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Original Article

Propolis polyphenolic compounds affect the viability and structure of *Helicobacter pylori in vitro*

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ABSTRACT

To evaluate the anti-*Helicobacter pylori* activity of the major polyphenol compounds of propolis and their cellular damage, both as single molecule or in combination. Honey bees propolis were fractionated by using CPC and preparative HPLC. Four major polyphenols (chrysin, pinocembrin, galangin and caffeic acid phenethyl ester) were identified by thin layer chromatography–mass spectroscopy and liquid chromatography–mass spectroscopy. These compounds inhibited both ATCC and clinical *H. pylori* strains, with caffeic acid phenethyl ester being the most active. The four compounds presented minimum inhibitory concentration in the range 256–1024 $\mu g\,ml^{-1}$ and a fractional inhibitory concentration of 64–512 $\mu g\,ml^{-1}$. In mixtures all compounds showed an indifference effect (FIC < 0.15) but chrysin + galangin which was synergistic (FIC = 2.0). Killing curves show a similar behavior as the antibiotic amoxycillin. On the other hand, analyses by transmission electron microscopy at sub inhibitory concentration show vesicle formation and cell lysis after exposition to both individual polyphenol compounds and in mixture. The major compounds of propolis show anti-*H. pylori* activity both as individual compounds and in mixture. When combined they present mainly indifference but exert a lytic activity upon *H. pylori*, suggesting a potential bactericidal activity of propolis.

Introduction

Infection by *Helicobacter pylori* continues to be a public health problem worldwide, especially due to inadequacies in eradication treatments under current therapy. According to the Maastricht V Consensus Report (Malfertheiner et al., 2017), conventional triple therapy, which includes a proton pump inhibitor (PPI) plus the antibiotic clarithromycin and amoxicillin or metronidazole, continues being the first-choice triple therapy. However, the World Health Organization informed recently that *H. pylori* was ranked as priority 2 on its global priority list of antibiotic-resistant bacteria, due to its increased resistance to clarithromycin (Tacconelli and Magrini, 2017). Nevertheless, when clarithromycin resistance of clinical isolates in a specific geographical area are above 20% the Maastricht V Consensus Report also established that this antibiotic must be replaced by levofloxacin. But this antibiotic presents adverse effects, including general malaise, headaches, nausea, vomiting and dizziness, which are all recognized as reasons for patient drop out the therapy (Camargo et al., 2014), pushing the focus in detecting new active compounds as alternative in therapeutic strategies for the management this bacterial infection (Malfertheiner et al., 2017).

Natural agents have been proposed as adjuvants in anti-*H. pylori* eradication therapy (Ayala et al., 2014; Venegas et al., 2016) and both *in vitro* and *in vivo* studies have reported the successful use of some natural products like mastic gum, broccoli, blueberries, propolis, cinnamon and curcumin (Murali et al., 2014; Shapla et al., 2018), but there are unsuccessful clinical trials too (Coelho et al., 2007). Special attention has conceited the polyphenols compounds such as phenolic acids and flavonoids, which are widely available in nature and particularly in propolis samples (Bankova et al., 2016; Shapla et al., 2018). Moreover, the assays carried out to detect bioactive molecules against *H. pylori* have shown that this antibacterial activity is associated with some plant species of ancestral medical and/or food uses (Parreira et al., 2014; Gulec et al., 2016). Among these products, propolis appears as one of the richest sources of polyphenols like caffeic acid phenethyl ester, chrysin, galangin, pinocembrin and quercetin (Yen et al., 2017).

Over 300 chemical compounds have been described in propolis of different origins (Castaldo and Capasso, 2002; Salatino et al.,

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2005). Chilean propolis, palynologically typified and identified by HPLC–ESI-MS/MS, also evidenced the presence of flavonoids, phenolic acids and their esters, with a relative abundance of caffeic acid phenethyl ester, chrysin, galangin, kaempferol, pinobanksin, pinocembrin and quercetin (Barrientos et al., 2013; Castro et al., 2014). These compounds have shown antibacterial activity upon Gram-negative and Gram-positive bacteria (Suleman et al., 2015), including *H. pylori* (Nostro et al., 2006; Villanueva et al., 2015; Baltas et al., 2016). However, any particular region – like the Biobío region in Chile – may have several botanical different micro-areas too and propolis produced by honey bees in this particular region also differs quantitatively in their evaluated biological properties (Venegas et al., 2016). In consequence, a precise description of the botanically characteristic of the sampling area must be done and should be geographically referenced too for comparison purpose and use.

Propolis shows other biological activities such as antioxidant, anti-inflammatory and anesthetic properties have been informed too (Bankova et al., 2000). Thus, nowadays propolis is considered one of the future therapies against *H. pylori* due to the content of a wide variety of biologically active compounds (Shapla et al., 2018). The aim of this work was to determine the anti-*H. pylori* activity of the major polyphenol compounds present in a Chilean propolis from a botanically characterized micro-area at the region of Biobío, and the effect of polyphenols upon the bacterial structure as single molecules or in mixture, both at inhibitory and sub-inhibitory concentrations. In addition, the antibacterial effect of the association of particular polyphenols from propolis and their death kinetic parameters were also evaluated.

Material and methods

Bacterial strains and growth condition

Strains of *Helicobacter pylori* ATCC 43504, ATCC J99, and the clinical strain UDEC-84C isolated from a gastric biopsy were used. All the strains were available at the Laboratorio de Patogenicidad Bacteriana, Universidad de Concepción, Chile. Strains were maintained at −80 °C in Columbia broth (Difco) with 20% glycerol. Prior to be used, the strains were activated by inoculating a Columbia agar medium supplemented with 5% horse blood (defibrinated) and DENT inhibitor followed by incubation for three to five days, under microaerophilic atmosphere (10% CO_2), at 37 °C. This culture medium and condition was also used for all assays.

Propolis samples

Propolis sample (code VIIICOR031215) was provided by the Laboratorio de Farmacognosia, Facultad de Farmacia, Universidad de Concepción. The sample was harvested three years ago (summer 2015) through the mesh method (Sales et al., 2006; Abu Fares et al., 2008) and stored at 4 °C in amber vials until use. The region of sampling – Biobío coast zone – is located 36°55′34.0″S (Venegas et al., 2016). Biobío coast zone is characterized for its template climate and has a botanical origin identified as *Populus* sp., *Salix humboldtiana* and *Eucalyptus globulus*. The total polyphenol content was 220.35 mg GAE/g (gallic acid equivalents per gram of sample) and the total flavonoids content, 37.15 mg quercetin/g (quercetin equivalents per gram of sample).

Isolation, identification and quantification of phenolic compounds in Chilean propolis

For the isolation of main propolis target compounds, a Spot-CPC-250-B Bio-Extractor centrifugal partition chromatograph (Armen, France) with a 250 ml total cell volume was used. The system has four-way switching valves that allows operation in either the descending or ascending modes. The CPC system was connected to a SPOT.PREP II system (Armen, France), with integrated UV detector and fraction collector. CPC separation was performed with a two-phase solvent system composed of hexane–ethyl acetate–methanol–water with a volume ratio 2:1:2:1 (v/v) (Arizona R system). The solvent mixture was automatically generated by the SPOT-PREP-II unit. The CPC rotor was first filled with 1.5 column volumes using the lower phase at 30 ml min^{-1} and 500 rpm rotation. Upper phase was pumped into the system in the ascending mode at a flow rate of 15 ml min^{-1} and rotation was increased from 0 to 2000 rpm. After equilibrium was reached, the sample (2 g of freeze-dried ethanol propolis extract) was dissolved in 10 ml 1:1 mixture of upper and lower layers and injected into the CPC system. Elution was monitored using scan 200–600; 280 and 350 nm wavelengths, collecting fractions in 32 ml tubes. Fractions with similar composition according with on-line UV spectra and TLC were combined and further purified by semi-preparative reverse-phase HPLC using a YL9111S binary pump system and a Kromasil C-18 (10.0 mm × 250 mm, 10 μm particle size) column, eluted with an isocratic program with 60% A (H_2O/formic acid 0.01%) and 40% B (100% ACN) in 35 min, flow rate 5 ml min^{-1}, detection at 280 and 350 nm. Samples were prepared at 5 mg ml^{-1} and injected in mobile phase using a loop of 500 μl.

The major polyphenols found were further identified and quantitated according to Pellati et al. (2011) using HPLC-UV/DAD through a Waters Alliance 2695 Separations Module – model 1100 (Milford, U.S.A.). As controls, commercial caffeic acid phenethyl ester, chrysin, galangin and pinocembrin compounds (Sigma, MO, USA) were used. Stock solutions of each compound were prepared at 1–5 mg in 10 ml methanol. Injection volume was 5 μl for both standards and samples. Calibration curves were prepared with the standards at five different concentrations: 150, 75, 37, 18, and 9 μg ml^{-1}. Peak areas were used to trace the calibration curves. Slope of the regression line and values of correlation coefficient (R^2) for each calibration plot were obtained by using GraphPad Prism 5 software package. The identity of compounds isolated was confirmed by TLC–MS by using a Camag TLC–MS interface connected to a LCMS-8030 (Shimadzu, USA). We analyzed masses in full-scale mode for positive and negative ions in the range 100–1000 *m/z*. The following parameters were established: capillary voltage: 4.5 kV; nebulizer pressure (N_2): 3 l min^{-1}; drying gas: 250 °C; flow: 15 l min^{-1}; collision gas: nitrogen, with a collision energy (CE) of 20 V. Fragmentation voltage was maintained at 135 V. Data was acquired with the software LabSolutions (Rev. B.02.01).

Agar diffusion, broth dilution and chess board assays of propolis

The agar diffusion test was performed according to the method described by Valgas et al. (2007), using 20 μl per well of a 100 μg ml^{-1} stock solution of the compound. The minimal inhibitory concentration (MIC) for each polyphenol was determined through broth micro dilution test using 92 wells microplate, according to the recommendations made by the Clinical and Laboratory Standards Institute for Gram-negative bacteria (CLSI, 2010). Also, the chess board assay was used to determine the presence of compounds interactionas recommended by CLSI (2010). Amoxicillin (2 μg ml^{-1}) was used as reference of an active drug upon *H. pylori*.

Fractional inhibitory concentrations (FIC) and FIC index

The FIC was determined as described by Gibriel et al. (2013). Briefly, MIC was determined for the dual mixture of polyphenols and for each compound alone. Then, the FIC index was calculated as the sum of (MIC of mixture divided by MIC of molecule 1 alone) plus (MIC of mixture divided by MIC of molecule 2 alone). When

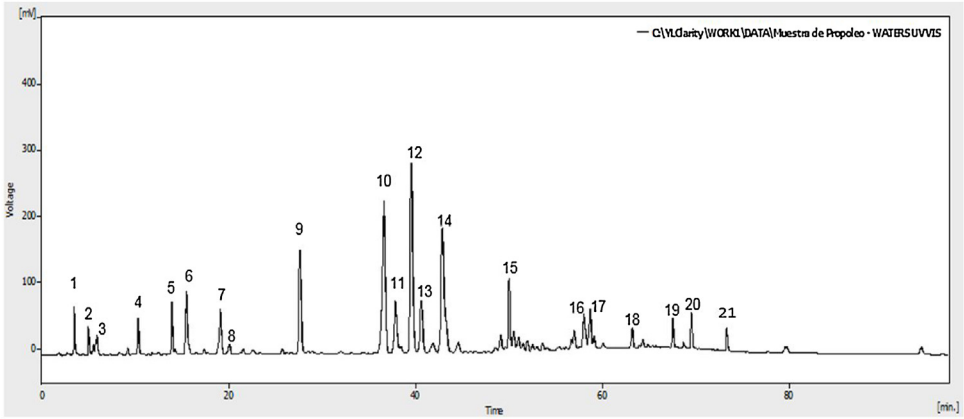

Fig. 1. HPLC trace ($\lambda_{280\,nm}$) of propolis sample used in this study. Identified compounds are: (1) caffeic acid, (2) *p*-coumaric acid, (3) ferulic acid, (4) 3,4-dimethyl-caffeic acid, (5) pinobanksin-5-methyl-ether, (6) kaempferide, (7) apigenin, (8) kaempferol, (9) cinnamilidenacetic acid, (10) caffeic acid prenyl ester, (11) chrysin, (12) pinocembrin, (13) galangin, (14) caffeic acid phenylethyl ester, (15) pinobanksin-3-*O*-acetate, (16) *p*-coumaric prenyl ester, (17) *p*-coumaric cinnamyl ester, (18) pinobanksin-3-*O*-butyrate, (19) pinobanksin-3-*O*-pentanoate, (20) pinobanksin-3-*O*-hexanoate, and (21) p-methoxy cinnamic acid cinnamyl ester (see Table 1 for details).

the FIC index was <0.5 synergy was present; for FIC index of 0.5–4 the mixture was indifferent, while FIC index over 4 was indicative of antagonism.

Killing curves

Selected propolis compound were assayed according to Flamm et al. (1996) and Pillai et al. (2005) protocol. Briefly, strains were cultivated in agar as previously described and a McFarland #2 suspension in saline solution was prepared to be used as inoculum. The assay was done by using Brain Heart Infusion Broth (BHIB) as a culture medium, supplemented with 1% yeast and viable count was evaluated through the microdrop method (Miles and Misra, 1938) in Columbia agar. The criteria defined by Pearson et al. (1980) were used to establish bacterial death. Viable bacterial counts were done in duplicate and the results are the average of two independent experiments.

Transmission electronic microscopy (TEM)

TEM was performed according to Goswami et al. (2012), with minor modifications. Briefly, 10 μl aliquots of the death kinetics tests obtained 0 and 12 h after exposure to polyphenols were centrifuged at 5000 × *g* for 10 min and the bacterial pellet was washed three times with 750 μl sterile distilled water. The pellet was fixed in 2.5% glutaraldehyde for 24 h at 4 °C, embedded in Araldite (Durcupan ACM, by Fluka, Switzerland) and then dehydrated in an ethanol gradient. Cuts of *ca.* 50–60 nm were deposited on 200-mesh collodion-carbon-coated grids, stained with 1% osmium tetroxide and were analyzed through a transmission electron microscope JEOL-JEM 1200 EX II (Jeol Technics Ltd, Tokyo, Japan).

Statistical analysis

When required, data were analyzed by using the statistical software GraphPad Prism 6 and one-way ANOVA. As a post-test, we also used Tukey's multiple comparison test and considered a statistical significance limit with value $p < 0.05$. Agar diffusion inhibition and death curve values correspond to the average between two independent experiments each performed in triplicate.

Table 1
Identification of polyphenols present in propolis.

Peak	Propolis compound	Retention time (min)	λ_{max} (nm)	Reference
1	Caffeic acid	3.6	292, 322	[a]
2	*p*-Coumaric acid	5.2	310	[b]
3	Ferulic acid	6.2	295sh, 322	[a]
4	3,4-Dimethyl-caffeic acid	10.9	295sh, 322	[b]
5	Pinobanksin-5-methyl-ether	14.7	286	[b]
6	Kaempferide	16.4	265, 335, 364	[a]
7	Apigenin	18.8	268, 337	[b]
8	Kaempferol	20.3	265, 364	[a]
9	Cinnamilidenacetic acid	29.1	256, 349	[b]
10	Caffeic acid prenyl ester	38.5	298, 325	[b]
11	Chrysin	39.6	268, 313	[a]
12	Pinocembrin	41.5	289	[a]
13	Galangin	42.6	265, 300sh, 358	[a]
14	Caffeic acid phenylethyl ester	44.7	295, 325	[a]
15	Pinobanksin-3-*O*-acetate	44.7	292	[b]
16	*p*-Coumaric prenyl ester	51.9	294, 310	[b]
17	*p*-Coumaric cinnamyl ester	58.5	294, 310	[b]
18	Pinobanksin-3-*O*-butyrate	59.1	292	[b]
19	Pinobanksin-3-*O*-pentanoate	64.3	292	[b]
20	Pinobanksin-3-*O*-hexanoate	65.7	292	[b]
21	*p*-Methoxy cinnamic acid cinnamyl ester	68.5	295, 325	[b]

[a] Confirmed with standard.
[b] Confirmed by reference (database). Peaks correspond to those compounds shown in Fig. 1.

Results

Identification and quantification of phenolic compounds in Chilean propolis

Twenty one polyphenols were detected and identified in the propolis sample studied (Fig. 1 and Table 1), which mostly belong to flavonoids and phenolic acids. Considering retention time, spectroscopic data and their proven anti-*H. pylori* activity, the main target compounds identified in the propolis sample were: caffeic acid phenethyl ester, chrysin, galangin and pinocembrin. Chromatographic analysis and quantification using HPLC-DAD allowed us to establish that caffeic acid phenethyl ester was the compound with the highest concentration in the sample, followed by pinocembrin, while the compound with the lowest was chrysin (Table 2). The equation and the correlation coefficients of the calibration curves observed indicated that were linear over the range of 9–150 $\mu g\,ml^{-1}$ used for all analytes. Other minor

Table 2
Identification of major polyphenols in propolis by HPLC-DAD and TLC–MS interface.

Polyphenol	Rt (min)	Exact mass	M-H^{exp}	Standard curve* (regression equation)	R^2	Proportion (%)
CRY	39.6	254.05	253.00	$y = 42.435x - 95.176$	0.9980	1.85
PIN	41.5	256.07	256.00	$y = 70.748x - 373.620$	0.9980	4.00
GAL	42.6	270.05	269.05	$y = 44.729x - 179.980$	0.9986	3.42
CAPE	44.7	284.10	285.10	$y = 31.446x - 15.007$	0.9993	8.02

Rt, retention time (in minutes); M-H^{exp}, experimental molecular ion (in negative ion mode); (·)*, peak area *vs* concentration in the extracts; CRY, chrysin; PIN, pinocembrin; GAL, galangin; CAPE, caffeic acid phenethyl ester.

compounds detected and identified too were ferulic acid, caffeic acid, kaempferol and kaempferide.

By CPC using the elution–extrusion method in ascending mode, the target polyphenols were obtained in fractions F7–F12 as detected by TLC using commercial pure compounds as reference (Fig. 2). Identity of these compounds was confirmed by direct MS analysis of each TLC band eluted using a TLC–MS interface. This procedure also allows to obtain the experimental mass and fragmentation patterns for each phenolic substance. Table 2 show the summary of the tentative identity assignment based on the experimental mass determined for each major polyphenolic compound. Kaempferol was not detected by TLC–MS.

Only caffeic acid phenethyl ester, galangin and pinocembrin were further purified by semipreparative HPLC, meanwhile kaempferol and chrysin were commercially acquired for the following analyses due to their low concentration in the propolis sample.

Agar diffusion method, minimum inhibitory concentration and chess board

All major propolis compounds showed inhibitory activity upon *H. pylori* (Table 3). Agar diffusion inhibition zones oscillated between 33 and 35 mm in diameter for the amoxicillin control, while polyphenols presented lower inhibition zones (between 18 and 25 mm). Additionally, the clinical strain *H. pylori* 84C was qualitatively and quantitatively more resistant than the reference ATCC strains. The *H. pylori* ATCC 43504 strain showed average MIC values of 256 μg ml^{-1} for chrysin, galangin and caffeic acid phenethyl ester, whereas the clinical strain showed average values of 512 μg ml^{-1}.

In dual mixture each compound's MIC remains unchanged (Table 4), but chrysin + pinocembrin mixture, which show a MIC 32–8 μg ml^{-1}, respectively, and a calculated FIC of 0.14, suggesting clearly the synergy between both compounds. Additionally, the galangin + caffeic acid phenethyl ester mixture showed a decrease in the MIC only for galangin, but the calculated FIC index was 1.25, which is indicative of indifference. Similar FIC index as galangin + caffeic acid phenethyl ester was obtained with the other four combinations assayed, which is indicative of an indifference effect for these combinations of polyphenols, too.

Killing curves

The combination of chrysin + pinocembrin and galangin + caffeic acid phenethyl ester showed a bactericidal effect upon *H. pylori* with similar death kinetic parameter as those observed with the amoxicillin control (Fig. 3). *D* values (time required for

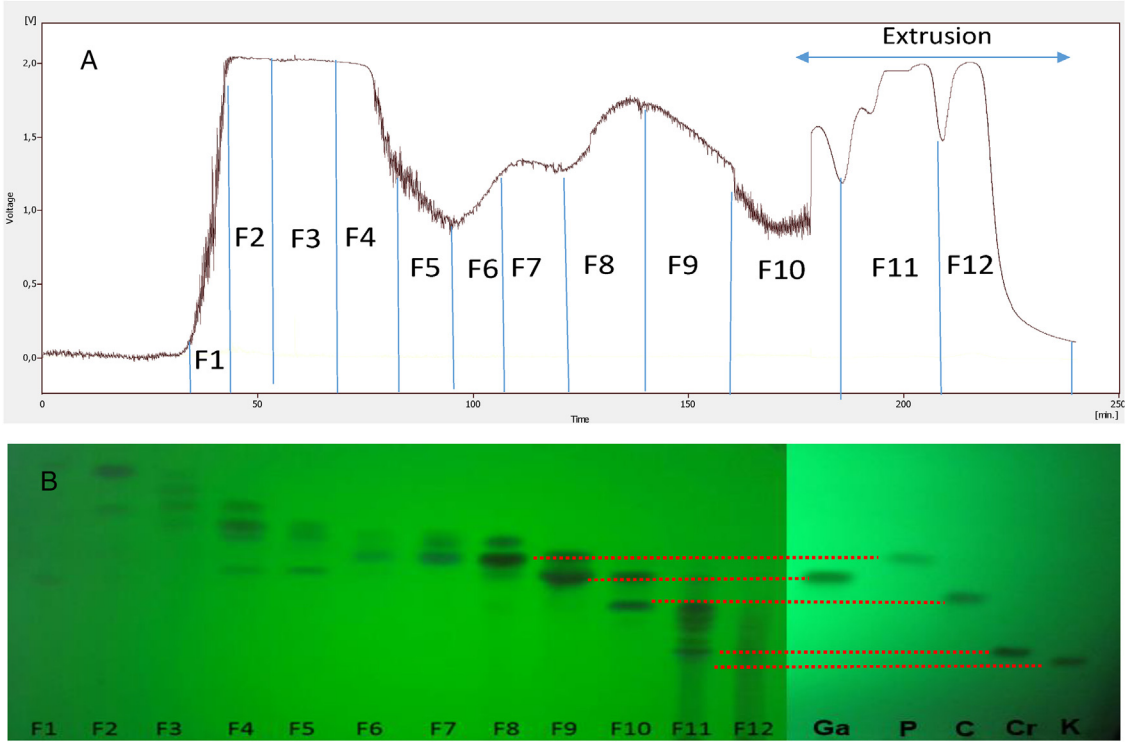

Fig. 2. (A) CPC trace of propolis extract in ascending mode (extrusion was performed at 175 min). TLC of different fractions of propolis obtained by CPC in ascending mode with the solvent system hexane–ethyl acetate–methanol–water with a volume ratio 2:1:2:1 (v/v). (B) TLC analysis of CPC fractions and comparison with standard of galangin (Ga), pinocembrin (P), caffeic acid phenylethyl ester (C), chrysin (Cr), and kaempferol(K).

Table 3
Antibacterial activity of major propolis polyphenols upon *Helicobacter pylori*.

Polyphenol assayed	Anti-bacterial activity upon strain					
	H. pylori ATCC 43504		*H. pylori* J99		*H. pylori* 84C	
	AD test	MIC	AD test	MIC	AD test	MIC
CRY	21 ± 1.16	256	20 ± 0.80	ND	18 ± 1.50	512
PIN	22 ± 1.32	512	22 ± 0.51	ND	19 ± 1.80	1024
GAL	21 ± 1.16	256	20 ± 1.04	ND	18 ± 1.90	512
CAPE	25 ± 1.41	256	24 ± 1.03	ND	20 ± 0.81	512

AD test, agar diffusion test expressed as millimeters of inhibition zone diameter – each value corresponds to the average of two independent experiments done in triplicate using 20 μl per well of a polyphenol stock solution (100 μg ml^{-1}); MIC, minimal inhibitory concentration (μg ml^{-1}) – each value corresponds to the average of two independent experiments; CRY, chrysin; PIN, pinocembrin; GAL, galangin; CAPE, caffeic acid phenethyl ester. For detail see "Material and methods" section.

Table 4
Fractional inhibitory concentration (FIC) and FIC index of the major propolis polyphenols upon *Helicobacter pylori* ATCC 43504.

Parameter	Polyphenols combined					
	CRY + PIN	GAL + CAPE	CRY + CAPE	GAL + PIN	CRY + GAL	PIN + CAPE
FIC	32 + 8	64 + 256	256 + 128	256 + 512	256 + 256	256 + 128
FIC index	0.14	1.25	1.5	2.0	2.0	1.0

Each value corresponds to the average of two independent experiments. Dual mixture of polyphenols was assayed for the MIC by using the chess board strategy and both FIC and Fix index were determined (for details see "Material and methods" section). CRY, chrysin; PIN, pinocembrin; GAL, galangin; CAPE, caffeic acid phenethyl ester.

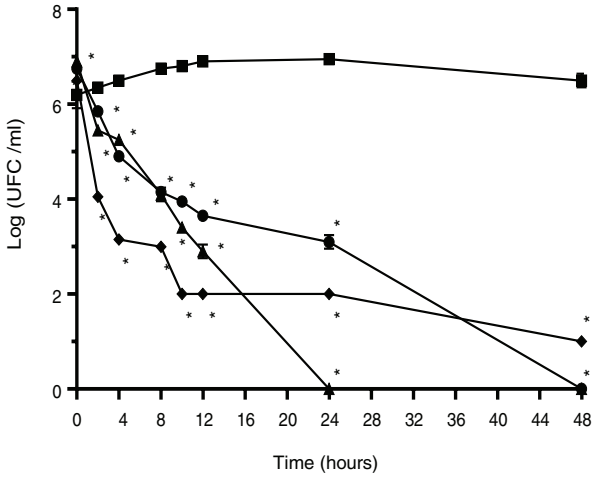

Fig. 3. *Helicobacter pylori* ATCC 43504 death kinetics with polyphenol mixtures. (■): control without compounds mixture, (▲): chrysin + pinocembrin mixture (32 μg ml^{-1} + 8 μg ml^{-1}), (●): galangin + caffeic acid phenylethyl ester mixture (64 μg ml^{-1} + 256 μg ml^{-1}), and (♦): antibiotic control (amoxicillin). *Time in which significant growth differences are observed ($p \leq 0.05$) as determined by variance analysis followed by Tukey's test. All points are the average of two independent experiments with three replicas each.

decreasing the viable count of a bacterial culture in one logarithm) calculated for the chrysin + pinocembrin mixture was 3.25 h and for galangin + caffeic acid phenethyl ester 4.04 h, while amoxicillin presented a *D* value of 3.15 h (data not shown). On the hand, bacterial death determined using the Pearson Criteria was observed at 10 h with chrysin + pinocembrin mixture and at 12 h with galangin + caffeic acid phenethyl ester combination, meanwhile amoxicillin causes *H. pylori* death at 4 h post-exposure.

Transmission electron microscopy

TEM analyses of *H. pylori* cells exposed to polyphenolic compounds in mixtures and their corresponding unmixed controls showed cellular structure alteration or lysis after 12 h incubation (Fig. 4). Conversely, the *H. pylori* control (untreated with propolis compounds), showed no alterations (Fig. 4A and B). Both chrysin + pinocembrin and galangin + caffeic acid phenethyl ester mixtures, and the corresponding individual polyphenols all produce cellular lysis (Fig. 4C–I). Additionally, exposing the bacteria to sub-inhibitory concentrations of chrysin (32 μg ml^{-1}) and pinocembrin (8 μg ml^{-1}) also induced alterations in the cell envelope of similar characteristic to those observed with the mixture (Fig. 4D). This morphological alteration includes the membrane vesicle formation or blebs (Fig. 4E), and lysis. On the other hand, galangin + caffeic acid phenethyl ester dual mixture produced cellular lysis but low evidence of vesicles formation (Fig. 4F), while caffeic acid phenethyl ester caused outer membrane detachment at an inhibitory concentration of 256 μg ml^{-1} (Fig. 4I).

Discussion

The results presented in this study show that the major propolis compounds exert anti-*H. pylori* activity. Hitherto, only the inhibitory effect of the total propolis extracts (from different zones of Chile) on *H. pylori* had been demonstrated (Villanueva et al., 2015). However, the authors did not carry out a comprehensive chemical analysis to ascribing the variation in the inhibition observed to the chemical and geographic differences of the samples. Our results demonstrate that in propolis not only the presence of canonical antibacterial compounds must be determined but also the ratio and potential interactions among them. We have demonstrated a generalized indifference effect among the major propolis compounds in mixture, except for the association chrysin + pinocembrin that show synergy. This kind of approach had not been previously reported in Chilean propolis. So, keeping in mind the anti-*H. pylori* effect, we suggest that in future studies it will be important to pinpoint samples with high amounts of chrysin + pinocembrin. In addition, recently Shapla et al. (2018) compiled evidence of the role that can play propolis as adjuvant in the therapy against *H. pylori* and its associated pathologies. The authors established clearly the protective role of propolis in diseases induced by *H. pylori* neutralizing the miss functioning of the host physiology as well as the bacterial physiology. Therefore, they suggest that propolis may be a useful complement or even alternative agent to antibiotics in the treatment of *H. pylori* associated gastrointestinal diseases. Furthermore, morphological study through transmission electron microscopy allowed us to establish diverse effects of propolis's compounds on the bacterial cell ultra-structure including lysis, membrane vesicle formation

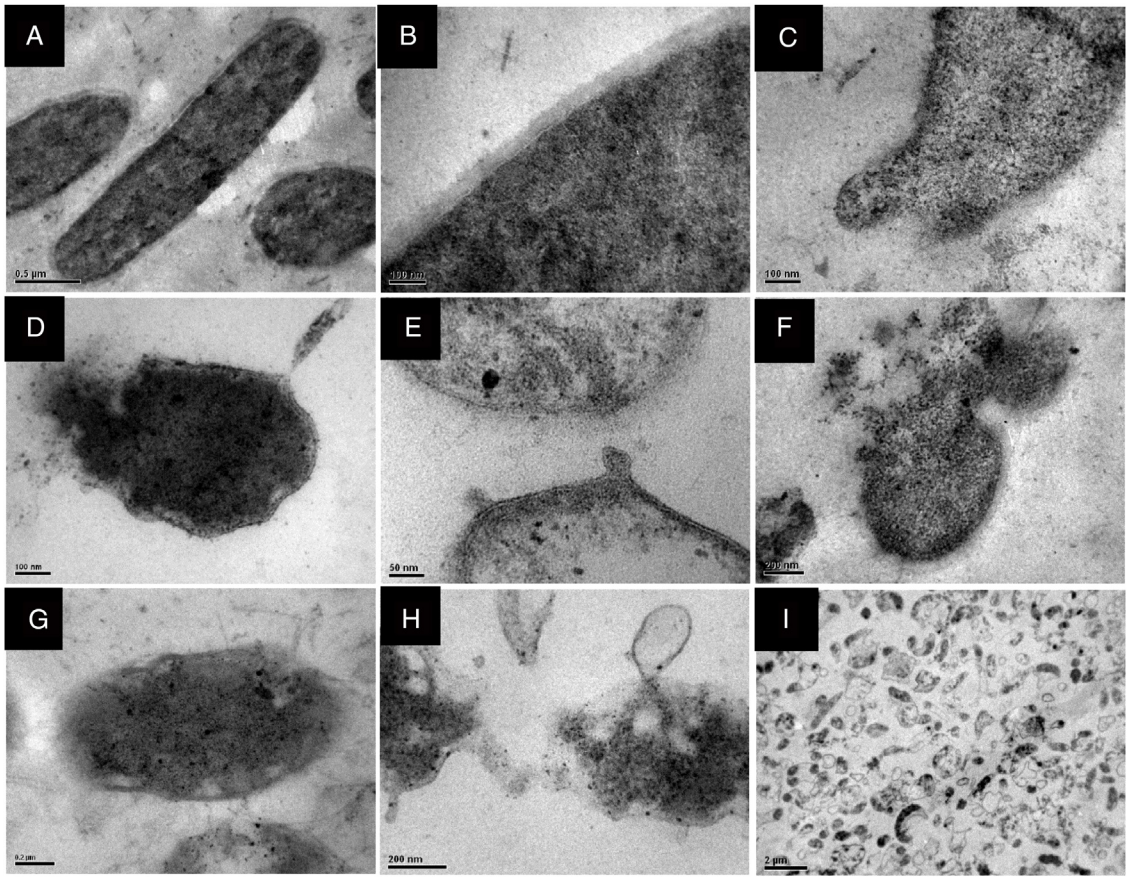

Fig. 4. Structural effect of polyphenols upon *Helicobacter pylori* ATCC 43504. Bacterial cells were exposed for 12 h with dual-mixed or single polyphenols and analyzed by transmission electron microscopy (see "Material and methods" section). A and B: control without polyphenols, C: chrysin + pinocembrin mixture (32 μg ml^{-1} + 8 μg ml^{-1}), D: chrycin (32 μg ml^{-1}), E: pinocembrin (8 μg ml^{-1}), F: galangin + caffeic acid phenylethyl ester mixture (64 μg ml^{-1} + 256 μg ml^{-1}), G: galangin (64 μg ml^{-1}), H and I: caffeic acid phenylethyl ester (256 μg ml^{-1}) at different magnification. The values of bars used were: A: 0.5 μm; B–D: 0.1 μm; E: 0.05 μm; C–F–G–H: 0.2 μm; I: 2 μm.

and membrane alterations which may account for the bactericidal action of this honeybee product.

Propolis is a very complex mixture of polyphenols, waxes and aromatic compounds with a wide range of polarities. Hence, we carried out an initial separation using centrifugal partition chromatography, in order to avoid irreversible loses observed in column chromatography with solid supports as stationary phases. This separation technology has been used previously for the isolation of diterpenes from an ethyl acetate extract of propolis (Jerz et al., 2014). In the present work, two chromatographic techniques (CPC and HPLC), were used to isolate the main propolis polyphenols in large amounts, enough to perform several *in vitro* assays. So, galangin, caffeic acid phenethyl ester and pinocembrin were successfully isolated after CPC/HPLC procedure. Moreover, as can be seen in Fig. 2, CPC methodology is a powerful tool that could be used to investigate the importance of minor compounds in the whole antimicrobial activity of propolis samples. In addition, HPLC-DAD and TLC–MS analytical tools made possible the direct characterization and quantitative analysis of the main target compounds present in the propolis samples (Tables 1 and 2). The results presented are in agreements with those obtained by Castro et al. (2014) and Valenzuela-Barra et al. (2015), who identified 37 polyphenols in six types of propolis from the Chilean central valley (Metropolitan region of Santiago). Among the flavonoid and phenolic acid compounds identified in this work it is noteworthy to mention chrysin, galangin, pinocembrin and caffeic acid phenethyl ester. The same compounds are reported by us as the major propolis polyphenols collected in the southern Chile.

Regarding antibacterial the activity of propolis and its extracts, many studies reports similar results to those observed in our study, such as in Brazil, Bulgaria, Poland, Turkey and Middle Eastern countries (Hashimoto et al., 1998; Boyanova et al., 2005; Ibrahim and Turab, 2011; Skiba et al., 2011; Baltas et al., 2016). However, using Wang's criteria (2014) for medicinal plants rich in polyphenols, it was observed that caffeic acid phenethyl ester, galangin, chrysin and pinocembrin presented weak to moderate anti-*H. pylori* ATCC 43504 activity (ranging from 100 to 1000 μg ml^{-1}). According to this classification, the clinical isolate *H. pylori* 84C strain behaved similarly to the ATCC 43504 strain, the only difference being that pinocembrin was less active (MIC > 1000 μg ml^{-1}). Nonetheless, the combination of chrysin + pinocembrin reduced MIC for each compound in mixture by 1/8 and 1/64, respectively, that it is considered synergic association according to the calculated FIC of 0.14 (Hernández et al., 2003; Kobayashi, 2005; Orhan et al., 2005; Shields et al., 2011).

Lysis and vesicle formation observed in the transmission electron microscopy when the *H. pylori* strain was exposed to polyphenolic compounds suggest an inhibition mechanism similar to that of amoxicillin, namely an inhibitory effect on peptidoglycan synthesis. These findings are similar to those reported by Eumkeb et al. (2011) for other species, who demonstrated that galangin and kaempferide flavonoids produced outer membrane detachment on *Escherichia coli*. This effect may be due to internal damage of the peptidoglycan layer. Although, there are several studies that demonstrate that anti-*H. pylori* activity of propolis correlate with the concentration of specific polyphenolic compounds,

just a few researches propose molecular mechanism for explaining this effect. For instance, Cui et al. (2013) reported that caffeic acid phenethyl ester can inhibit the activity of *H. pylori* peptide deformylase (HpPDF) enzyme, required for removing the formyl group from the *N*-terminus of the nascent polypeptide chains, which is key for *H. pylori* survival.

An aspect that should be considered in the analysis of the results is the stability of polyphenols in cell culture media, particularly in kinetic studies where long incubation times are required. Xiao and Högger (2015) concluded that the rank of stability for flavones and flavonols were: resorcinol-type > catechol-type > pyrogallol-type. Recently, Ma et al. (2018) assessed the stability of 77 natural flavonoids in cell culture medium. They observed that only 36 flavonoids remain over than 70% active after 15 h of incubation at 37 °C. Unfortunately, none of the above-mentioned publications report stability evaluation of propolis-derived polyphenols. Nevertheless, the results presented allow to conclude that the major polyphenolic compounds present in propolis show anti-*H. pylori* activity as single molecules and that in mixtures they have indifference effect but some of them act synergistically, *i.e.* chrysin + pinocembrin. This last result suggests that effectiveness of propolis against *H. pylori* should rely on specific polyphenols association instead of a large amount of any particular one. Analysis of interactions between other molecules, even if they are minor compounds (Choules et al., 2018), is needed to better characterize the potential antibacterial use of a particular propolis. Moreover, when *H. pylori* is the target pathogen, our result suggest that those propolis enriched in chrysin and pinocembrin should be selected.

Based on the morphological effects of polyphenols assayed upon the *H. pylori* cellular structure, the *in vitro* anti-*H. pylori* activity and the bactericidal capacity of propolis because of its lytic effect were confirmed, but this property cannot be extrapolated to all propolis.

Authors contributions

MR-RM: major contributor in the work with propolis as M.Sc. student; JF: second major contributor in the work with propolis as M.Sc. student; EP: support intellectually the manuscript and gave advises in the work with propolis; AG: support intellectually the work with *H. pylori* and gave advise in the assays with this pathogen; MA: support the work of quantitation of propolis and participated in the discussion of the results; CG: wrote critically the manuscript, main responsible of the whole work.

Conflicts of interest

The authors declare no conflicts of interest.

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