *E. coli* (Table 3). The control (ethanol 70% and propylene glycol 100%, v/v) showed no inhibitory zone against any bacteria or fungi tested. The data reported in the literature reveals that propolis samples from Brazil showed inhibition diameters against *S. aureus* within the 8–13 mm range (Gonsales et al. 2006), comparable with results obtained by Prytzyk et al. (2003) from Bulgarian propolis. Argentinean propolis measured inhibition zones of over 10 mm for *S. aureus* and of under 10 mm for *S. pyogenes* (Nieva Moreno et al. 1999). Chinese and Japanese propolis gives inhibition zones ranging from 5.5 to 6.8 mm for *S. mutans* (Ikeno et al. 1991). Stepanovi et al. (2003) discovered that the inhibition zone of propolis from different locations of Serbia ranged from 18 to 23 mm. These results are in the same range as those reported in this study, but the literature indicates that the sensitivity of microorganisms and differences in the active compounds of propolis that possess antibacterial and antifungal activities are greatly affected by variations in geographical origins (Bankova et al. 2000). The low sensitivity detected for *E. coli* was in agreement with several publications, where it was concluded that this bacterium showed very low susceptibility to the bactericidal action of propolis (Kujumgiev et al. 1999; Nieva Moreno et al. 1999; Bonvehí and Coll 2000). The most plausible explanation for the lack of sensitivity shown by Gram-negative bacteria could be attributed to their outer membrane that inhibits and/or retards the penetration of propolis at lower concentrations, but this effect is as yet not fully explained. Another possible reason might be the presence of multidrug resistance pumps (MDRs), which extrude amphipathic toxins across the outer membrane (Tegos et al. 2002). Furthermore, results of tests evaluating propolis from other parts of the world could be difficult to compare because by the low hydro-solubility of active compounds in the agar layer (Sawaya et al. 2004).

The results of MIC's against different microbial agents are shown in Table 4. These values were determined by the agar-well diffusion method, applying breakpoint tables for their interpretation (EUCAST 2011). According to the results obtained, all the Gram-positive bacteria tested were highly sensitive to a lower concentration of propolis (MIC ranged between 0.6 and 1.3 mg/ml) especially against *S. mutans* and *S. aureus*, exhibiting a moderate effect on *S. pyogenes* (MIC ranged between 17 and 26 mg/ml). *Escherichia coli* displayed a negligible inhibition by most of the EEP and PEP samples, and was insensitive at any of the concentrations tested (1–100 mg/ml). Different researchers reported that propolis had no effect on standard *E. coli* (Kujumgiev et al. 1999; Stepanovi et al. 2003; Gonsales et al. 2006). In contrast to this strain, the samples showed efficient antibacterial action against *S. enterica*

Table 3 Inhibition zone diameters of ethanolic and propylene glycol extracts of propolis against bacterial and fungal strains

Sample no.	Inhibition zones (mm)							
	S. mutans	*S. pyogenes*	*S. aureus*	*H. pylori*	*S. enterica*	*E. coli*	*C. albicans*	*S. cerevisae*
1	5/5	4/5	10/11	14/14	12/12	0	8/8	9/8
2	8/8	10/9	14/13	18/17	16/15	0	4/5	5/6
3	7/6	9/9	13/12	17/15	14/13	0	6/6	7/6
4	8/7	10/9	15/14	18/17	17/16	0	6/7	7/6
5	9/8	12/11	16/14	19/17	18/17	0	9/8	10/9
6	9/9	11/10	16/15	20/19	18/11	0	10/8	9/9
7	8/7	9/8	12/11	15/14	13/12	0	6/7	7/6
8	6/7	12/11	14/13	18/17	16/15	0	6/7	8/7
9	7/7	11/10	15/14	19/17	17/16	0	8/7	9/9
10	6/7	9/8	14/14	19/17	17/16	0	8/9	10/9
11	6/6	8/7	13/12	17/16	14/13	0	5/5	8/7
12	5/5	5/6	10/9	14/15	11/10	0	6/7	8/8
13	10/9	8/7	15/14	18/17	16/14	0	10/8	9/8
14	8/7	7/7	14/13	15/15	14/13	0	5/6	6/6
15	7/6	5/5	12/11	15/14	13/13	0	6/7	7/6
16	6/6	5/6	11/11	14/14	13/12	0	6/6	7/7
17	7/7	9/8	13/12	15/14	15/14	0	7/6	8/7
18	5/5	5/5	10/10	14/13	12/12	0	5/6	6/5
19	7/6	7/6	13/12	15/14	15/14	0	6/7	8/8
Control	0	0	0	0	0	0	0	0
Tetracycline			22			30		
Ampicilline			32			31		
Mean	7.1/6.9	8.2/7.7	13.2/12.5	16.5/15.6	14.8/13.6	0	6.7/6.8	7.8/7.2
Range	5–10/5–9	4–12/5–11	10–16/9–15	14–20/13–19	11–18/10–17	0	4–10/5–9	5–10/5–9

Sample nos. 1–9 (year of sampling—2005); Sample nos. 10–19 (year of sampling—2008); Results for different extracts are separated by (/); first value in ethanol extract propolis (EEP); second value in propylene glycol extract propolis (PEP); Control: 70% ethanol/100% propylene glycol

(MIC ranged between 0.6 and 1.4 mg/ml) and *H. pylori* (MIC ranged between 6 and 14 mg/ml) such as Gram-negative bacteria. As Table 4 shows, in the antifungal assay, EEP/PEP showed a strong sensitivity against *C. albicans* and *S. cerevisae* (MIC ranged between 0.6 and 1.5 mg/ml). However, it was found that a concentration of 15–30 mg/ml of propolis was needed to inhibit the growth of *C. albicans* (Pepeljnjak et al. 1982). No significant differences were detected between EEP/PEP extracts and years of collection ($P \leqslant 0.05$).

These results are in the same range as those reported with Brazilian propolis (Santos et al. 2002). Sonoran propolis (collected in northwestern Mexico) showed very high growth-inhibitory activity towards Gram-positive bacteria, particularly against *S. aureus* (MIC of 0.1 mg/ml) (Velazquez et al. 2007). According to Hegazi et al. (2000), Austrian propolis has exhibited high activity against *C. albicans* and German propolis has been very active against *S. aureus* and *E. coli*. This difference in the MIC values of propolis was related to the different constituents of propolis collected from different geographical regions (Bankova et al. 2000). The results of this work, as well as the results of other authors, indicate that agar-well diffusion is an appropriate method for evaluating the antimicrobial activity of solvent propolis samples.

Variations in the flavonoid content of propolis were mainly due to the difference in the preferred regional plants visited by honeybees and also to the raw propolis cleaning and extraction process (Ahn et al. 2007; Bonvehi and Gutiérrez 2011). In previous study, the amounts of total phenolic content and flavonoid contents (flavones and flavanones) determined in Basque propolis varied widely and ranged from 200 to 340 and 72–161 mg/g, respectively (Bonvehi and Gutiérrez 2011). They also varied according to the geographic regions. Furthermore, all the results confirmed that the extracts analysed possessed strong antioxidant activity in the different free radicals studied, and flavonoid compounds are the best candidates to assessing the quality of Basque propolis, due to their different biological properties and predominance in the phenolic fraction. Similarly, the higher the content of phenolic and flavonoids compounds, the more active was the

Table 4 Minimum inhibitory concentration against bacterial and fungal strains

Sample no.	Minimum inhibitory concentration (MIC) (mg/ml)							
	S. mutans	*S. pyogenes*	*S. aureus*	*H. pylori*	*S. enterica*	*E. coli*	*C. albicans*	*S. cerevisae*
1	0.9/0.9	18/19	0.8/0.8	7/8	0.8/0.9	n.d..	0.8/0.8	0.9/0.9
2	0.6/0.7	24/26	0.6/0.7	6/8	0.7/0.8	n.d.	0.7/0.8	0.8/0.9
3	0.8/0.9	20/21	0.8/0.9	9/11	0.9/1.0	n.d.	0.8/0.9	0.8/0.9
4	0.7/0.7	22/22	0.8/0.8	7/8	0.8/0.8	n.d.	0.8/0.9	0.7/0.8
5	0.7/0.8	22/24	0.7/0.8	8/8	0.6/0.6	n.d.	0.6/0.7	0.7/0.7
6	0.8/0.9	21/22	0.9/1.0	8/9	0.8/0.9	n.d.	0.9/1.0	0.9/1.0
7	1.1/1.1	25/26	1.0/1.1	11/13	1.0/1.2	n.d.	1.2/1.1	1.3/1.4
8	0.8/0.8	20/21	0.8/0.9	9/10	0.9/1.0	n.d.	0.8/0.8	0.9/0.9
9	0.7/0.8	18/19	0.7/0.7	6/6	0.6/0.6	n.d.	0.9/1.0	0.9/1.0
10	0.8/0.9	19/19	0.9/1.1	7/8	0.8/0.9	n.d.	0.8/0.9	0.9/0.9
11	1.2/1.3	26/25	1.3/1.3	13/14	1.2/1.4	n.d.	1.3/1.3	0.9/0.9
12	0.7/0.8	19/20	0.8/0.9	8/9	0.7/0.8	n.d.	0.8/0.9	1.4/1.5
13	0.9/1.0	21/22	0.9/1.0	10/11	1.0/1.0	n.d.	1.0/1.1	0.8/0.9
14	0.7/0.7	18/19	0.5/0.6	8/9	0.9/1.0	n.d.	0.8/0.9	0.9/1.0
15	0.6/0.7	17/18	0.9/0.9	6/6	0.8/1.0	n.d.	0.7/0.8	0.9/0.9
16	0.8/0.9	18/18	0.7/0.8	9/9	0.8/0.9	n.d.	0.9/1.0	1.1/1.2
17	0.9/1.0	18/19	0.7/0.8	8/9	0.9/1.0	n.d.	0.8/0.9	0.9/1.0
18	0.9/1.0	21/22	0.8/0.9	10/11	1.0/1.1	n.d.	1.0/1.2	1.2/1.3
19	0.7/0.8	18/19	0.8/0.9	8/9	0.8/0.9	n.d.	0.8/0.9	0.9/0.9
Control	0	0	0	0	0	0	0	0
Mean	0.8/0.9	20/21	0.8/0.9	8/9	0.8/0.9	n.d.	0.9/0.9	0.9/1.0
Range	0.6–1.2/0.7–1.3	17–26/18–26	0.6–1.3/0.6–1.3	6–13/6–14	0.6–1.2/0.6–1.4	n.d.	0.6–1.3/0.7–1.3	0.7–1.4/0.7–1.5

Sample nos. 1–9 (year of sampling—2005); Sample nos. 10–19 (year of sampling—2008); Results for different extracts are separated by (/); first value in ethanol extract propolis (EEP); second value in propylene glycol extract propolis (PEP); n.d.: indicates that no inhibitory concentration was detected in the range tested (1–100 mg/ml)

antimicrobial activity detected in the samples analysed (Tables 2 and 4).

In general the strong antimicrobial activity of Basque propolis may be because of the presence of diverse phytochemical compounds, mainly flavonoids, this confirming the traditional reputation of propolis as a powerful antibacterial agent. Flavonoids and esters of phenolic acids are normally associated with the antibacterial activity of bee glue (propolis origin, honeybee species and extract preparation), especially in European propolis (Kosalec et al. 2003, 2004; Boyanova et al. 2005; Popova et al. 2009). However, although tropical propolis does not contain these kinds of compounds, they show similar antibacterial activity and this suggests that synergy between different compounds is essential for its biological activity (Kujumgiev et al. 1999).

Recently, we found that the flavonoid levels of aged propolis were 20% lower that those in fresh propolis, and that some labile propolis compounds are highly degraded (Bonvehí and Coll 2000). Thus, antibacterial activity was proposed as a quality criterion for propolis freshness (Dolci and Ozino 2003; Mohammadzadeh et al. 2007) and a significant negative correlation between the amount of total flavonoids and MIC value was observed in this study. The results showed that all extracts (i.e., EEP and PEP) of Basque propolis tested were very active, and the samples with highest total phenolic content also showed the best antimicrobial activity. We can conclude from this study that Basque propolis extracts show antimicrobial activity that acts mainly on Gram-positive bacteria and yeasts, having a positive correlation with flavonoid content.

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