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Evaluation of propolis polyphenols absorption in humans by liquid chromatography/tandem mass spectrometry

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Propolis has various biological activities such as antibacterial, antiviral, antioxidant, immunostimulating and antiinflammatory, which are generally ascribed to the polyphenolic fraction. The aim of this study was to evaluate the absorption of the main polyphenols [caffeic acid (CA), pinobanksin-5methyl ether (P-5ME), pinobanksin (Pb), chrysin (C), pinocembrin (P), galangin (G), pinobanksin-3-acetate, pinobanksin esters and caffeic acid phenylethyl ester (CAPE)] from a dewaxed and standardized extract of propolis (EPID®). Fifteen healthy volunteers consumed 5 mL EPID® in water, corresponding to 125 mg of flavonoids. Blood samples were collected before, each hour for 8 h and 24 h after EPID® intake. After deconjugation by β-glucuronidase/sulfatase the plasma samples were analyzed by a selective liquid chromatography/tandem mass spectrometry (LC/MS/MS) method using morin as internal standard (I.S.). A kinetic profile characterized by two t_{max}, respectively at 1 h and about 5 h post-ingestion, was observed in all the subjects. The two peaks may be due to enterohepatic cycling. Among the various polyphenols ingested, only P-5ME, Pb, C, P and G were detected in plasma and $C_{max}t_{1h}$ were 65.7 ± 13.3, 46.5 ± 12.7, 79.5 ± 18.6, 168.1 ± 16.3 and 113.7 ± 16.8 ng/mL, respectively. These levels decreased significantly after 8 h and were no longer detectable 24 h after EPID® intake. The recovery of the extraction for CA, Pb, C, P, G and I.S. from spiked plasma was 95.2 ± 3.1, 93.1 ± 3.6, 91 ± 2.5, 96.4 ± 4.2, 93.4 ± 2.4 and 85.5 ± 2.4%, respectively. The results of this study evidence that flavonoids from EPID® are absorbed, metabolized and Pb-5ME and G seem to have apparent absorption, measured as (AUC/dose), higher than C, P and Pb.

Propolis is a chemically complex resinous honeybees (*Apis mellifera*) product containing material collected from buds or exudates of different plants (resin), volatile substances and beeswax. It contains, depending on the region of collection, different chemicals; at least 200 compounds have been identified in different samples of propolis, with more than 100 being present in any given sample. These include fatty and phenolic acid and esters, substituted phenolic esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols, chalcones), terpenes, aromatic aldehydes and alcohols, and derivatives of sesquiterpenes and stilbenes.[1,2] Propolis is used by bees to seal holes in their honeycombs, smooth out the internal walls, and protect against outside invaders and enemies. However, bees also take advantage of its biological action. Propolis is a traditional remedy in folk medicine, and recently has been attracting attention due to its various biological activities and therapeutic properties.[3] Specifically, the ethanolic extract of propolis has been reported to possess antibacterial,[4] antiviral,[5] antifungal,[6] antiinflammatory,[7] local-anesthetic, antioxidant,[8] immunostimulating,[9] cariostatic,[10] antitumoral[11] and anti *Helicobacter pylori* [12,13] activities.

These effects are mostly ascribed to the polyphenolic fraction. For this reason, the raw propolis must be purified to obtain an extract containing mainly the polyphenols. EPID® is a commercial water-dispersible and wax-free extract of propolis obtained by a patented procedure based on multistep extraction. Moreover, EPID® flavonoid content is standardized at about 1.8%. This means that each batch, apart from the starting raw propolis, has the same fingerprint and content of major polyphenols. This feature is relevant to ensure therapeutic reproducibility and safe use.

Many authors have studied the chemical composition of propolis from different sources, and different analytical procedures to evaluate their polyphenols are available.[14–16] By contrast, the studies on the uptake of propolis polyphenols are rare, performed using high amounts of pure compounds, like chrysin,[17] and in animal models.[18,19]

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The purpose of this study was to investigate the absorption of polyphenols, especially flavonoids, in healthy volunteers after ingestion of EPID®. For this purpose, a sensitive method using liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) was for the first time developed in order to detect low plasma levels of polyphenols and their metabolites after ingestion of EPID®. Moreover, the pharmacokinetic profile of different flavonoids in human plasma after oral propolis administration was investigated.

EXPERIMENTAL

Chemicals

Chrysin (C), galangin (G), pinocembrin (P), quercetin (Q) and morin (M) were from Extrasynthese (Genay, France). Caffeic acid phenylethyl ester (CAPE), β-glucuronidase/sulfatase from *Helix pomatia* and caffeic acid (CA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, formic acid, and sodium acetate were from Merck (Darmstadt, Germany). Water was obtained from a MilliQ apparatus (Millipore, Milford, MA). The propolis ethanolic extract (EPID®) and Pinobanksin (Pb) were kind gifts from Specchiasol s.r.l. (Bussolengo, Vr, Italy).

Subject and study design

Healthy subjects (9 men, 6 women, age = 24.6 ± 3.0 years, BMI = $22.9 \pm 3.1\,kg/m^2$) were selected after completing a questionnaire concerning their dietary habits and lifestyle. They were not taking any supplements, drugs or medication. Informal, written consent was obtained from each participant and the local ethical committee approved the protocol. Volunteers refrained from consuming propolis for 3 days prior to the study. On the day of the experiment subjects were confined to the laboratory. Fasted volunteers consumed a standard breakfast together with 5 mL EPID® in water (200 mL, accounting for 125 mg total flavonoids) between 8:30 and 9:00 a.m. The standard breakfast consisted of brioches (234 kcal), biscuits (69 kcal for women and 138 kcal for men), milk (100 mL), sugar (10–20 g). Lunch consisted of bread (70 g), cheese (20 g), ham (50 g) and ice-cream (80 g). The dinner consisted of pasta (80 g), olive oil (10 g), parmigiano cheese (15 g), meat (100 g) and bread (70 g). Eight hours after propolis intake the volunteers were allowed to leave the laboratory, returning the next morning for the final blood sampling. Blood samples were collected in tubes containing lithium-heparin before, each hour for 8 h and 24 h after EPID® administration. Time zero was set at 30 min just before propolis ingestion.

Polyphenols determination in EPID®

Qualitative and quantitative analysis of phenolic acids and flavonoids in EPID® sample was performed as previously reported.[16] Briefly, EPID® was diluted 200-fold in methanol and the resulting solution was sonicated for 10 min and centrifuged at 1000 *g* for 1 min. One aliquot of the supernatant was filtered through a Millipore 0.2 μm disk and 10 μL were used for analysis. Calibration curves were obtained dissolving 20 mg of standard powder in 200 mL methanol. CA, internal standard (I.S.), Pb, C, P, G and CAPE were measured in the range of 2–50 μg/mL.

Plasma preparation

Polyphenols conjugates were hydrolyzed by incubating 100 μL of heparinized plasma with 100 μL glucuronidase/sulphatase (1 U/μL) and 50 μL of 0.1 mol/L sodium acetate buffer, pH 5.2, containing morin (50 ng/mL) as I.S., at 37°C for 18 h. The reaction mixture was extracted with 500 μL ethyl acetate, vortexed and centrifuged at 1000 *g* for 1 min. The supernatant (400 μL) was dried under N_2 and the residue dissolved in 100 μL methanol. To evaluate unconjugated polyphenols, a plasma sample (100 μL) was incubated with 100 μL 0.1 mol/L sodium acetate buffer, pH 5.2, and 50 μL of 0.1 mol/L buffer, pH 5.2, containing I.S. (morin, 50 ng/mL), at 37°C for 18 h. The reaction mixture was then treated as described above.

LC/MS/MS conditions

The chromatographic system consisted of an Alliance 2695 (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer mod. Quattromicro (Micromass, Beverly, MA, USA). A 3.5 μm C_{18} Symmetry column (150 × 2.1 mm, Waters) was used for the separation at a flow rate of 0.25 mL/min. The column was maintained at 30°C. The separation was performed by means of a linear gradient elution (eluent A, 0.1% formic acid; eluent B, acetonitrile). The gradient was as follows: 20% B for 6 min, 20 to 30% B in 4 min, 30 to 40% B in 30 min, 40 to 60% B in 20 min, 60 to 90% B in 20 min and 90% B for 10 min. The capillary voltage was set to 3.0 kV; the cone voltage and the collision energy were specific for each compound. The source temperature was 130°C, the desolvating temperature was 350°C and argon was used at 2.5×10^{-3} mbar to improve fragmentation in the collision cell. Data were acquired by Masslinx 4.0 with Quan-Optimize option for fragmentation study. The fragmentation transitions for the multiple reaction monitoring (MRM) were $(m/z)^-$ 179 → 135 for CA, $(m/z)^+$ 303 → 153 for the IS, $(m/z)^+$ 287 → 91 for Pb-5ME, $(m/z)^+$ 273 → 153 for Pb, $(m/z)^+$ 255 → 153 for C, $(m/z)^+$ 257 → 153 for P, $(m/z)^+$ 271 → 153 for G, $(m/z)^-$ 313 → 253 for Pb-acetate, $(m/z)^-$ 283 → 179 for CAPE, $(m/z)^-$ 327 → 253 for Pb-propionate, $(m/z)^-$ 341 → 253 for Pb-butanoate, $(m/z)^-$ 353 → 253 for Pb-pentenoate and $(m/z)^-$ 355 → 253 for Pb-pentanoate, with a dwell time of 0.3 s per transition.

Preparation of working solutions

The primary stock solutions of CA, Pb, C, P, G, CAPE and I.S. (0.1 mg/mL) were prepared in methanol and diluted to give working solutions of 10, 20, 50, 100 and 200 ng/mL in methanol. All stock solutions and the working solutions were stored at −20°C and 4°C, respectively.

Pinobanksin esters and Pb-5ME were assayed using pinobanksin calibration curves and their amounts were normalized taking into account the molecular mass ratios.

Method validation

The LC/MS/MS method was validated for linearity, limit of quantification and detection, peak purity, precision and repeatability. Limits of quantification (LOQs) and limits of

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detection (LODs) (signal-to-noise (S/N) ratio of 3) were determined by serial dilutions of standard solutions. The accuracy (matrix effect) was evaluated according to Matuszewski *et al*.[20] Specifically, three sets with different CA, Pb, C, P, G and CAPE concentrations (2, 10, 50, 100, 200 ng/mL) and I.S. (50 ng/mL) were prepared. The first set consisted of standards plus I.S.; the second set was represented by a plasma sample containing standards and I.S. subjected to enzymatic treatment; and the third was obtained by extracting a plasma sample subjected firstly to enzymatic treatment and then added of standards plus I.S. All the tests and LC/MS/MS analyses were carried out in triplicate. Peak purity and identity were confirmed by LC/MS/MS experiments. Intra- and inter-day precision of the assay were verified by analyzing sample sets three times on five consecutive days. Repeatability was confirmed by evaluating standard deviations of the retention times.

Statistical analysis

Statistical analyses were performed with the STATISTICA software (Statsoft Inc, Tulsa, OK, USA). A one-way repeated measure analysis of variance (ANOVA) with the time as the dependent factor was used. The peak plasma concentration (C_{max}) and the time to reach peak concentrations (t_{max}) have been expressed as mean values and standard deviations.

The elimination rate constant (K) was determined in the terminal phase from the slope of the regression line of the logarithmic concentrations (logC/t) in plasma samples. Three time points, starting with the time taken to reach peak concentration, were included in the estimation of K. The apparent terminal half-life ($t_{1/2}$) was calculated as $t_{1/2} = 0.693/K$. The area under the plasma concentration time curve ($AUC_{0\text{-}t}$) was calculated using the linear trapezoidal rule in the range 0–8 h and 0–24 h, although between 8 and 24 h there was no plasma sample.

RESULTS AND DISCUSSION

Polyphenols in EPID®

We have previously reported a reversed-phase LC-DAD-MS/MS method for the evaluation of phenolic acids and flavonoids in propolis raw materials.[16] By this method about 36 different polyphenols were separated and identified in the EPID® sample and the main flavonoids detected were pinocembrin, pinobanksin-3-*O*-acetate, chrysin, pinobanksin and Pb-5ME. Regarding the phenolic acid fraction, CA and its derivatives represent the most abundant constituents. The content of polyphenols in EPID® is given in Table 1, while their chemical structures are reported in Fig. 1.

LC/MS/MS optimization

In order to optimize ESI conditions for flavonoids, CA and CAPE, quadrupole full scans were carried out both in positive and negative ion mode. Precursor ion, capillary, extractor and RF lens voltages were optimized by direct infusion experiments, while product ions, cone voltage and collision energy were evaluated by Quan-optimize option. The ESI positive collision-induced dissociation MS (CID-MS/MS) of galangin, pinobanksin, chrysin, pinocembrin and morin (I.S.) gave a product ion with $(m/z)^+$ 153. The

Table 1. Qualitative and quantitative[a] composition of polyphenols in EPID®

Compound	mg/100 mL	Ingested amount (mg)
CA	60.2 ± 1.4	3.0 ± 0.1
CA derivative (as CA)	152.5 ± 2.1	7.6 ± 0.1
p-Coumaric acid	30.5 ± 0.7	1.5 ± 0.0
Ferulic acid	10.8 ± 0.8	0.5 ± 0.0
iso-Ferulic acid	10.5 ± 0.7	0.5 ± 0.0
3,4-Dimethyl-CA	50.5 ± 0.6	2.5 ± 0.0
CAPE	49.6 ± 1.9	2.5 ± 0.1
Pb-5ME	60.4 ± 0.7	3.0 ± 0.2
Pb	150.1 ± 1.1	7.5 ± 0.1
C	487.5 ± 3.5	24.4 ± 0.2
P	506.0 ± 5.7	25.3 ± 0.3
G	212.5 ± 2.1	10.6 ± 0.1
Flavonols (as Q)	408.0 ± 5.7	20.4 ± 0.3
Pb-acetate	403.5 ± 4.9	20.2 ± 0.2
Pb-propionate	180.1 ± 5.7	9.0 ± 0.3
Pb-butanoate	59.5 ± 3.5	3.0 ± 0.2
Pb-pentenoate	119.5 ± 3.5	6.0 ± 0.2
Pb-pentanoate	39.5 ± 2.1	2.0 ± 0.1

[a] Values are expressed as mean ± SD.

$(m/z)^+$ 153 is a common product ion for flavonoids and is believed to result from the retro-Diels-Alder reaction of the flavonoid A ring. In negative ion mode, chrysin, galangin and pinobanksin gave a product ion with $(m/z)^-$ 151 while

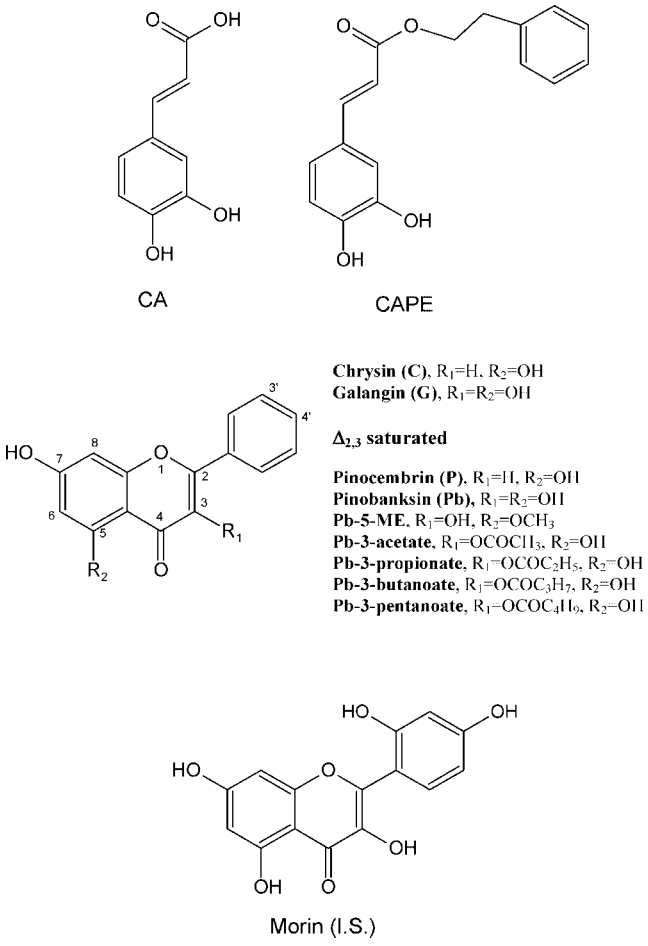

Figure 1. Chemical structures of CA, CAPE, I.S., flavonoids and their esters found in EPID®.

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Table 2. Calibration equations ($Y = m X + q$)[a] for the quantification of different polyphenols in human plasma after EPID® intake. Calibration range: 10–200 ng/mL ($n = 5$)

Standard	slope	intercept	r	LLOD (ng/mL)
CA	25.1	11.2	0.999	3
Pb	17.2	−9.1	0.999	5
C	35.2	−7.2	0.999	3
P	43.5	145	0.997	2
G	19.1	50	0.998	4
CAPE	17.7	23.7	0.999	5

[a] Y = area/1000, X = ng/mL.

pinobanksin and Pb-5ME gave a product ion with $(m/z)^-$ 253 ($[M–H_2O]^-$) and $(m/z)^-$ 255 ($[M–CH_3]^-$), respectively. The positive ESI mode was selected for Pb, Pb-5ME, C, P, G and morin as it was more sensitive. Pinobanksin esters, CA and CAPE gave different typical product ions with $(m/z)^-$ 253 ($[M–acid\ moiety–H_2O]^-$), $(m/z)^-$ 135 ($[M–COOH]^-$) and $(m/z)^-$ 179 ($[M–phenylethyl\ moiety]^-$), respectively.

Method validation

Calibration curves were constructed for each standard at five concentration levels and three independent determinations were performed at each concentration. Regression analysis was employed to determine the linearity of the calibration graphs and the calculated equations and the lower limits of detection (LLODs) are reported in Table 2. The accuracies of the extraction for CA, Pb, C, P, G and I.S. from spiked plasma samples were 95.2 ± 3.1, 93.1 ± 3.6, 91 ± 2.5, 96.4 ± 4.2, 93.4 ± 2.4 and $85.5 \pm 2.4\%$, respectively. The stability of these polyphenols to enzymatic hydrolysis and the extraction process was determined by comparing the differences in the precision of peak area for sample sets 1 and 2 [CV set 2–CV set 1]. The differences were <4%, showing that CA, Pb, C, P, G and I.S. were stable in biological matrix at 37°C for 18 h.

The precision of the method was tested by both intra-day ($n = 3$) and inter-day (5 days, $n = 3$) reproducibility, and the coefficient of variation (CV) was below 7.4%. Regarding repeatability, a maximum relative standard deviation (RSD) of 2.2% (for pinocembrin, galangin, Pb-acetate and CAPE) was observed for triplicate injections.

Absorption of polyphenols from EPID

A typical chromatogram of a plasma sample collected 1 h after the ingestion of EPID® is shown in Fig. 2. Among the various polyphenols ingested and monitored by LC/MS/MS, Pb-5ME, Pb, C, P and G were detected in plasma and C_{maxt1h} values were 65.7 ± 13.3, 46.5 ± 12.7, 79.5 ± 18.6, 168.1 ± 16.3 and 113.7 ± 16.8 ng/mL, respectively. Another compound (Fig. 2, peak 3a) with the same fragmentation

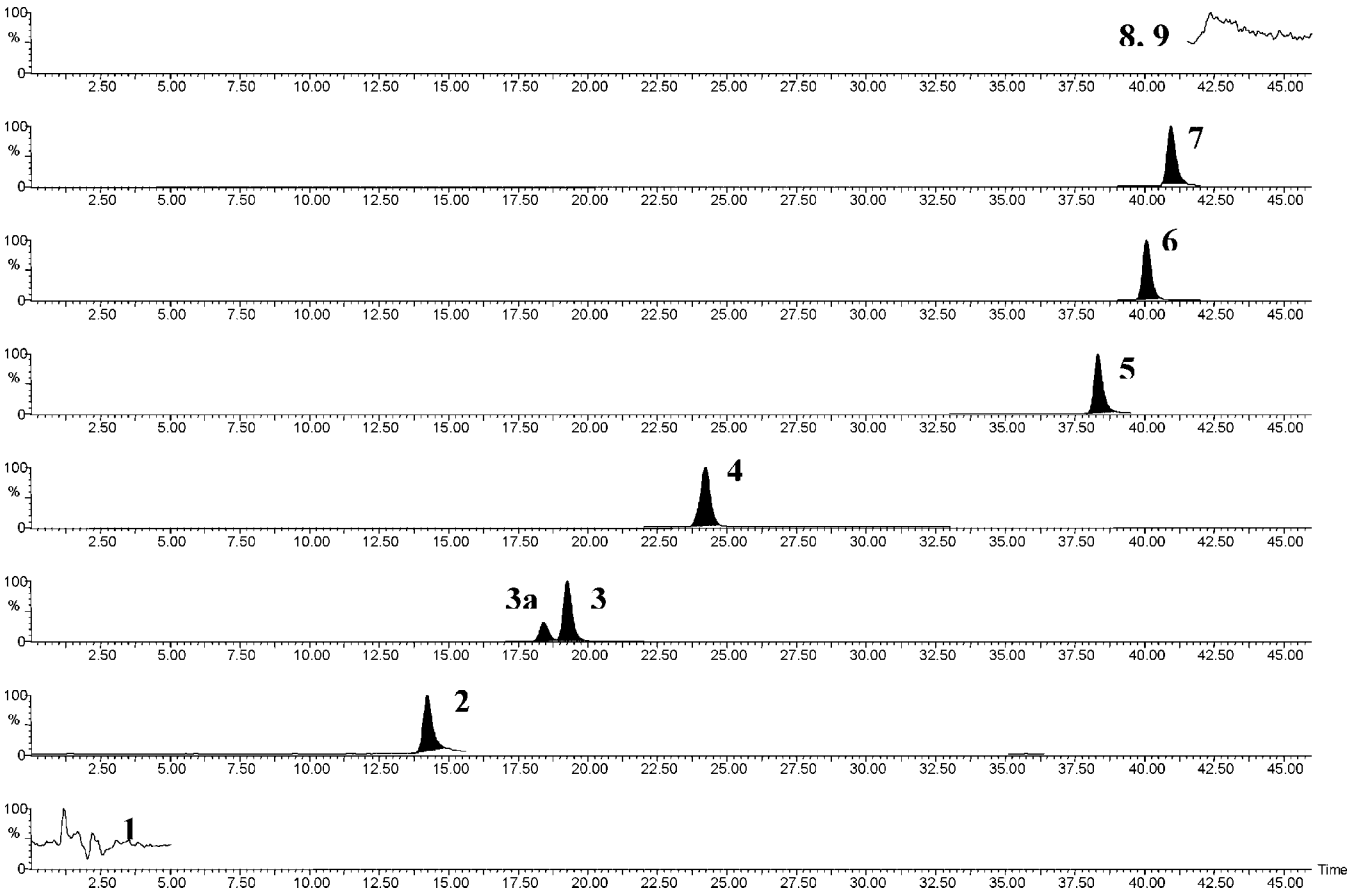

Figure 2. Typical LC/MS/MS chromatogram (MRM mode) of a plasma sample (subject 3) collected 1 h after the intake of 5 mL EPID® in 200 mL of water. 1, CA $(m/z)^-$ 179 → 135 (not found); 2, I.S. $(m/z)^+$ 303 → 153 (morin); 3a, unknown; 3, Pb-5ME $(m/z)^+$ 287 → 91; 4, Pb $(m/z)^+$ 273 → 153; 5, C $(m/z)^+$ 255 → 153; 6, P $(m/z)^+$ 257 → 153; 7, G $(m/z)^+$ 271 → 153; 8, Pb-acetate $(m/z)^-$ 313 → 253 (RT 42.5 min, not found); 9, CAPE $(m/z)^-$ 283 → 179 (RT 43.1 min, not found).

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Table 3. Flavonoid concentrations[a] in plasma sample (ng/mL) before (t = 0) and after the EPID® intake

t (h)	Pb-5ME	Pb	C	P	G
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1	81.6 ± 32.0	93.3 ± 28.6	167.6 ± 57.4	315.7 ± 125.6	119.4 ± 24.5
2	53.3 ± 13.3	60.6 ± 15.2	102.4 ± 29.4	237.5 ± 82.2	93.0 ± 29.8
3	28.7 ± 8.1	39.0 ± 6.2	81.9 ± 34.5	169.5 ± 53.6	68.1 ± 25.5
4	19.0 ± 9.6	25.6 ± 8.5	56.3 ± 16.7	148.9 ± 62.2	43.3 ± 21.1
5	34.6 ± 8.8	57.0 ± 29.9	111.7 ± 44.6	219.9 ± 66.7	86.5 ± 43.3
6	30 ± 13.0	42.0 ± 20.0	85.2 ± 41.5	202.7 ± 59.7	68.3 ± 39.7
7	16.2 ± 6.1	28.1 ± 10.4	62.2 ± 33.3	153.1 ± 56.6	42.3 ± 29.5
8	14.6 ± 12.7	12.1 ± 3.1	35.4 ± 21.1	97.2 ± 41.8	12.4 ± 10.5
24	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

[a] Values are expressed as mean ± SD.

transition as Pb-5ME was evidenced in all subjects. This peak was absent in EPID® and at time zero, and its identity was not investigated because of its low level. To detect these compounds the enzymatic hydrolysis was mandatory, since they were present in plasma mainly (>90%) as conjugates (glucuronides e/o sulfates). Concerning Pb-esters and CAPE, they were not detected, likely due to their hydrolytic cleavage in the gastrointestinal tract to produce Pb and CA, respectively. Pinobanksin was found in the plasma of all subjects while CA (retention time (RT) 3.1 min) was not detected in any plasma samples. This is probably due to different attendant causes: one is that the amount of CA ingested of about 4.5 mg (3 mg from CA and 1.5 mg from CAPE) was low to be able to detect an increase in plasma; the other possibility is that CA was quickly absorbed and rapidly eliminated. Consequently, after 1 h its plasma concentration was under the LLOD.

The mean plasma levels of the absorbed compounds following the intake of 5 mL EPID®, corresponding to 125 mg of flavonoids, are reported in Table 3. These levels decreased significantly after 8 h and were not detected 24 h after EPID® intake.

It should be mentioned that Pb-5ME, Pb, C, P and G were not present in plasma at time zero. This is due to the typical composition of propolis, which contains a range of flavonoids not present in vegetables, fruits and beverages. Furthermore, all these compounds were absorbed by all the subjects with low (CV < 12% for C, P and G) or moderate (CV ≈ 22% for Pb, Pb-5ME) inter-individual differences, and their AUC_{0-24h} values were comparable with those reported in the literature.[21]

A typical time course (subject 3) of plasma concentrations of Pb-5ME, Pb, C, P and G after ingestion of EPID® is shown in Fig. 3. These kinetic profiles were observed in all the subjects, and were characterized by two t_{max}, respectively, at 1.0 ± 0.0 h and 5.5 ± 0.6 h post-propolis ingestion. The appearance of the first peak at a relatively short time (1 h) may be ascribed to the fact that propolis does not contain glycosylated polyphenols, but only aglycones that are more rapidly absorbed.[22] Concerning the two-peak phenomenon, this cannot be explained by the two different times of nourishment (breakfast at 8.30 a.m. and lunch at 12.30 a.m.),

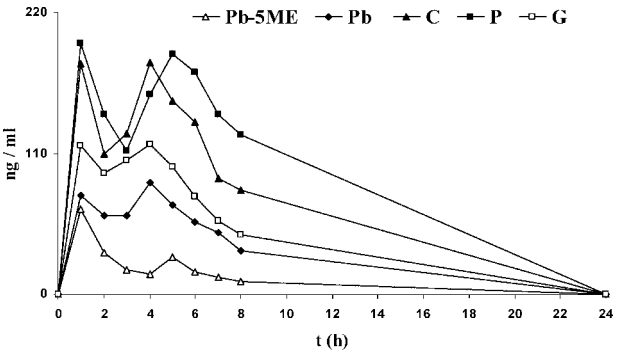

Figure 3. Individual plasma concentration-time profile of Pb-5ME, Pb, C, P and G (subject 3) after a single oral dose (5 mL) of EPID®.

Table 4. Plasma kinetic indices in 15 healthy volunteers for Pb-5ME, Pb, C, P and G after ingestion of 5 mL EPID®, corresponding to 125 mg of flavonoids

	Pb-5ME	Pb	C	P	G
Ingested amount (I.A., mg)	3.0 ± 0.2	7.5 ± 0.1	24.4 ± 0.2	25.3 ± 0.3	10.6 ± 0.1
$t_{max\ 1}$ (h)	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
$t_{max\ 2}$ (h)	5.2 ± 0.4	5.3 ± 0.5	5.1 ± 0.7	5.3 ± 0.6	5.6 ± 0.5
C_{max} $t_{max\ 1}$ (ng/mL)	65.7 ± 10.5	46.5 ± 3.7	79.5 ± 11.6	168.1 ± 14.3	113.7 ± 13.8
C_{max} $t_{max\ 2}$ (ng/mL)	38.1 ± 6.5	41.9 ± 9.2	89.7 ± 12.3	183.3 ± 21.2	121.7 ± 19.4
AUC_{0-8h} (ng*h)/mL	228 ± 36	230 ± 36	420 ± 66	1003 ± 158	608 ± 96
AUC_{0-24h} (ng*h)/mL	386 ± 61	434 ± 68	930 ± 146	2166 ± 340	1028 ± 162
AUC_{0-8h}/I.A. [(ng*h)/mL]/mg	76 ± 12	31 ± 5	17 ± 3	40 ± 6	57 ± 9
AUC_{0-24h}/I.A. [(ng*h)/mL]/mg	129 ± 20	58 ± 9	38 ± 6	86 ± 13	97 ± 15
$t_{1/2}$ (h)	2.7 ± 0.5	4.1 ± 0.8	3.7 ± 0.4	5.1 ± 0.4	2.1 ± 0.3

Pb-5ME: pinobanksin-5-methyl ether; Pb: pinobanksin; C: chrysin; P: pinocembrin; G: galangin.

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which could suggest that an aliquot of the ingested polyphenols is absorbed along with breakfast, and another aliquot is absorbed within the lunch. In fact, the same double-peak behavior was observed in volunteers consuming only the breakfast (data not reported). Hence, a possible explication is that some polyphenols, after EPID® ingestion, are firstly conjugated within the intestinal epithelium with subsequent appearance in plasma (first peak); then, the polyphenol conjugates in liver undergo enterohepatic cycling, with the second peak corresponding to the re-absorption of the conjugates secreted in bile.[23]

Table 4 summarizes the mean values of the kinetic indices calculated for the flavonoids plasma levels and expressed as apparent absorption measured as area under the curve (AUC) and dose-adjusted (AUC/dose). For Pb-5ME, P and G the ratio AUC_{0-24h}/mg ingested was higher than for pinobanksin and chrysin. On the other hand, half-lives ($t_{1/2}$) of Pb-5ME and galangin were lower than Pb, P and C, indicating that the clearance of Pb-5ME and G from plasma was more rapidly. Higher AUC/dose and lower $t_{1/2}$ may suggest that Pb-5ME and G are better absorbed than the others flavonoids of propolis. However, Pb, P and C have lower AUC/dose values but it may be they have a higher bioavailability. In fact a lower plasma level can be due to an extensive distribution in tissues.

CONCLUSIONS

A sensitive and specific LC/MS/MS method was validated to guarantee the determination of different polyphenols in human plasma after propolis intake. The results of this study evidence for the first time the presence of several flavonoids (Pb-5ME, Pb, C, P and G) in human plasma after ingestion of a purified and dewaxed propolis extract (EPID®). This indicates that polyphenols from this extract are absorbed, metabolized and, therefore, may exert systemic effects, such as protection of lipid membranes from oxidative injury and immunostimulation.

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