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Metabolic products of European-type propolis. Synthesis and analysis of glucuronides and sulfates

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ABSTRACT

Ethnopharmacological relevance: Propolis is a bee-derived product used since antiquity for its general health-giving properties and is especially noted for its anti-bacterial activity. In more recent times, propolis has been employed against more specific targets such as antiproliferative effects vs cancer cells, wound healing and type-2 diabetes.

Aim of the study: European (poplar)-type propolis from New Zealand contains a number of hydroxy cinnamic acid esters and a set of aglycone flavonoid compounds, mainly chrysin, galangin, pinocembrin and pinobanksin. Propolis is usually taken orally and propolis metabolites quickly appear in the plasma of the ingested. In this work we aimed to identify the major flavonoid plasma metabolites by direct analysis of the plasma.

Materials and methods: After consumption of a large dose of propolis in a single sitting, blood samples were taken and analysed using LCMS/MS. The major flavonoid metabolites identified were also synthesised using chemical (sulfates) or enzymatic methods (glucuronides).

Results: Both the sulfate and glucuronide conjugates of the four major propolis flavonoids are readily detected in human plasma after propolis ingestion. Preparation of the sulfates and glucuronides of the four major flavonoids allowed the relative proportions of the various metabolites to be determined. Although the sulfates are seen as large peaks in the LCMS/MS, the glucuronides are the dominant conjugate species.

Conclusions: This study shows most of the flavonoids in the plasma are present as 7-O-glucuronides with only galangin showing some di-glucuronidation (3,7-O-diglucuronide). No evidence was found for hydroxy cinnamic acid type metabolites in the plasma samples.

1. Introduction

Metabolomic transformation of xenobiotics in human systems often involves conversion of ingested compounds into more polar metabolites such as glucuronides and sulfates. This process facilitates removal of these compounds via liver detoxification. Identification and analysis of these metabolites is critical to understanding the bioactivity of drugs and bioactive foods so it is important to obtain purified and characterised samples of the glucuronide and sulfate metabolites of drugs for further toxicity and/or bioactivity studies (Gonzales et al., 2015).

Propolis is a bee-derived product used since antiquity for its general health-giving properties and is especially noted for its anti-bacterial activity (Braakhuis, 2019). In more recent times, propolis has been employed against more specific targets such as antiproliferative effects vs cancer cells (Catchpole et al. 2015, 2018), wound healing (Kwiecińska-Piróg et al., 2019) and type-2 diabetes (Kitamura 2019; Karimian et al. (2019); Xue et al., 2019). The composition of propolis depends on the plant species the bees visit. New Zealand propolis can be classified as 'European' type, which is collected mainly from poplar species (*Populus* spp.) (Catchpole et al., 2015) whereas the composition of propolis collected in tropical regions is much more diverse. The most well-known type of tropical propolis is Brazilian green propolis, which contains resin collected from *Baccharis dracunculifolia* trees. The main components in European-type propolis from poplars are aglycone

Abbreviations: LCMS/MS, liquid chromatography with tandem mass spectrometric detection; NMR, nuclear magnetic resonance; SPE, solid phase extraction; DMSO, dimethyl sulfoxide; SAX, strong anion exchange; HRMS, high resolution mass spectrometry; UDPGT, Uridine 5′-diphospho-glucuronosyltransferase; UDPGA, Uridine 5′-diphosphoglucuronic acid; HPLC/UPLC, high- or ultra-performance liquid chromatography; MRM, multiple reaction monitoring in tandem mass spectrometric detection.

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flavonoids (mainly chrysin, galangin, pinocembrin and pinobanksin) and hydroxycinnamic acids and esters. The most actively researched molecules are chrysin and caffeic acid phenethyl ester (CAPE) (Sawicka et al., 2012; Watanabe et al., 2011). It is generally accepted that aglycone flavonoids are not absorbed into the blood stream and must first undergo phase-II metabolism to form glucuronides and sulfates (Gonzales et al., 2015). Propolis has limited water solubility and a number of alternative delivery systems have been looked at to increase the bioavailability of the propolis components (e.g. Elbaz et al., 2016; Cavalu et al., 2018).

There have been a limited number of studies looking at plasma concentrations of propolis metabolites following propolis ingestion. Generally, sample workup includes a deconjugation step using a glucuronidase/sulfatase enzyme to cleave any metabolic conjugation products (Gardana et al., 2007; Kumazawa et al., 2004). Experiments involving *p*-coumaric acid and artepillin C (the main constituent of Brazilian propolis) tested on rats showed these two compounds are quite different in their bioavailability and degree of conjugation (Konishi et al., 2005). A study with human volunteers fed European-type propolis showed the presence of flavonoids in plasma sample with the levels measured over several hours. The samples were analysed after enzymatic deconjugation and no caffeic acid, CAPE or pinobanksin 3-O-acetate were detected (Gardana et al., 2007). In plasma analysis of rats fed a European-type propolis, only conjugated forms of components such as pinobanksin were detected (Kumazawa et al., 2004).

Glucuronidation is the most studied phase-II metabolite for flavonoids and the regio-specificity of the glucuronidation is dependent on the structure of the flavonoid. Galangin can be 3-O, 5-O or 7-O glucuronidated by human liver microsome (Otake et al., 2002). By contrast only one sulfated derivative has been reported. Two glucuronides and one sulfate were identified in the plasma of rats exposed to galangin (Chen et al., 2015). Galangin was preferentially glucuronidated after oral dosing but sulfated after intravenous medication. Chrysin 7-O-glucuronide is known as a natural product and is the main chrysin glucuronide detected in plasma samples with only minor amounts of the di-glucuronide (Galijatovic et al., 1999). Incubation of chrysin with Caco-2 and Hep G2 cells showed faster formation of chrysin sulfate versus the mono-glucuronide. The metabolic fate of pinobanksin and pinocembrin is less well studied; glucuronides and sulfates have not been specifically detected but assumed to be present because of the appearance of the unmodified molecules after enzyme treatment. A rat study on the fate of CAPE after propolis consumption assumed rapid plasma hydrolysis as CAPE was not detected in the plasma but both CAPE and CAPE glucuronide were detected in the urine (Celli et al., 2004).

Previously we have attempted to analyse propolis metabolites in human plasma samples from a trial with a cohort of subjects, some of whom consumed propolis capsules at typical recommended dosage. We found the analysis challenging due to low plasma levels of the metabolites and difficulties in optimising the analysis (unpublished data). In this current work we decided to analyse plasma samples from a single subject using a megadose of propolis. Using the plasma samples with high levels of metabolites we could then compare analysis after enzymatic degradation of conjugates of the four major flavonoids present in European-type propolis and compare the products with those from direct analysis of sulfate and glucuronide conjugates. To further assist this work, it was necessary to prepare and characterise a set of glucuronides and sulfates from the four major flavonoids. The 7-O-glucuronides of the four flavonoids were prepared from the aglycones using an enzymatic method (from porcine liver microsomes) whilst the 7-O-sulfates were chemically synthesised using a previously reported sulfation method. These conjugates are readily detected by LCMS. The response factors of the metabolites were determined and used to show that most of the flavonoids in European-type propolis are present in plasma as 7-O-glucuronides.

2. Material and methods

2.1. Chemical and materials

Capsules containing New Zealand propolis were supplied by Manuka Health NZ Ltd (product Bio30). The capsules were stored at RT. Chrysin (97%), pinocembrin (95%), D-glucuronic acid sodium salt, uridine 5′-diphosphoglucuronic acid (UDPGA), quinidine hydrochloride and tetrabutylammonium hydrogen sulfate (TBAHS) were purchased from Sigma Aldrich, galangin (>97%) from Fluka. *N,N′*-dicyclohexylcarbodiimide (DCC) was obtained from Acros Organics (USA). Pinobanksin was isolated from crude propolis (Han et al., 2010). Bovine serum albumin (Fraction V IgG free) was purchased from Life Technologies (New Zealand). NMR spectra were collected using a Bruker Avance 500 MHz NMR spectrometer (Rheinstetten, Germany). Solid phase extraction (SPE) was performed using Agilent Bond Elut C18 SPE columns (500 mg, Agilent Technologies, USA) or Phenomenex Strata SAX (strong anion exchange) SPE column (500 mg, Phenomenex NZ, Auckland). Solvents for HPLC and LCMS were LCMS grade acetonitrile and methanol from Fisher (Thermo Fisher Scientific, Auckland, NZ).

2.2. Analysis of capsules containing propolis

A commercial sample of propolis capsules (Manuka Health New Zealand; Bio30) was analysed. Briefly, 6 capsules were weighed, partially cut to expose contents and extracted twice with ethanol using vortex shaking and sonication. The combined extracts were made up to 50 ml and analysed to determine the presence and amount of 16 components that we routinely measure for propolis-containing samples, i.e.: caffeic acid; *p*-coumaric acid; cinnamic acid; pinobanksin (Pb); pinocembrin (Pc); 3-methyl-3-butenyl caffeate; benzyl caffeate; 1,1-dimethyl allyl caffeate; pinobanksin-3-O-acetate (PbOAc); CAPE; chrysin (Chry); galangin (Gal); cinnamyl caffeate; pinocembrin 7-methyl ether; tectochrysin; and galangin 7-methyl ether. Analysis was performed on duplicate extractions. Details of the analytical methods are provided below. Structural identification of propolis metabolites is based on work performed previously in this and other laboratories (Catchpole et al., 2015; Pellati et al., 2011).

2.3. Consumption of propolis capsules and plasma sample preparation

A male volunteer consumed 21 Bio30 capsules in a single sitting (without food, approx. 2 hrs after breakfast) and samples of whole blood were taken 1.5 and 3.5 h later. The samples were heparinised and centrifuged then frozen (−21 °C) until required for analysis. Samples were prepared for analysis using acetonitrile addition as a protein-precipitation step. Plasma samples were either used directly or concentrated in an SPE step. Analytical samples were stored at 4 °C. Ethics approval was not required for this work performed within Callaghan Innovation.

2.3.1. Enzymatic treatment of plasma samples

The glucuronidase/sulfatase enzyme used was purchased from Sigma [β-glucuronidase from *Helix pomatia* G0751-500KU, 1634 K units/G solid]. 500 µl of plasma was incubated with 500 µl glucuronidase/sulfatase enzyme (1U/µl) in pH 5.2 sodium acetate buffer. After overnight incubation at 37 °C the reaction mixture was worked up by the addition of 1 ml acetonitrile and the sample centrifuged. The sample was then either filtered (0.2 µm) or evaporated to dryness under vacuum and then taken up in 200 µl DMSO.

2.3.2. Direct samples of plasma

100 µl of plasma was deproteinised by adding 200 µl of acetonitrile, vortexing and centrifuging. The supernatant was then filtered using a Thomson SINGLE StEP filter vial (0.2 µm nylon) and the filter vial used directly for LCMS analysis.

2.3.3. SPE treatment of plasma

500 µl of plasma was diluted with 1 ml of water containing 0.05% formic acid. This was passed through a C18 SPE column (500 mg), washed with water and the retained metabolites eluted with ethanol. The ethanol eluate was dried under N_2 and taken up in 200 µl ethanol.

2.4. Preparation of flavonoid sulfates

The method used was similar to that described by Barron and Ibrahim (1987). Briefly, chrysin (25 mg) and DCC (200 mg) were dissolved in pyridine (5 ml) and stirred at 4 °C while TBAHS (38 mg) was added. The reaction mixture was stirred at 4 °C for 3 days. The sulfated product was recovered after diluting the reaction mixture with methanol (25 ml) and passing through a SAX SPE column (500 mg), the column was washed with methanol and the retained sulfate eluted with ammonium acetate (0.8 M aq. ammonium acetate:methanol (2:8)). The sulfates of pinobanksin, pinocembrin and galangin were prepared in a similar fashion. No further chromatography was required. The yield of the sulfates was ca. 5–8 mg from 25 mg aglycone.

Pinobanksin 7-O-sulfate. ^{1}H NMR (500 MHz, DMSO-d_6) 4.68 (d, J = 11.1, H3) 5.24 (d, J = 11 Hz, H2) 6.34 (d, J = 2, H6) 6.38 (d, J = 2, H8) 7.42 (3H, m, H3',4', 5') 7.54 (2H, d, J = 7.6, H2',6'). ^{13}C NMR (125 MHz, DMSO-d_6) 72.3 (C3), 83.4 (C2), 98.8 (C8), 100.0 (C6), 103.0 (C10), 128.5 (C3',5'), 128.7 (C2',6'), 129.1 (C4'), 137.6 (C1') 162.1 (C9), 162.5 (C7), 162.6 (C5), 199.0 (C4). LC-HRMS [M-H]$^-$ calc for $C_{15}H_{11}O_8S$: 352.0180; found: 352.0228.

Pinocembrin 7-O-sulfate. ^{1}H NMR (500 MHz, DMSO-d_6) 5.64 (dd, J = 12.8, 3.1, H2) 2.84 (dd, J = 17, 3.3 Hz, H3a), 3.4 (obsc., H3b), 6.34 (d, J = 2, H6), 6.39 (d, J = 2, H8), 7.40 (dd, J = 7.3, H-4'), 7.44 (2H, dd, J = 7.5, H2',6'), 7.54 (d, J = 7.3, H2',6'). ^{13}C NMR (125 MHz, DMSO-d_6) 42.3 (C3), 78.4 (C2), 98.3 (C8), 99.3 (C6), 103.6 (C10), 126.6 (C3',5'), 128.5 (C2',6'), 128.5 (C4'), 138.6 (C1') 161.9 (C9), 162.0 (C7), 162.3 (C5), 196.9 (C4). LC-HRMS [M-H]$^-$ calc for $C_{15}H_{11}O_7S$: 335.0231; found: 335.0228.

Chrysin 7-O-sulfate. ^{1}H NMR (500 MHz, DMSO-d_6) 7.04 (s, H3), 6.62 (d, J = 2.3, H6), 7.07 (d, J = 2, H8), 7.40 (3H, m, H-3',4',5'), 8.11 (d, J = 7.1, H2',6'). ^{13}C NMR (125 MHz, DMSO-d_6) 98.0 (C8), 102.4 (C3), 105.4 (C6), 105.9 (C10), 126.5 (C3',5'), 129.2 (C2',6'), 130.6 (C1'), 132.5 (C4'), 156.5 (C9), 159.8 (C7), 160.5 (C5), 163.7 (C2), 182.3 (C4). LC-HRMS [M-H]$^-$ calc for $C_{15}H_9O_7S$: 333.0074; found: 333.0079.

Galangin 7-O-sulfate. ^{1}H NMR (500 MHz, DMSO-d_6) 6.22 (d, J = 2.2, H6) 6.47 (d, J = 2.0, H8), 7.50 (dd, J = 7.5, H4'), 7.56 (2H, dd, J = 7.8, H3', 5'), 8.15 (2H, d, J = 8, H2',6'). ^{13}C NMR (125 MHz, DMSO-d_6) 93.6 (C8), 98.3 (C6), 103.2 (C10), 127.5 (C3',5'), 128.5 (C2',6'), 130.0 (C4'), 137.1 (C1') 137.1 (C3), 145.8 (C9), 156.4 (C7), 160.8 (C5), 164.2 (C2), 176.3 (C4). LC-HRMS [M-H]$^-$ calc for $C_{15}H_9O_8S$: 349.0024; found: 349.0228.

2.5. Preparation of flavonoid glucuronides

2.5.1. Microsome preparation

Dried porcine liver microsome suspension (porcine UDPGT) was prepared by a modification of a literature procedure (Gibson and Skett 1994), from fresh pig livers obtained locally. The pellets were suspended in 50 mM Tris/HCl, pH 8.0 (containing 0.1 M KCl, and 2 mM dithiothreitol) and freeze-dried.

2.5.2. Enzymatic glucuronide conjugation reactions

Enzymatic glucuronide conjugation reactions were carried out in screw-capped glass Duran flasks using porcine UDPGT at 150-ml reaction scale. Standard reactions conditions used were: Tris buffer 12.5 mM, calcium chloride 1.75 mM, bovine serum albumin 3% wt./vol., glucuronic acid 12.5 mM, dithiothreitol 0.25 mM, quinidine hydrochloride 0.1 mM, dried microsomes 3% wt./vol., uridine 5′-diphosphoglucuronic acid 6 mM, flavonoid aglycone 1.5 mM. All reagents apart from UDPGT, UDPGA and aglycone were combined and diluted with distilled water to the required concentration. The pH of the solutions was then adjusted by addition of aqueous HCl or NaOH to pH 8.5. The UDPGT and UDPGA were added with stirring and the pH again checked and adjusted to pH 8.5. The aglycone was dissolved in 5 ml DMSO and then added to the reaction solution dropwise with vigorous stirring to disperse the aglycone. Argon was then bubbled through the solution for 3 min before the reaction flask was sealed. Reactions were carried out for 24 h at 37 °C with shaking. Reactions were terminated after 24 h by addition of 150 ml water and centrifuged at 8000 g for 10 min.

2.5.3. Purification of glucuronide conjugates

Glucuronide conjugates were initially purified using C18 SPE. The filtered solutions from above, containing the dissolved target glucuronide were each applied to a 10-g C18 (2.5 × 4 cm, Chromabond C-18, Macherey-Magel, Duren, Germany) SPE cartridge, washed with water and eluted using 20–40% EtOH in water. These fractions were concentrated and further purified using preparative HPLC (Phenomenex C-12 column, 25 cm × 30 mm, 5 µm, flow rate of 20 ml/min). The solvent was a gradient of 0.05% aqueous formic acid (solvent A) and acetonitrile (solvent B). The gradient used was 5% B through to 50% B. Peaks were detected by UV absorption at 268 and 327 nm and collected automatically based on slope detection. Fractions were assessed by LCMS and those containing the pure glucuronide conjugates were pooled and evaporated under vacuum to remove the solvent. The NMR spectral data for galangin 3-O and 7-O-glucuronide was consistent with that reported by Zhang et al., (2016), and that for chrysin-7-O-glucuronide by Popova et al., (1977).

Pinobanksin 7-O-glucuronide. ^{1}H NMR (500 MHz, DMSO-d_6) 3.31 (3H, m, H2'', 3'', 4''), 3.98 (d, J = 9.6, H5''), 4.71 (m, H3) 5.16 (d, J = 7.5, H1''), 5.28 (d, J = 11.6 Hz, H2), 6.19 (d, J = 2, H6 or 8), 6.21 (d, J = 2, H8 or 6), 7.42 (3H, m, H3',4', 5'), 7.54 (2H, d, J = 7.6, H2',6'). ^{13}C NMR (125 MHz, DMSO-d_6) 71.2 (C4''), 71.6 (C3), 72.7 (C2''), 75.2 (C4''), 75.2 (C5''), 75.6 (C3''), 83.0 (C2), 95.2 (C8), 96.7 (C6), 98.9 (C1''), 102.2 (C10), 128.1 (C2',6'), 128.2 (C3',5'), 128.7 (C4'), 137.0 (C1'), 162.3 (C5, C9), 162.7 (C5, C9), 164.9 (C7), 170.0 (C6''), 198.4 (C4). HMBC correlation (5.16 H-1'' to 164.9 C-7) LC-HRMS [M-H]$^-$ calc for $C_{21}H_{19}O_{11}$: 447.0927; found: 447.0930.

Pinocembrin 7-O-glucuronide. ^{1}H NMR (500 MHz, DMSO-d_6) 2.85 (dd, J = 17.4, 3.2 Hz, H3a), 3.31 (3H, m, H2'', 3'', 4''), 3.31 (m, H3b), 3.95 (d, J = 9.6, H5''), 5.15 (d, J = 7.6, H1''), 5.67 (dd, J = 12.4, 3.1, H2), 6.16 (d, J = 1, H6), 6.24 (d, J = 1, H8), 7.43 (3H, m, H-4',5',6'), 7.54 (2H, dd, J = 7.4, H2',6'), 7.54 (2H, d, J = 7.4, H2',6'). ^{13}C NMR (125 MHz, DMSO-d_6) 42.1 (C3), 71.3 (C4''), 71.3 (C4''), 72.7 (C2''), 75.1 (C5''), 75.6 (C3''), 78.6 (C2), 95.3 (C8), 96.5 (C6), 98.9 (C1''), 103.4 (C10), 128.5 (C3',4', 5'), 128.6 (C2',6'), 138.4 (C1'), 162.6 (C5, C9), 162.9 (C5, C9), 164.9 (C7), 170.1 (C6''), 196.8 (C4). HMBC correlation (5.15 H-1'' to 164.9 C-7). LC-HRMS [M-H]$^-$ calc for $C_{21}H_{19}O_{10}$: 439.0978; found: 439.0982.

2.6. LCMS analysis

The LCMS analysis was performed on a Shimadzu Nexera UPLC with 8040 triple quad MS system (Kyoto, Japan). Chromatographic separation was achieved using either of two systems. System 1 – chromatography with a Phenomenex Omega PS C18 column (2.1 mm i.d. X 150 mm) maintained at 30 °C. The UPLC mobile phase was acetonitrile (B) and water containing 5% formic acid (A). The solvent gradient programme was: initial 15% B; 3 min 15% B; 18 min 55% B; 21 min 60% B; 27 min 65% B; 30 min 75% B; 38 min 100% B; 40 min 100% B and then return to starting conditions. Flow rate was 0.3 ml/min. System 2 – rapid analysis using a Waters Acquity C18 BEH column (50 × 2 mm) with methanol as solvent B and 1% formic acid as solvent A. The solvent gradient programme was: initial 5% B; 0.5 min 5% B; 2.5 min 50% B; 9 min 100% B; 10 min 100% B; 10.2 min return to starting conditions. For analysis, the MS was operated with an ESI probe with selected multiple reaction monitoring (MRM) for selected metabolites (see below). The

ionisation settings were 15 l/min drying gas, heating block at 400 °C and desolvation line at 250 °C. High resolution MS was performed using direct infusion with ESI ionisation and a Waters Xevo G2-XS QToF instrument (Milford, USA).

MRM settings for unconjugated propolis compounds; Caffeic acid (m/z 179.1000 > 135.2000), CAPE(m/z 283.1000 > 179.2000(−)), Pb–5Me (m/z 287.000 > 91.200(+)), Pinobanksin (m/z 273.000 > 153.200(+)), Chrysin (m/z 255.000 > 153.200(+)), Pinocembrin (m/z 257.000 > 153.200(+)), Galangin (m/z 269.000 > 153.200(−)), Pb-acetate (m/z 312.8000 > 253.2000(−)), Pb-propionate (m/z 327.100 > 253.200(−)), Pb-butanoate (m/z 341.100 > 253.200(−)), Pb-pentanoate (m/z 353.100 > 253.300(−)).

MRM settings for conjugated propolis compounds (all -ve mode): Pinobanksin sulfate (m/z 351.0000 > 271.0000), pinobanksin glucuronide (m/z 447.0000 > 271.0000), galangin sulfate (m/z 349.0000 > 269.0000), galangin glucuronide (m/z 445.0000 > 269.0000), chrysin sulfate (m/z 333.0000 > 253.0000), chrysin glucuronide (m/z 429.0000 > 253.0000), pinocembrin sulfate (m/z 335.0000 > 255.0000), pinocembrin glucuronide (m/z 431.0000 > 255.0000).

2.7. Relative response determination for conjugates vs aglycones

Solutions of the conjugate (in methanol/water, 10 µl), an internal standard (catechin in methanol/water, 10 µl) were combined with 100 µl pH 5.2 sodium acetate buffer or 100 µl glucuronidase/sulfatase enzyme (1U/µl) in buffer. The control buffer solutions were kept at 4 °C while the enzyme solutions were subjected to overnight incubation at 37 °C. All samples were prepared for analysis by addition of 200 µl acetonitrile, centrifugation and the supernatants analysed by LCMS. The relative response was calculated from the peak area difference of the conjugate sample in the control and the aglycone in the enzyme treated sample.

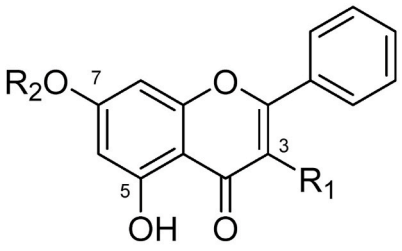
3. Results

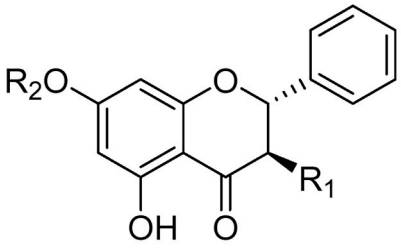
3.1. Analysis of commercial propolis sample

Manuka Health New Zealand Bio30 propolis capsules were chosen for this work as they contained high levels of propolis phenolics and are wax free. The capsules contain 16 components that we routinely measure for propolis-containing samples. The LCMS-MRM analysis of the Bio30 propolis sample is shown in Fig. 2A. The set of pinobanksin esters (acetate, propionate, butanoate and pentanoate) were seen as major peaks, probably due to the facile loss of the ester groups in MS-Q2 giving a strong MRM. The peaks for galangin and pinobanksin were much smaller than those for pinocembrin and chrysin indicating a lower sensitivity of the MRM method for these less well fragmented compounds. The amounts (mg/capsule, duplicate analysis) were: caffeic acid 4.5, *p*-coumaric acid 2.8, cinnamic acid 10, pinobanksin (Pb) 16.3, pinocembrin (Pc) 65, 3-methyl-3-butenyl caffeate 30, benzyl caffeate 29, 1,1-dimethyl allyl caffeate 30, pinobanksin-3-O-acetate (PbOAc) 49, CAPE 6, chrysin (Chry) 23, galangin (Gal) 27, cinnamyl caffeate 17, pinocembrin 7-methyl ether 5.9, tectochrysin 2.7 and galangin 7-methyl ether 2.1.

3.2. Identification of conjugates in plasma

The major components in the propolis were flavonoid aglycones, and the four compounds: pinobanksin, pinocembrin, galangin and chrysin comprise the set of basic examples of a 3,5-dihydroxy flavanol, flavanone, flavonol and flavone respectively (Fig. 1). A fifth major compound was the 3-O-acetate of pinobanksin. Propolis has also been reported to contain a set of caffeic acid esters of which CAPE is the most well-known (Catchpole et al., 2015). Analysis of plasma samples is more complex due to other components in the sample, so a new LCMS method was developed for the analysis of plasma samples. A subset of 10 of the 16 routinely tested compounds was chosen to validate the new method. This set comprised the major compounds mentioned above





Chrysin R_1= R_2=H
Chrysin 7-O-glucuronide
R_1= H, R_2=glucuronic acid
Chrysin 7-O-sulfate
R_1= H, R_2= SO_3H
Galangin R_1= OH, R_2= H
Galangin 7-O-glucuronide
R_1= OH, R_2= glucuronic acid
Galangin 3-O-glucuronide
R_1= O-glucuronic acid, R_2= H
Galangin 7-O-sulfate
R_1= OH, R_2= SO_3H

Pinocembrin R_1= R_2=H
Pinocembrin 7-O-glucuronide
R_1= H, R_2= glucuronic acid
Pinocembrin7-O-sulfate
R_1= H, R_2= SO_3H
Pinobanksin R_1= OH, R_2= H
Pinobanksin 7-O-glucuronide
R_1= OH, R_2= glucuronic acid
Pinobanksin 7-O-sulfate
R_1= OH, R_2= SO_3H

Fig. 1. Structures of the four flavonoids and their conjugates.

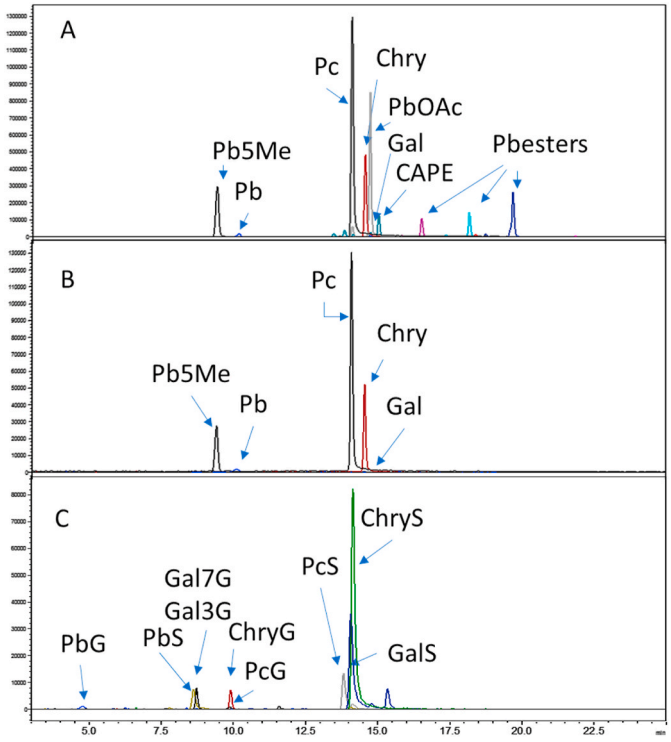


Fig. 2. LCMS chromatograms of: propolis resin (A); enzyme-treated plasma (B); and plasma (C) at 1.5 h after ingestion. Pc = pinocembrin, Pb = pinobanksin, Chry = chrysin, Gal = galangin, G = glucuronide, S = sulfate. Pb5Me = pinobanksin 5-O-methyl ether, PbOAc = pinobanksin 3-O-acetate.

(>10mg/capsule) as well as 5-O methyl pinobanksin, and a set of further alkyl esters of pinobanksin (pinobanksin-propionate, -butanoate and -pentanoate). The caffeoyl type esters were excluded except for CAPE. These choices were based on the relatively low levels of some of the other compounds and the high probability that any esters will be hydrolysed in the gastric system, which should yield caffeic acid (included). Also, tectochrysin was included based on likely methylation.

A single volunteer was used to provide blood samples in order to demonstrate the typical plasma profile after consumption of propolis. The subject consumed 21 Bio30 capsules in a single sitting and samples of whole blood were taken 1.5 and 3.5 h later. This dose was deliberately much higher than normal usage and was designed to give high circulating levels of propolis metabolites to facilitate analysis.

LCMS-MRM analysis of the enzyme-treated plasma samples showed a somewhat different profile to the propolis resin sample (Fig. 2A/B). The most obvious difference was the absence in the plasma of the pinobanksin esters. The one example of a caffeic acid ester, CAPE, was also missing in the plasma. The four propolis flavonoid aglycones (Pb, Pc, Chry and Gal) were all present at around the same ratio as seen in the propolis itself. There appeared to be no increase in Pb, which might be expected after hydrolysis of the Pb-3-O-Ac and other esters during sample preparation rather than as a consequence of metabolic activity in the volunteer, but this difference might be due to the poor detector response to pinobanksin. The similarity in the flavonoid aglycone ratio between the plasma and propolis can also be seen in the UV chromatogram of the two samples (Fig. 3). Analysis using UV detection was only possible due to the high dose of propolis used in this experiment. No evidence was found for hydroxy cinnamic acid type metabolites in the plasma samples.

Plasma analysed directly after protein precipitation showed no detectable propolis aglycone compounds (Fig. 2C). Also, none of the other acylated flavonoids (e.g. pinobanksin 3-O-acetate) or phenolic acid esters are seen. Extra MRMs were included in the analysis methods to detect the expected mono-glucuronides and mono-sulfates of Pb, Pc, Chry and Gal as well as mono- and di-glucuronide or sulfate conjugates of CAPE and caffeic acid. These MRMs were based on the facile loss of the conjugate group. The four flavonoid-mono-glucuronides were detected, with large peaks seen for the Pb, Pc and Gal glucuronides and a smaller peak for the Chry-glucuronide. Peaks were observed that corresponded to two galangin glucuronides. Large peaks were also seen for the sulfated conjugates, consistent with a single metabolic sulfate for each of the four flavonoids. This result shows that the major propolis compounds are converted to mono-glucuronide and -sulfate conjugates either during intestinal absorption or by hepatic conversion.

3.3. Preparation of glucuronides and sulfates

Sulfate derivatives of the four major non-acylated aglycone flavonoids in propolis were prepared according to the method described by Barron and Ibrahim (1987). As observed in the earlier work the 7-sulfates were the major products from the reaction of all four flavonoids with only trace amounts of the 3,7-disulfate being formed. The NMR and UV data for the sulfates were consistent with 7-O-sulfation. In particular, the C13 NMR shifts of the carbon signals for the C-6, -7 and -8 carbons showed upfield shifts for C-7 and down field shifts for C-6 and -8 (relative to the aglycone) which are characteristic for C-7 substitution (Zhang et al., 2016). The LCMS analysis showed that synthetic sulfates matched the observed sulfates seen in the plasma sample (Fig. 4).

Glucuronidation of various metabolites of interest can be achieved using chemical methods (usually by reacting with acetobromo-α-D-glucuronic acid methyl ester, e.g. Fan et al., 2011) or by employing an enzymatic method. The chemical method is useful when the conjugation

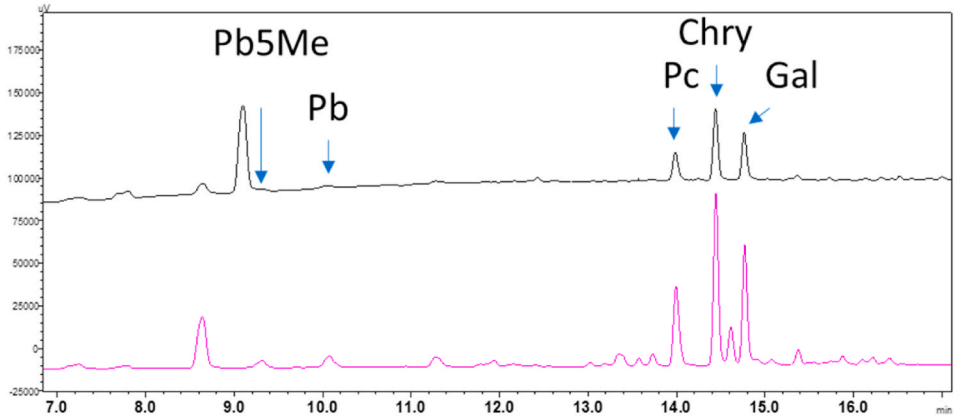


Fig. 3. UV (268 nm) chromatogram of enzyme treated plasma 1.5 h after propolis ingestion (top) and propolis resin (bottom). Pb5Me is pinobanksin 5 methyl ether. Pc = pinocembrin, Pb = pinobanksin, Chry = chrysin, Gal = galangin.

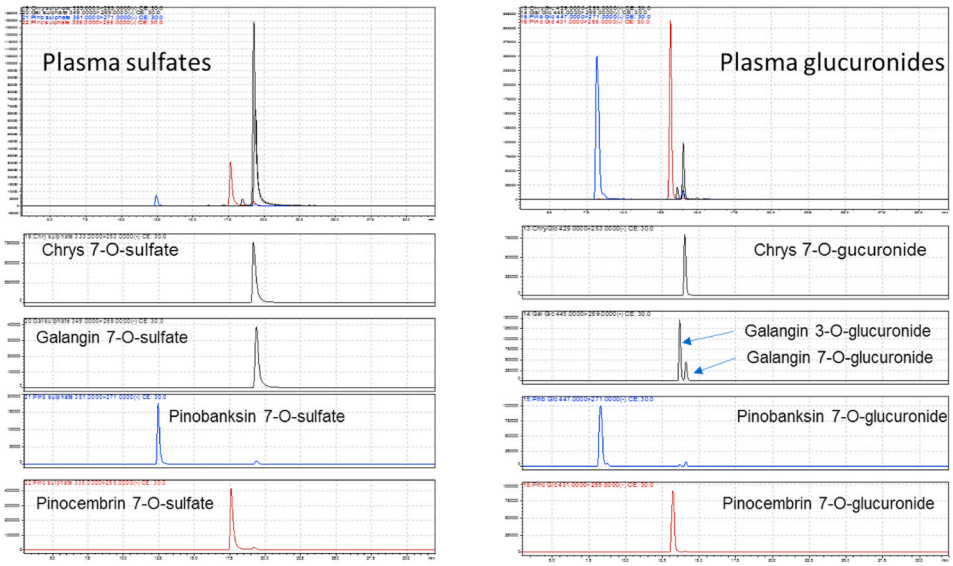


Fig. 4. LCMS chromatograms of chemical synthesised (sulfates) and biochemical synthesised (glucuronides) found in plasma following propolis ingestion.

site is unhindered by surrounding groups and only one site of conjugation is possible. An enzymatic method using a liver microsome preparation is more likely to mimic the *in vivo* conjugation and can work when the chemical method fails. However, the enzymatic method has a lower yield and higher reagent cost. For this work, we used a preparation of porcine liver microsomes. Other liver microsome preparations can be used (ovine, human) but, in this case, good results were obtained with the more readily available porcine material. The dried microsomes were incubated with the flavonoid aglycone in the presence of a glucuronide donor (UDPGT). For pinocembrin and chrysin the expected 7-O-glucuronides were isolated and purified. For pinobanksin the major glucuronide produced was the 7-O-glucuronide. Galangin was the exception showing mono-glucuronidation occurring at either the 7- or the 3- position. Confirmation of the site of glucuronidation was by NMR (Long range C–H correlation between C-7 of the flavonoid and H-1 of the glucuronic acid).

3.4. Quantitative analysis of conjugates

The relative peak areas for the conjugates versus the aglycones was assessed by preparing samples of the conjugates in buffer and comparing the samples incubated overnight with glucosidase/sulfatase versus untreated samples. This process revealed widely varying response factors for the conjugates. The sulfate derivatives gave the highest response factors, (sulfate:aglycone peak area ratio: chrysin 28.5, pinocembrin 7.9, pinobanksin 62.4 and galangin 216.7) while those for the glucuronides were smaller and <1 for the pinocembrin and chrysin glucuronides (glucuronide:aglycone peak area ratio; chrysin 0.6, pinocembrin 0.2, pinobanksin 3.8 and 17 and 35 for galangin 3- and 7-glucuronide respectively).

A standard curve prepared for each of the aglycones then allowed conversion of the conjugate peaks in the plasma to approximate aglycone concentrations. The peaks areas for the plasma sample (6 μl injection, equivalent to 2 μl plasma) are shown in Fig. 5 along with the converted values for the aglycones presented as ng injected.

4. Discussion

The aim of this work was to identify the propolis derived conjugates in plasma (with and without enzymatic deconjugation) by comparison with synthetically prepared conjugates prepared in this work. In addition, some information has been obtained regarding the quantities of some of the flavonoids circulating in the blood stream following propolis ingestion. In general, the detector response using MRM transitions is expected to be much higher for the conjugated flavonoids versus the aglycones and so direct analysis of conjugates is desirable. These results will facilitate the plasma analysis work in any future human study with multiple subjects consuming lower doses than the megadose and single subject used in this study.

The key components in propolis are a set of flavonoids (predominantly pinobanksin, pinocembrin, galangin and chrysin), as well as a group of caffeic acid esters. No evidence is seen in the plasma samples for the presence of any caffeic acid type metabolites. The LCMS analysis of possible metabolites from these two sets of components shows that

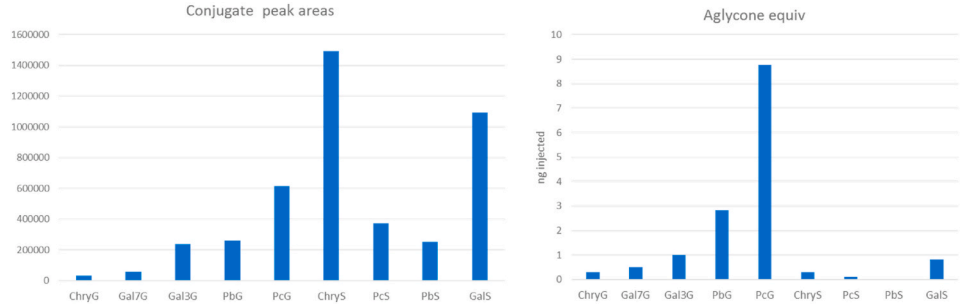


Fig. 5. Comparison of peak areas for MRM detection of conjugates versus aglycones (presented as injected equivalents (ng)). Left-hand columns show peak areas for conjugates and right-hand columns show corresponding values (ng) for amounts of each conjugate determined as aglycone equivalents. Pc = pinocembrin, Pb = pinobanksin, Chry = chrysin, Gal = galangin, G = glucuronide, S = sulfate.

only the flavonoid metabolites appear at detectable quantities in the plasma.

The set of four propolis aglycones are basic representatives of the flavone, flavonol, flavanone and flavanol classes of the flavonoids. Conjugation of these compounds is limited to available phenolic sites (positions 3, 5 and 7, Fig. 1) and generally the 5-O conjugates are less favoured. So, for chrysin and pinocembrin only the 7-O-sulfates and glucuronides were found in the plasma. Previous studies with chrysin have shown 7-O-conjugation to be favoured with faster formation of the sulfate versus the glucuronide in cell incubation studies (Galijatovic et al., 1999). Chrysin 7-O-glucuronide is also a known natural product (Popova et al., 1977).

For pinobanksin and galangin the presence of a 3-hydroxy group introduces a second likely site for conjugation. For galangin, the plasma samples showed two mono-glucuronides but only one (mono-) sulfate. The two plasma glucuronides have UV spectra characteristic of 3-O-substitution (reduced absorption maximum at 359 nm) and 7-O-substitution (only minor difference compared with the aglycone). This was confirmed by comparison with the synthetically prepared samples of the two glucuronides. Similarly, the galangin sulfate was determined to be the 7-O-sulfate based on UV data and confirmed by comparison with the synthetic sample. This conforms to earlier work where two glucuronides and one sulfate were seen in rats exposed to galangin (Chen et al., 2015). Galangin was preferentially glucuronidated after oral dosing but sulfated after intravenous medication. For pinobanksin, 3-O-conjugation does not appear to be favoured as only one glucuronide and one sulfate are seen in the plasma consistent with 7-O-conjugation.

5-Methyl pinobanksin is a minor compound in New Zealand propolis and it was observed in the plasma after enzyme treatment at about the same relative level as in the initial propolis resin. However, no evidence was found for the presence of 5-methyl pinobanksin conjugates or the aglycone in the plasma samples.

Although the major peaks in the LCMS chromatogram are the those due to chrysin- and galangin-sulfate, this work shows that the majority of the flavonoids are present as their glucuronide conjugates. The response factors vary across the various metabolites but in general the factors determined for the sulfates are much higher than the corresponding glucuronides. This finding qualitatively agrees with a previous observation that flavonoids are potent inhibitors of human sulfotransferases (Wong and Williamson, 2013). Assuming a plasma volume of 2.9 l (60% of 5 l) these values then yield total aglycone amounts in the circulating plasma of 0.9 mg chrysin, 12.9 mg pinocembrin, 4.1 mg pinobanksin and 3.8 mg galangin. The “megadose” of propolis capsules ingested in one sitting in the current study represented a dose of 1365 mg of pinocembrin and 42 mg of chrysin, so at the time of blood sampling less than 1% of the pinocembrin and around 0.2% of the chrysin was in circulation. A previous study with mice using chrysin and its conjugates showed the circulating plasma levels of the sulfates and glucuronides were of the same order (Ge et al., 2015). A previous study with humans orally ingesting a 400-mg dose of chrysin concluded that chrysin aglycone has low oral bioavailability into blood (Walle et al., 2001). Free aglycone reached a maximum average concentration of 6 ng/ml in plasma, whereas chrysin sulfate reached a maximum average concentration of 200 ng/ml. Chrysin glucuronide was the main metabolite detected in urine, while the majority of the chrysin ingested appeared unchanged in faeces.

This study has shown the four main flavonoid compounds present in a commercial propolis product appear in human plasma following ingestion and they are present in the form of sulfate and glucuronide derivatives. Each of these derivatives has been prepared and characterised. The metabolites of pinobanksin and pinocembrin have not been prepared before and the availability of these compounds will enable further studies on the bioavailability and drug interaction of propolis. For example, a study of the interaction of chrysin sulfate and chrysin glucuronide with the major plasma protein serum albumin showed the sulfate had higher affinity for the protein than the glucuronide, an interaction which may distort the analysis (Mohos et al., 2018).

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