Antibacterial activity of propolis extracts from the south of Portugal

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Abstract: To examine the antibacterial activity of diverse extracts of propolis harvested at winter and spring from several locations of Algarve, Portugal, against Gram-negative and Gram-positive bacteria was the main goal of the present work. For such, the antibacterial activity was determined by agar diffusion. The results showed that all tested bacterial strains showed susceptibility to diluted propolis extracts and in a dose-dependent manner. Two propolis samples collected at springtime showed higher antibacterial activity, in comparison with samples harvested at wintertime. Ethanolic and methanolic extracts have a very similar activity ($P<0.05$). *Helicobacter pylori* strains J99 and 26695 were the most susceptible strains to the tested extracts (33.67±2.52 mm and 35.67±0.58mm, respectively). This study constitutes the first approach of the biological activities of Portuguese propolis from the Algarve region and evidences its potential use to combat bacterial infections, in particular against the gastric pathogen *H. pylori*.

Keywords: Antibacterial activity; harvesting time; Portuguese propolis.

INTRODUCTION

Propolis is a natural resinous substance collected by honeybees (*Apis mellifera* L.) from various plant sources (Cardinault *et al*., 2012). The chemical composition of propolis is very complex and depends on the specificity of the local flora and thus on the geographic and climatic characteristics of this site. This fact results in the striking diversity of propolis chemical composition (Popova *et al*., 2010). A wide range of biological activities, namely antibacterial, anti-inflammatory, antioxidative, hepatoprotective effects and anti-tumoral activities have been reported (Miguel *et al*., 2010; Miguel *et al*., 2011; Teixeira *et al*., 2010; Cardinault *et al*., 2012). The presence of antioxidant compounds, namely phenolic constituents, especially flavonoids and phenolic acids are linked to these activities (Miguel *et al*., 2010; Teixeira *et al*., 2010; Miguel *et al*., 2011; Sforcin and Bankova, 2011; Miguel *et al*., 2014).

There is an increasing interest in propolis use for therapeutic purposes, and a better understanding of its activity will provide the scientific basis for its proper utilization, associated or not with conventional treatments (Orsi *et al*., 2006; Zaden *et al*., 2009). Galangin, pinocembrin and pinostrombin have been recognized as the most effective flavonoid agents against bacteria (Teixeira *et al*., 2010). Ferulic and caffeic acid also contribute to bactericidal action of propolis (Teixeira *et al*., 2010; Sforcin and Bankova, 2011; Cardinault *et al*., 2012). Different methods of extraction and different solvents employed for the extraction may also cause variability on biological activities (Sforcin and Bankova, 2011; Cardinault *et al*., 2012). The most appropriate season to collect propolis is also an important factor. The edafoclimatic conditions of the South of Portugal are particularly beneficial to the development of beekeeping and related activities such as the production of propolis.

It is known that crude propolis cannot be used and its purification is done by extraction with solvents that are mainly ethanol and methanol (Daher and Gülaçar, 2008). In our study the use aqueous, ethanolic and methanolic extracts was tested in order to investigate possible differences on the antibacterial activity related with extraction variation.

MATERIALS AND METHODS

Propolis samples

Propolis samples given by the beekeeper were obtained from four different locations: Barrocal Norte (B.N.) Arrodeios, B.N. Pé da Serra, Barrocal Sul (B.S.) Ameijoafra and Transição Norte (T. N.) Madeira in Salir situated in the Algarve region (South of Portugal). Samples were collected manually at two different times, winter and spring and stored in a dark cabinet at room temperature.

Three types of propolis extractions were tested: aqueous (Aq), ethanolic (Et) and methanolic (Me). The aqueous, methanolic and ethanolic extracts were prepared as described previously (Popova *et al*., 2004; Midorikawa *et al*., 2001). The propolis extracts were then diluted in *n*-propanol and used on the antimicrobial assays.

Antibacterial activity

The bacteria used in this study included two Gram-positive namely *Staphylococcus aureus* CFSA2,

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Streptococcus pneumoniae D39 and four Gram-negative, *Salmonella enterica* subspecie *enterica* serovar Thyphimurium ATCC 14028, *Helicobacter pylori* strains J99 and 26695 and *Haemophilus influenza* TD-4.The antimicrobial activity was determined by the agar diffusion method as previously described (Hazzit *et al*., 2009; Dandlen *et al*., 2011). Sterile filter paper discs (Oxoid, Basingstoke, UK) containing 5, 10, 15 and 20 µL of diluted propolis extract (1:10) in *n*-propanol, sterile *n*-propanol (used as negative control) and 30 µg of the antibiotic chloramphenicol or 10 µg of penicillin G per disc (used as positive control). The assays were done in triplicate. The diameter of the inhibition zone (mm) was measured after incubation for 24-48 h at 37ºC.

High performance liquid chromatography (HPLC) analysis

All standards were of high degree of purity (99%). The solutions of these standards were prepared in the appropriate solvent and filtered (syringe filter 0.45 µm PTFE membrane, VWR International) prior to the analysis by HPLC. Such standards were: benzoic acid, vanillic acid, rosmarinic acid, p-hydroxybenzoic acid, D-(-)-quinic acid, 3,4-dihydroxybenzoic acid, naringin, chlorogenic acid, (±)-naringenin, taxifolin, caffeic acid, gallic acid, diosmin, *p*-coumaric acid, quercetin, *trans*-cinnamic acid, sinapic acid, apigenin, syringic acid, galangin, carnosol, ferulic acid, pinocembrin, carnosic acid. The conditions of analysis were followed as described by Croci *et al*. (2009).

STATISTICAL ANALYSIS

Data were subjected to analysis of variance (ANOVA) by using the SPSS programme version 16.0. Duncan Post-hoc tests were performed when significant differences occurred at 5% level. Statistically significant differences between the activities of the micro organisms found in wintertime and springtime were evaluated using Student's *t*-test.

Correlations between the amounts of phenol or flavonoid and individual components of aqueous extracts and antimicrobial activities were achieved by Spearman correlation coefficient (*r*) at a significance level of 99% ($P<0.01$) and 95% ($P<0.05$).

Hierarchical cluster analysis was utilized to investigate the similarities and dissimilarities among the activities with respect to extracts. For classification, the Ward's Minimum Variance Method was utilized. The squared Euclidean distance was used as the dissimilarity measure for Ward's method.

RESULTS

Chemical composition

In wintertime, the concentrations of total phenols, flavone and flavonols, flavanones and dihydroflavonols changed according to the place of propolis collection as well as the type of solvent used (table 1). Independent on the solvent of extraction, propolis from TNM had always-lower amounts of total phenols and flavones and flavonols than the remaining samples. When ethanol 70% was used as solvent of extraction, this sample had the highest amounts of flavanones and dihydroflavonols and the lowest concentration in the aqueous extract, although without significant difference when compared to the remaining aqueous extracts (table 1).

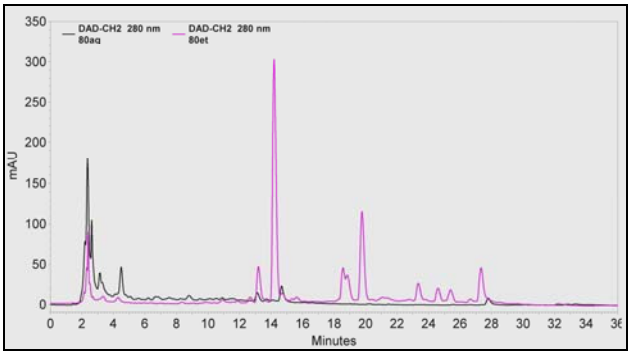

Fig. 1A: Matched HPLC chromatograms with an ethanolic extract (pink) with an aqueous extract (black)

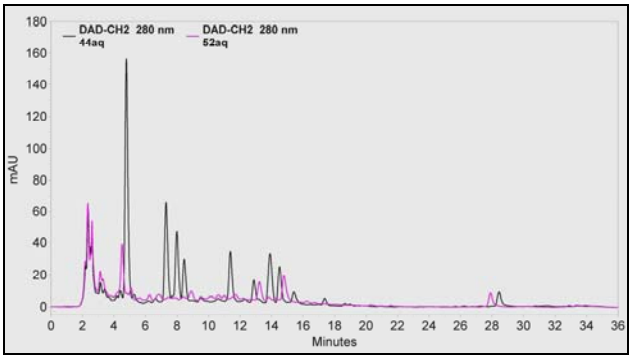

Fig. 1B: Matched HPLC chromatograms of the aqueous extract from B.N. Arrodeios (black) and T. N. Madeira (pink).

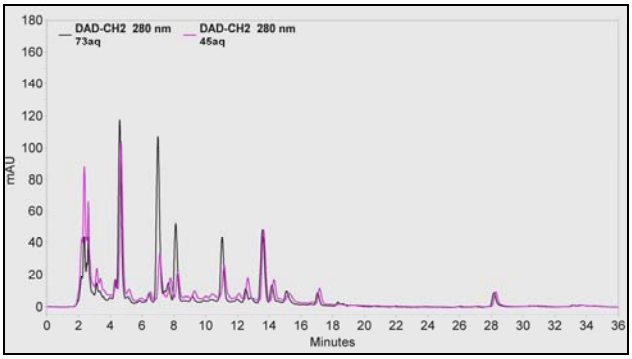

Fig. 1C: Matched HPLC chromatograms of the B. N. Pé da Serra collected in spring (black) and winter (pink).

Water was the poorest solvent for extracting phenols and flavonoids from all propolis samples (table 1 and fig. 1A). From fig. 1A, it is possible to compare the chromatographic profiles of propolis extracted with water

and ethanol 70%. Even in the absence of identification of compounds the chromatogram shows a great quantitative difference of phenols between the ethanolic and aqueous extracts of propolis.

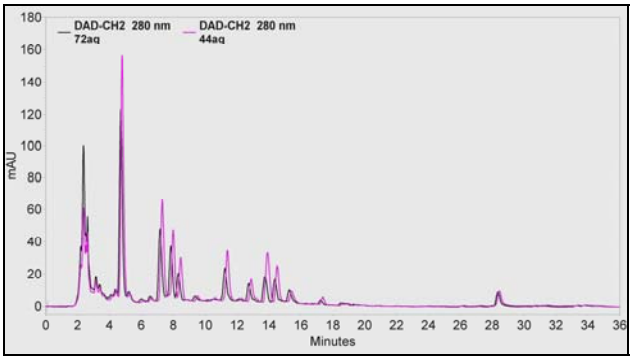

Fig. 1D: Matched HPLC chromatograms of the aqueous sample of B. N. Arrodeios collected in spring (black) and winter (pink).

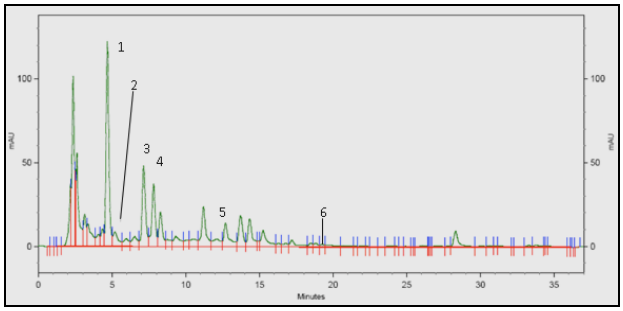

Fig. 1: HPLC chromatogram for a sample of a propolis aqueous extract, identified compounds: 1-caffeic acid, 2-syringic acid, 3-taxifolin, 4-ferulic acid, 5-apigenin and 6-galangin.

As reported for samples collected in wintertime, those harvested in springtime had also different amounts of total phenols, flavone and flavonols, flavanones and dihydroflavonols depending on the place of propolis collection as well as the type of solvent used (table 1).

Generally, propolis extracts collected in wintertime had higher amounts of total phenols than those from springtime. The exception was the sample from BNP, in which the extracts obtained from propolis collected in springtime possessed higher concentrations of phenols, however, the methanolic extract had higher content of phenols in wintertime than in springtime (table 1). Concerning flavonoids, the samples of BNP sample had also higher amounts of these compounds in this period than in winter. TNM also had higher amounts of phenols and flavonoids in springtime than in wintertime, but such was only observed in the aqueous extracts. Such may means the presence of higher amounts of hydro-soluble phenols and flavonoids than the remaining samples. The sample TNM was also the poorest in phenolic compounds (table 1). This propolis is therefore somehow different from the remaining samples and such may be supported by the fig. 1B which compares two aqueous extracts (TNM and BNA) obtained from propolis collected in wintertime.

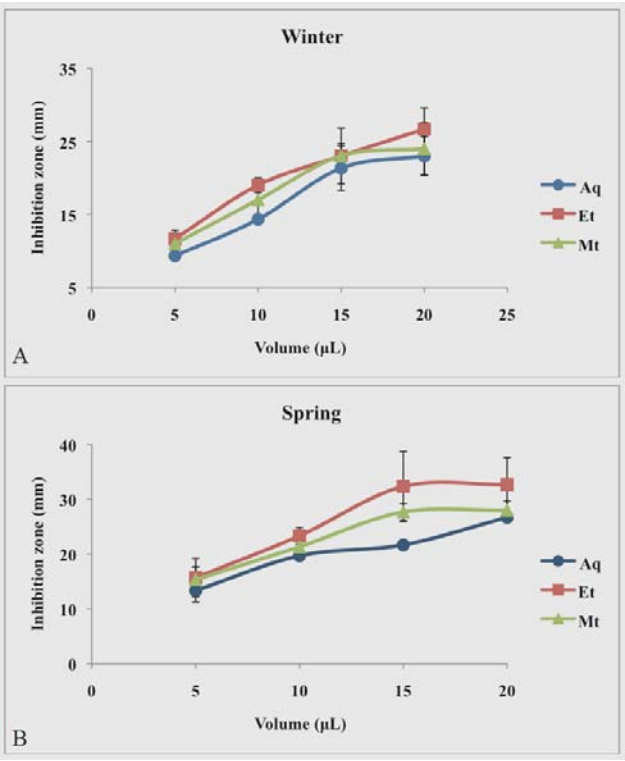

Fig. 2: Antibacterial activity of propolis extracts from B. N. Arrodeios collected in winter (A) and springtime (B) against *H. pylori* J99; Aq-aqueous, Et-ethanolic and Mt - methanolic extracts.

Fig. 1C depicts the chromatographic profile of the aqueous extracts of propolis from BNP which supports the quantitative data presented in table 1. Nevertheless some compounds, not identified, are present in similar amounts in both collection periods.

Fig. 1D is an aqueous extract of propolis from BNA in which higher amounts of phenols were found in wintertime than in springtime as supported through the data depicted in table 1.

Very few components were identified and quantified in the aqueous extracts of propolis (fig. 1E and table 2, respectively). Although the identification of syringic acid and galangin in the aqueous extracts, they were not quantified due to their very low amounts.

From the identified compounds in the present work, caffeic acid predominated in all samples of propolis collected in both time periods followed by taxifolin (table 2). It is noteworthy to refer the great increase of taxifolin concentration in the aqueous extract of propolis from BNP from winter (7.14µg/mL) to spring (22.93µg/mL)

(table 2). In the same sample, the concentration of caffeic acid also increased in the spring but not as pronounced as observed for taxifolin. In BNA samples, apigenin concentration decreased sharply from winter to spring. In the same sample, caffeic acid and taxifolin also decreased. In springtime, the amounts were almost half of those observed in winter; nevertheless the decrease of apigenin was much more important (table 2).

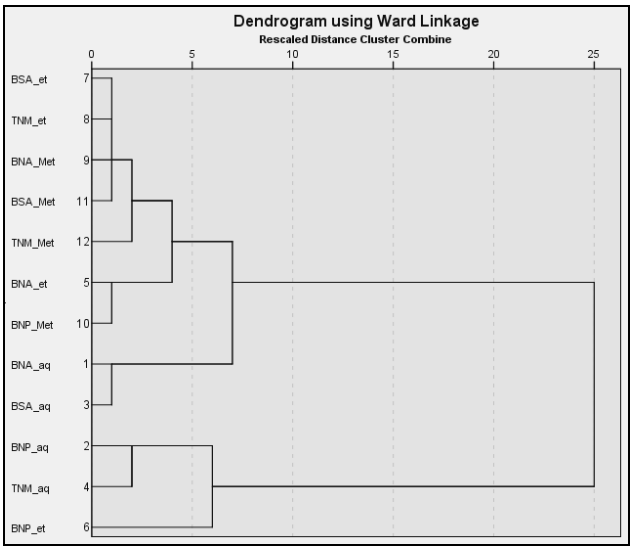

Fig. 3: Dendrogram for the classification of extracts of propolis collected in winter according to their activities against six microorganisms.

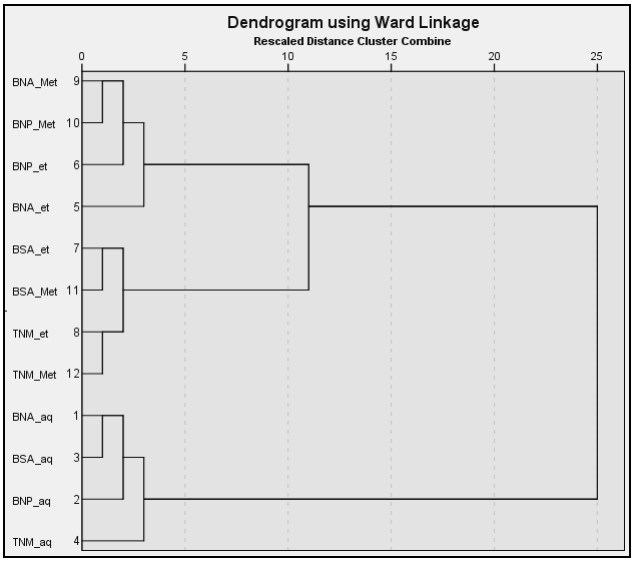

Fig. 4: Dendrogram for the classification of extracts of propolis collected in spring according to their activities against six micro organisms

The highest concentrations of total phenols, flavone and flavonols, flavanones and dihydroflavonols observed for the aqueous extracts of TNM in springtime did not correspond to the highest concentrations of the phenols identified and quantified in the present work. Other components were responsible for such results, and not yet identified.

Antibacterial activity

In the present study the antimicrobial activity of diverse extracts of propolis collected at Algarve region of Portugal is reported for the first time. Moreover, the impact of harvesting time and collection site on the biological activities was evaluated.

After testing different volumes of diluted propolis extracts (1:10) it was observed that all extracts exhibited antibacterial activity (table 3).

The bacterial susceptibility to propolis varied and considering the highest growth inhibition zone measured for each one when 20 µL of extract was applied, *S. Thyphimurium* was one of the most susceptible when the methanolic extract BNP-Me was used (27.33±1.53 mm) followed by *S. aureus* when exposed to the TNM-Et extract (28.33±2.89 mm), *H. influenza* under the activity of the BSA-Et extract (31.00±1.73 mm), *S. pneumoniae* D39 under the exposition of the BNP-Et extract (32.33± 2.52 mm) and *H. pylori* J99 when exposed to the TNM-Et extract (33.67±2.52 mm) (table 2). The most susceptible was *H. pylori* 26695 (35.67±0.58 mm) when the ethanolic extract BNA-Et was used (table 3). The results showed that both ethanolic and methanolic extracts have a very similar activity, may be partly due to their similar polarities, whereas the aqueous extracts showed a similar or a slightly minor activity in comparison to the ethanolic and methanolic extracts (table 3, fig. 2). For example, all three extracts of samples collected from B. N. Arrodeios in wintertime have similar antibacterial activity, except for *H. pylori* 26695, *S. pneumoniae* and *H. influenza* for which the aqueous extracts displayed a minor inhibition activity.

By applying cluster analysis of the antimicrobial activities, three groups were possible to detect, independent on the collection period of samples. Such groups were more closely related with the type of solvent, as the dendrograms permit to see than to the collection location (figs. 3 and 4). In winter, two clusters with all aqueous extracts and one ethanolic sample (BNP-Et) were found, and the third cluster with the remaining alcoholic samples (methanol and ethanol). According to the fig. 4, it is possible to see a cluster with all aqueous extracts, whereas the second cluster had BNA-Et, BNA-Met, BNP-Et and BNP-Met samples, and the third cluster groups the remaining BSA-Et, BSA-Met, TNM-Et and TNM-Met.

Methanol and ethanol extracts were significantly better than the aqueous ones for prevent the growth of *H. pylori* 26695 and *Staph. pneumoniae* in both collection periods (table 4). In contrast, the type of solvent was not important in the activities found for *H. pylori* J99 either in

Table 1: Concentrations (mg/mL) of phenols, flavone and flavonols, flavanones and dihydroflavonols in ethanolic, methanolic and aqueous extracts of propolis collected in Algarve (Portugal): Barrocal Norte Arrodeios (BNA), Barrocal Norte Pé da Serra (BNP), Barrocal Sul Ameijoafra (BSA) and Transição Norte Madeira (TNM) in winter and springtime

				Winter					
Sample	Phenol-Et	Phenol-Met	Phenol-Aq	Flavone-Et	Flavone-Met	Flavone-Aq	Flavanone-Et	Flavanone-Met	Flavanone-Aq
BNA	9.98±0.88^{a}	8.48±0.78^{a}	2.48±0.24^{a}	1.98±0.12^{a}	2.53±0.28^{a}	0.023±0.002^{ab}	1.72±0.12^{ab}	2.09±0.26^{a}	0.69±0.11^{a}
BNP	8.48±0.88^{a}	8.08±0.78^{a}	1.53±0.24^{b}	1.78±0.12^{a}	2.58±0.28^{a}	0.021±0.002^{bc}	1.33±0.12^{c}	2.00±0.26^{a}	0.54±0.11^{a}
BSA	10.87±0.88^{a}	9.85±0.78^{a}	3.18±0.24^{a}	1.69±0.12^{a}	2.51±0.28^{a}	0.030±0.002^{a}	1.52±0.12^{bc}	1.67±0.26^{a}	0.75±0.11^{a}
TNM	5.42±0.88^{b}	4.94±0.78^{b}	0.79±0.24^{b}	1.29±0.12^{b}	1.73±0.28^{a}	0.014±0.002^{c}	1.96±0.12^{a}	1.71±0.26^{a}	0.45±0.11^{a}
				Spring					
BNA	9.57±0.81^{a}	5.31±0.63^{b}	2.06±0.36^{a}	1.67±0.10^{ab}	1.97±0.18^{ab}	0.023±0.004^{a}	2.15±0.26^{a}	0.91±0.51^{c}	0.49±0.09^{b}
BNP	9.81±0.81^{a}	6.69±0.63^{ab}	1.97±0.36^{a}	1.82±0.10^{a}	2.21±0.18^{a}	0.028±0.004^{a}	2.10±0.26^{a}	3.19±0.51^{a}	0.69±0.09^{ab}
BSA	9.17±0.81^{a}	7.71±0.63^{a}	3.07±0.36^{a}	1.40±0.10^{b}	1.92±0.18^{ab}	0.033±0.004^{a}	1.09±0.26^{b}	2.66±0.51^{ab}	0.78±0.09^{ab}
TNM	3.85±0.81^{b}	3.23±0.63^{c}	2.72±0.36^{a}	0.96±0.10^{c}	1.43±0.18^{b}	0.025±0.004^{a}	1.47±0.26^{ab}	1.14±0.51^{bc}	0.86±0.09^{a}

Values expressed are the mean value of three replicates ± the standard deviation. Values within each column in the same collection period followed by different letters are statistically different (P<0.05)

Table 2: Concentration (μg/mL) of some phenolic compounds detected in the aqueous extracts of propolis collected in winter and springtime at different places of Algarve (Portugal): Barrocal Norte Arrodeios (BNA), Barrocal Norte Pé da Serra (BNP), Barrocal Sul Ameijoafra (BSA) and Transição Norte Madeira (TNM)

	Winter			
Sample	Caffeic acid	Taxifolin	Ferulic acid	Apigenin
BNA	40.52±0.02^{a}	21.53±0.00^{a}	4.50±0.00^{a}	8.56±0.00^{a}
BNP	22.49±0.02^{c}	7.14±0.00^{c}	1.63±0.00^{c}	1.31±0.00^{c}
BSA	24.84±0.02^{b}	13.7±0.00^{b}	2.45±0.00^{b}	2.69±0.00^{b}
TNM	11..35±0.02^{d}	1.78±0.00^{d}	0.05±0.00^{d}	0.06±0.00^{d}
		Spring		
BNA	27.50±0.00^{a}	10.57±0.00^{c}	3.88±0.00^{a}	0.09±0.00^{c}
BNP	25.29±0.00^{b}	22.93±0.00^{a}	1.11±0.00^{c}	1.00±0.00^{b}
BSA	18.72±0.00^{c}	11.98±0.00^{b}	1.26±0.00^{b}	2.33±0.00^{a}
TNM	10.89±0.00^{d}	1.35±0.00^{d}	0.05±0.00^{d}	0.06±0.00^{d}

Values expressed are the mean value of three replicates ± the standard deviation. Values within each column in the same collection period followed by different letters are statistically different (P<0.05)

winter or springtime. The activities against *Salmonella* and *Staph. aureus* of aqueous extracts of propolis collected in spring were significantly inferior to those of ethanolic and methanolic extracts, nevertheless in winter such differences were not evident (table 4).

Antimicrobial activities of propolis extracts collected in winter and springtime were not generally significant different according to Student's *t*-test. The exceptions were *H. pylori* J99 and *Salmonella Thyphimurium*, which were more sensitive to the extracts obtained from propolis collected in the springtime than in wintertime (P<0.05 and P<0.01, respectively) (table 5).

H. pylori 26695 was the most sensitive to the extracts of propolis in both collection periods (table 5). Propolis extracts exhibit a dose dependent antibacterial activity, as illustrated for *H. pylori* (fig. 6A and 6B).

The correlation of activities and amounts of phenols and flavonoids was performed in the present work and such is presented in table 6. In some cases this correlation was found, although in other cases such was not found, deserving further studies to clarify these results.

According to the results, the activity of *H. pylori* J99 seems to be due to other components other than phenols, nevertheless flavones and flavonols such as flavanones and di-hydroflavonols present in the extracts of propolis seem to contribute to the activity against *H. pylori* 26695 and *S. pneumoniae*. Such as for *H. pylori* J99, the activity of propolis extracts against *Haemophilus influenza* cannot be attributed to flavanones and dihydroflavonols and flavonols and flavones or even other phenol components, particularly in samples of springtime. Concerning *Staph. aureus* CFSA2, flavanones and di-hydroflavonols and flavones and flavonols are responsible for the activity of the extracts. Difficult to explain is the fact that the activity against *Salmonella Thyphimurium* of propolis extracts of wintertime cannot be correlated with the presence of phenols in contrast to that observed in springtime.

Table 3: Antibacterial activity (inhibition zone, mm) of propolis extracts from winter and spring samples

Collection time	Sample[1]	*Salmonella* Thyphimurium	*Staph. aureus* CFSA2	*H. pylori* J99	*H. pylori* 26695	*S. pneumoniae*	*Haemophilus influenza*
Winter	BNA-Aq	24.66±0.57^{cdef}	23.00±1.00^{defg}	23.00±2.65^{bcd}	26.00±1.00^{fg}	21.33±3.21^{efghij}	24.67±0.58^{defghi}
	BNP-Aq	20.67±1.15^{hi}	22.00±0.00^{ghi}	9.50±0.71^{cde}	23.67±2.08^{gh}	18.67±2.52^{ij}	17.67±3.05^{j}
	BSA-Aq	20.67±1.15^{hi}	20.00±0.00^{i}	25.33±0.58^{abc}	24.67±0.58^{gh}	20.50±0.71^{fghij}	21.33±0.58^{hij}
	TNM-Aq	15.00±2.00^{l}	20.00±2.83^{i}	7.67±1.15^{de}	27.00±0.00^{efg}	19.67±1.15^{ghij}	23.33±2.08^{fghi}
	BNA-HA	25.00±0.00^{bcde}	25.00±0.00^{bcde}	26.67±2.89^{abc}	35.67±0.58^{ab}	30.00±4.36^{abc}	28.33±0.58^{cdefg}
	BNP-HA	22.67±0.58^{fgh}	24.67±0.58^{cdef}	7.00±1.73^{e}	32.00±0.00^{bcd}	32.33±2.52^{ab}	29.00±1.00^{cde}
	BSA-HA	16.00±0.00^{kl}	20.33±1.15^{hi}	30.00±0.00^{ab}	33.00±1.00^{bcd}	24.00±1.00^{cdefghi}	31.00±1.73^{c}
	TNM-HA	18.33±1.53^{j}	23.00±0.00^{defg}	30.33±1.15^{ab}	35.33±3.05^{ab}	25.33±4.93^{cdefg}	29.67±0.58^{cd}
	BNA-Mt	24.00±1.73^{cdefg}	25.33±0.58^{bcd}	24.00±3.60^{bc}	33.67±1.15^{bcd}	30.00±2.83^{abc}	28.67±1.15^{cdef}
	BNP-Mt	23.00±1.00^{efg}	25.33±0.58^{bcd}	27.00±2.65^{ab}	34.00±1.73^{abc}	23.00±2.65^{defghij}	26.00±2.00^{cdefgh}
	BSA-Mt	17.50±0.71^{jk}	21.00±0.00^{ghi}	28.33±8.50^{ab}	33.67±0.58^{bcd}	29.33±3.05^{abc}	28.33±2.08^{cdefg}
	TNM-Mt	19.00±2.00^{ij}	20.00±0.00^{i}	25.67±1.15^{abc}	27.33±8.08^{efg}	29.67±2.52^{abc}	30.50±0.71^{c}
Spring	BNA-Aq	22.67±0.58^{fgh}	22.33±4.62^{fghi}	26.67±0.58^{abc}	26.00±1.00^{fg}	19.00±2.65^{hij}	23.67±0.58^{efghi}
	BNP-Aq	24.00±1.00^{cdefg}	17.17±1.47^{j}	26.00±1.00^{abc}	23.67±2.08^{gh}	22.00±2.65^{defghij}	23.33±0.58^{fghi}
	BSA-Aq	23.33±0.58^{defg}	23.33±0.58^{defg}	27.67±1.15^{ab}	25.00±1.67^{gh}	20.00±1.73^{ghij}	20.00±0.00^{ij}
	TNM-Aq	22.00±1.00^{gh}	22.67±0.58^{efgh}	23.67±1.15^{bc}	21.33±2.31^{h}	17.33±1.53^{j}	27.00±0.00^{cdefg}
	BNA- HA	25.00±0.00^{bcde}	25.00±0.00^{bcde}	26.67±2.89^{abc}	35.67±0.58^{ab}	30.00±4.36^{abc}	28.33±0.58^{cdefg}
	BNP- HA	24.33±1.15^{cdef}	22.33±4.62^{fghi}	27.67±4.62^{ab}	27.00±7.07^{efg}	26.33±1.15^{bcdef}	29.00±1.73^{cde}
	BSA- HA	25.67±2.52^{abc}	26.00±0.00^{abc}	29.67±1.53^{ab}	27.00±4.58^{efg}	25.33±2.08^{cdefg}	20.33±0.58^{ij}
	TNM- HA	24.00±1.00^{cdefg}	28.33±2.89^{a}	33.67±2.52^{ab}	30.00±0.00^{de}	27.00±2.65^{bcde}	23.00±1.73^{ghi}
	BNA-Mt	25.33±1.15^{c}	26.33±0.58^{abc}	28.00±1.73^{ab}	31.33±1.15^{cd}	25.00±3.60^{cdefgh}	28.33±1.15^{cdefg}
	BNP-Mt	27.33±1.53^{a}	27.33±0.58^{ab}	27.00±1.00^{ab}	31.00±2.00^{cd}	27.00±1.00^{bcde}	30.00±1.00^{cd}
	BSA-Mt	27.00±2.65^{ab}	26.33±0.58^{abc}	31.33±2.52^{ab}	29.83±1.60^{def}	26.33±2.08^{bcdef}	19.67±1.15^{ij}
	TNM-Mt	24.33±1.15^{cdef}	26.00±1.00^{abc}	31.67±0.58^{ab}	33.00±2.00^{bcd}	27.67±1.53^{abcd}	23.00±2.65^{ghi}
	Chloramphenicol	24.67±0.58^{cdef}	21.67±1.63^{ghi}	42.25±16.50^{a}	37.67±2.52^{a}	22.83±2.56^{defghij}	37.67±0.58^{b}
	Penicillin	Nd.	N.d.	N.d.	N.d.	33.17±6.24^{a}	43.67±7.70^{a}

[1]Values expressed are the mean value of three replicates ± the standard deviation using 20µL of extracts of propolis. Values within each column followed by different letters are statistically different (P<0.05); BNA -B. N. Arrodeios, BNP -B. N. Pé da Serra, BSA - B. S. Ameijoafra and TNM - T. N. Madeira; Aq-Aqueous, HA – Hydro-Alcoholic and Mt – Methanolic extracts. N.d- not determined.

When correlating the amounts of those compound quantified in the aqueous extracts with the antimicrobial activities, such was not found, unless the activity against *H. pylori* 26695 which correlated well with the amounts of taxifolin and ferulic acid (table 6). These results suggest that the antimicrobial activities found in the aqueous extracts in the majority of cases cannot be attributed to caffeic acid, ferulic acid, taxifolin or apigenin.

DISCUSSION

Chemical composition

The amounts of total phenols, flavones and flavonols, flavanone and dihydroflavonols of the extracts were similar to those already reported by some authors (Miguel *et al*., 2010; Miguel *et al*., 2011; Miguel *et al*., 2014) for samples collected in the same areas. Nevertheless in the present work, the samples were harvested in specific zones. For example, in the case of samples from Ameijoafra and Pé da Serra, both from Barrocal of Algarve, and Madeira from Transição zone, the samples were collected on the northern slopes. Concerning the samples of Ameijoafra from Barrocal, they were collected on the southern slopes. Due to the proximity of locations, it was expected similar results.

Only few compounds were identified in the aqueous extracts by HPLC for the first time as far as we know and the results show that much more compounds are present in such extracts due to the differences between the total content of phenol, flavones and flavonols, flavanones and dihydroflavonols and the respective amounts of caffeic and ferulic acids, taxifolin, and apigenin, respectively. Syringic acid and galangin was also detected in all samples but in such low concentrations that hampered their quantifications. The identification of the compounds was done by comparing the retention times of the standard samples with those from the samples of the aqueous propolis extracts and simultaneously by co-elution of the standard samples with the samples of propolis. The standard samples used were selected based on the compounds that had been previously identified on samples of European propolis from poplar origin (Medana *et al*., 2008; Popova *et al*., 2010).

Table 4: Antibacterial activity (inhibition zone, mm) of propolis aqueous, ethanolic and methanolic extracts from winter and spring samples

				Winter		
Sample	*Salmonella* Thyphimurium	*Staph. aureus* CFSA2	*H. pylori* J99	*H. pylori* 26695	*S. pneumoniae*	*Haemophilus influenza*
Water	20.25±2.76^{a}	21.25±1.56^{a}	16.38±5.90^{a}	25.36±1.70^{b}	20.04±2.23^{b}	21.75±1.58^{b}
Ethanol 70%	19.92±2.76^{a}	22.58±1.56^{a}	23.42±5.90^{a}	33.42±1.70^{a}	27.58±2.23^{a}	29.83±1.58^{a}
Methanol	19.71±2.76^{a}	22.33±1.56^{a}	27.83±5.90^{a}	32.58±1.70^{a}	26.83±2.23^{a}	28.63±1.58^{a}
				Spring		
Water	23.00±0.74^{b}	21.38±1.56^{b}	26.00±1.73^{a}	24.00±1.94^{b}	19.58±1.24^{b}	23.50±2.84^{a}
Ethanol 70%	24.75±0.74^{a}	25.42±1.56^{a}	29.42±1.73^{a}	29.92±1.94^{a}	27.17±1.24^{a}	25.17±2.84^{a}
Methanol	26.00±0.74^{a}	26.50±1.56^{a}	29.50±1.73^{a}	31.29±1.94^{a}	26.50±1.24^{a}	25.25±2.84^{a}

Values expressed are the mean value of three replicates ± the standard deviation. Values within each column in the same collection period followed by different letters are statistically different (P<0.05)

Table 5: Antimicrobial activities (inhibition zone, mm) of propolis extracts collected in winter and springtime

Microorganism	Winter	Spring
Salmonella Thyphimurium	20.00±1.02**^{d}	24.58±0.46^{b}
Staph. aureus CFSA2	22.06±0.60^{cd}	24.43±0.88^{b}
H. pylori J99	22.54±2.60*^{cd}	28.31±0.81^{a}
H. pylori 26695	30.45±1.26^{a}	28.40±1.19^{a}
S. pneumoniae	24.42±1.31^{bc}	24.42±1.13^{b}
Haemophilus influenza	26.74±1.22^{ab}	24.64±1.08^{b}

Each value indicates the mean ± standard error of three independent experiments. *P<0.05 and **P<0.01 versus springtime by Student's t-test. Values within each column in the same collection period followed by different letters are statistically different (P<0.05)

Antibacterial activity

In our study, differences in the antibacterial activity according to the type of extract tested were observed. These differences also have been found by others and can be associated to the use of different solvents, which consequently extract diverse compounds (Croci *et al*., 2009). Our results showed that both ethanolic and methanolic extracts have similar activity, as expected since both solvents have similar polarity and therefore the resulting extract is constituted by the same components. The aqueous extracts showed a similar or a slightly lower activity in comparison to the ethanolic and methanolic extracts. This slight diminution of activity cannot only be explained by the amounts of the phenols in the samples, because a great difference was found in the amounts of these compounds, which did not correspond with the same strength in the antibacterial activities. The type of compounds seems to be more adequate for explaining these results. In fact, caffeic acid and its phenetyl esters, flavonoids (e.g. galangin) and the combined action of several components, sometimes none of which alone are effective have been reported as being very important in the antimicrobial activities found for propolis extracts (Grange and Davey, 1990; Popova *et al*., 2005). The absence of correlation between activity and apigenin, taxifolin, caffeic acid and ferulic acid amounts quantified in the aqueous extracts may show that the antimicrobial activities depended on the whole action of those compounds and/or other components not identified. Diterpenic acids were also found to contribute to the antibacterial activity of propolis, mainly against *Staph. aureus* (Bankova *et al*., 1996). However, the amounts of phenolic compounds, flavones and flavanones have been reported very important for the antibacterial activity of poplar propolis (Popova *et al*., 2005). The absence of correlation between activity of propolis samples against some microorganisms and amounts of phenols found in our work may be explained by the presence of other components responsible for the activities but not yet identified.

As previously reported (Miguel *et al*., 2010), the plant species found in the regions where propolis were collected include: *Quercus suber*, *Arbutus unedo* and *Cistus ladanifer*, along with several herbaceous plants such as *Lavandula luisieri*, *L. viridis*, *Tuberaria guttata*, *Trifolium campestre*, *T. stellatum*, among many others. The presence of diterpenes in some of these extracts may be responsible for the activity against *H. pylori* J99. In recent studies, the volatile fraction of some propolis of the same region presented in relative high amounts diterpene alcohols characteristic of *C. ladanifer* (Miguel *et al*., 2014).

Table 6: Spearman correlations between antimicrobial activity and amounts of phenols, flavones, flavonols, flavanones and dihydroflavonols

	Salmonella Thyphimurium	*Staph. aureus* CFSA2	*H. pylori* J99	*H. pylori* 26695	*S. pneumoniae*	*Haemophilus influenza*
			Winter			
Total phenols	-	-	-	-	-	0.639*
Flavones and flavonols	-	0.633*	-	0.718**	0.708*	-
Flavanones and dihydroflavonols	-	0.615*	0.594*	0.823**	0.788**	-
Caffeic acid	-	-	-	-	-	-
Taxifolin	-	-	-	-	-	-
Ferulic acid	-	-	-	-	-	-
Apigenin	-	-	-	-	-	-
			Spring			
Total phenols	0.722**	-	-	0.579*	0.640*	-
Flavones and flavonols	0.884**	0.605*	-	0.704*	0.625*	-
Flavanones and dihydroflavonols	0.751**	0.622*	-	0.648*	0.800**	-
Caffeic acid	-	-	-	-	-	-
Taxifolin	-	-	-	0.961*	-	-
Ferulic acid	-	-	-	0.960*	-	-
Apigenin	-	-	-	-	-	-

**Correlation is significant at the $P<0.01$; *Correlation is significant at the $P<0.05$; – not significant

The differences observed in antibacterial activity between collection times particularly for *Salmonella Thyphimurium* and *H. pylori* J99 may be related to the concentration of the bioactive compounds. These findings were previously reported with other samples of propolis (Castro *et al*., 2007; Bonvehí and Gutiérrez, 2012). Flavonoids and esters of phenolic acids in European propolis are generally associated with the antimicrobial activities (Popova *et al*., 2009), although other components can also show similar antibacterial activities alone or in association by synergism (Aga *et al*., 1994; Bankova *et al*., 1996; Kujumgiev *et al*., 1999; Bonvehí and Gutiérrez, 2012).

H. pylori strains showed a significant susceptibility to the tested propolis extracts. Bonvehí and Gutiérrez (2012) also reported anti-helicobacter activity of Basque propolis, attributing such activity to the flavonoids. The presence of this kind of compounds in our aqueous samples (taxifolin, apigenin and galangin) may partly explain the susceptibility of *H. pylori* 26695 to the propolis extracts, but other components such labdane-type diterpenes and some prenylated phenolic compounds constituting methanolic extracts of Brazilian propolis have been also reported by some authors (Banskota *et al*., 2001), as possessing antibacterial activity against *H. pylori.* Considering that our samples also possess diterpenes as reported in previous studies in the volatile fractions of the same samples (Miguel *et al*., 2014), the activity may be attributed to a joint action of diterpenes and flavonoids.

CONCLUSION

Water had lower capacity for extracting phenols and flavonoids from all propolis samples than ethanol or methanol. Caffeic acid followed by taxifolin predominated in all samples of propolis collected either in winter or springtime. Ethanolic and methanolic extracts presented similar antimicrobial activities and only slightly higher than those of aqueous extracts. Antimicrobial activities of propolis extracts collected in winter and springtime were not generally significant different, with the exceptions of *H. pylori* J99 and *Salmonella Thyphimurium* which were more sensitive to the extracts obtained from propolis collected in the springtime than in wintertime The susceptibility of all tested bacterial strains to diluted propolis extracts was dose-dependent. Among the Gram-negative and Gram-positive bacteria, *Helicobacter pylori* strains J99 and 26695 were the most susceptible strains to the tested extracts.

These results are promising to follow-up the use of propolis to combat this important gastrointestinal pathogen. The present study contributed to the first biological characterization of propolis produced in the South of Portugal supporting its future application for natural medicinal purposes, in the cosmetic and food industry.

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