



Antiproliferative activity of New Zealand propolis and phenolic compounds vs human colorectal adenocarcinoma cells



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ABSTRACT

New Zealand propolis is a “European” type propolis obtained by honey bees mainly from exudates of poplar. European type propolis is known to have anti-inflammatory and anti-cancer properties and this activity has been attributed to some of the main constituents such as chrysin and CAPE (caffeic acid phenethyl ester). As part of our studies on how New Zealand propolis might benefit gastro-intestinal health, we carried out *in vitro* bioactivity-guided fractionation of “Bio30™” propolis using both anti-inflammatory (TNF- α , COX-1, COX-2) and anti-colon cancer (DLD-1 colon cancer cell viability) assays; and determined the phenolic compounds responsible for the activity. The New Zealand wax-free Bio30™ propolis tincture solids had very high levels of the dihydroflavonoids pinocembrin and pinobanksin-3-O-acetate, and high levels of the dimethylallyl, benzyl and 3-methyl-3-butenyl caffeates relative to CAPE. The DLD-1 assays identified strong anti-proliferative activity associated with these components as well as chrysin, galangin and CAPE and a number of lesser known or lower concentration compounds including benzyl ferulate, benzyl isoferulate, pinostrobin, 5-phenylpenta-2,4-dienoic acid and tectochrysin. The phenolic compounds pinocembrin, pinobanksin-3-O-acetate, tectochrysin, dimethylallyl caffeate, 3-methyl-3-butenyl caffeate, benzyl ferulate and benzyl isoferulate also showed good broad spectrum activity in anti-proliferative assays against three other gastro-intestinal cancer cell lines; HCT-116 colon carcinoma, KYSE-30 oesophageal squamous cancer, and NCI-N87 gastric carcinoma. Activity is also observed in anti-inflammatory assays although it appears to be limited to one of the first cytokines in the inflammatory cascade, TNF- α .

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1. Introduction

Propolis is a heterogeneous material consisting of resin collected by honey bees from the leaf, buds and bark of certain tree species, which is then admixed with beeswax produced from the hypopharyngeal glands of the worker bees. Propolis is used by bees to defend the hive against invaders, and to reduce air flow into the hive to retain heat. In modern bee-keeping practice, the beekeeper places mats into the hives with grid slots ideally sized for propolis to be deposited. These mats are then removed from the hives to recover the propolis for commercial use.

Propolis has been categorised into seven major classes based on the plant source [1–3], of which the best known are “Poplar”, i.e. European, Chinese, North and Southern South American, New Zealand; “Brazilian

green” containing artepillin-C; “Birch” from Russia, “Red” propolis from Cuba, Brazil, Mexico; Mediterranean (Greece, Sicily, Crete, Malta) sourced from conifers; “Clusia” from Cuba and Venezuela, and “Pacific” from Okinawa, Taiwan, Indonesia, parts of Japan containing ‘propolins’. The composition of New Zealand-sourced propolis has not been extensively reported. New Zealand propolis tincture has been described as consisting largely of flavonoids, particularly dihydroflavonoids such as pinocembrin and pinobanksin-3-O-acetate [4]. Chrysin and galangin were the next most abundant components.

Propolis has been used by humans since antiquity for treating diseases. It is well known anecdotally and through *in vitro* bioassay for its antimicrobial, antioxidant, anti-inflammatory and more recently, anti-proliferative activity. European type propolis is currently incorporated into a wide range of complementary health care products, including tinctures, throat sprays, lozenges, toothpastes, soaps which require a broad range of activities. Its use for health and wellness applications by oral ingestion has been limited due to its highly variable and partly unknown composition [5], lack of clinical data [5], and apparently low

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absorption into the blood in the form of metabolites after oral ingestion [6,7]. However, low levels of absorption into the blood, and *in vivo* trials with rats [8,9] imply that the bioactives could benefit gastro-intestinal health if they pass through the gastro-intestinal tract without being metabolized. Two significant gastro-intestinal health issues are inflammation and cancer. Chronic inflammatory diseases such as inflammatory bowel disease often progress to colon cancer [10].

There are a number of review articles on the anti-cancer and anti-inflammatory activity of propolis, which also include a discussion on individual components in Poplar propolis, particularly chrysin and caffeic acid phenethyl ester (CAPE) and also polyphenols found in honey and propolis, and polyphenols in general [11–17]. CAPE has been extensively investigated for its anti-cancer activity, particularly colon cancers [8,9,18,19]. Chrysin is recognised as having a broad range of anti-cancer activity, and it is discussed in most review papers along with CAPE [11,14]. It was shown to have anti-proliferative activity against murine colon cancer cell line 26-L5, but an order of magnitude weaker than benzyl, phenethyl and cinnamyl caffeates [15]. Chrysin has also been shown to sensitise human colorectal cancer cell line HCT-116, liver cancer cell line HepG₂ and nasopharyngeal line CNE-1 to TNF- α induced apoptosis and HCT-116, HepG₂, cervical cancer cell line HeLa and CNE-1 to TRAIL induced apoptosis [20]. Galangin is another propolis component which has also been shown to inhibit proliferation and induce apoptosis in *in vitro* testing against a gastric cancer cell line SNU-484 [21] while extracts of *Alpinia officinalum* rich in galangin show activity against human uterine carcinoma and human colorectal adenocarcinoma [22]. Pinocembrin has been shown to be active against human adenocarcinoma cell line HCT 116. The mechanism of action is by triggering Bax-dependent mitochondrial apoptosis, as Bax deficient HCT116 cells were immune to pinocembrin [23]. Three Mexican propolis samples from the semiarid region Sonora rich in pinocembrin, pinobanksin, pinobanksin-3-O-acetate and chrysin were tested against a range of human cell lines [24], along with CAPE, galangin, chrysin, pinocembrin, and pinobanksin-3-O-acetate. One propolis sample was active against all cell lines, while the other two were active against all lines except A-549. Pinocembrin, pinobanksin and pinobanksin-3-O-acetate were inactive against all cell lines. Benzyl caffeate is reported as being a constituent of Netherlands [15] and Chinese [25] propolis that has anti-proliferative activity against murine colon cancer cell line 26-L5.

Propolis compounds have also demonstrated anti-inflammatory activity. Pinocembrin was found to provide *in vitro* and *in vivo* protection against LPS induced inflammation [26]. It has also been demonstrated to have positive effects in reducing reactive oxidation species and inflammatory agents in diabetic mice [27]. Chrysin has been shown to alleviate dextran-sulphate induced colitis in mice and also reduced weight loss in a dose dependent manner [28]. Chrysin also inhibited TNF- α induced NF- κ B in rat intestinal epithelial IEC-6 cells. An inhibitory effect on nitrous oxide production by LPS-activated murine macrophages was also shown by a benzyl caffeate, CAPE and some synthesized caffeates [29]. CAPE is also reported to inhibit differentiation of mouse fibroblasts to adipocytes (fat storage cells) by suppressing production of leptin, TNF- α and resistin and thus may have potential as an antiobesity agent [30,31].

In this work we present the results of a study of New Zealand propolis, using bioactivity-guided fractionation vs DLD-1 colon adenocarcinoma cells and LPS-induced inflammation in neutrophils to establish a first indication of the gastro-intestinal health potential of such propolis in ameliorating inflammation, and preventing or retarding the proliferation of gastro-intestinal cancers, i.e. as a 'chemo-preventative' agent. The use of propolis as an anti-inflammatory [29] and a chemo-preventative agent has been previously proposed [11]. Determination of the phenolic compounds responsible for the activity also allows crude propolis to be selected on the basis of its composition for making dewaxed propolis resin, and new propolis resin-based product formulations to be established. One such product, Bio100 Propolis with CycloPower™, has been undergoing *in vitro* anti-colon cancer bioassays with other apoptosis-inducing agents [32,33].

2. Materials and methods

2.1. Chemicals

Chrysin, tectochrysin, pinobanksin, pinocembrin, CAPE, 1,1-dimethylallyl caffeate (DMAC), cinnamic acid, galangin, were purchased from Sigma-Aldrich. Pinocembrin 7-methyl ether was purchased from Apin Chemicals, United Kingdom. Caffeic, ferulic and isoferulic acids were from commercial samples held at Callaghan Innovation. Pinobanksin-3-O-acetate was from a laboratory collection held at Callaghan Innovation. Pinostrobin chalcone was isolated from propolis (see supplemental material).

2.2. Propolis feed material

Propolis ethanolic tincture provided by Manuka Health New Zealand ('Bio30™') had the ethanol/water solvent removed under vacuum firstly using a 20 l Büchi rotary vacuum evaporator and secondly using a freeze dryer. Bio30™ propolis tincture was commercially manufactured by the ethanol/water extraction of crude propolis (200–1000 kg batch sizes) selected on the basis of having at least 30 mg/g on a wax free basis of the compounds pinocembrin, galangin, chrysin and CAPE. The extraction process results in a wax free propolis resin dissolved in an ethanol/water solvent mixture. The large batch size extracted ensures that the composition is remarkably consistent, even though the crude propolis is sourced from a number of regions in New Zealand. The sticky resinous product obtained from the freeze dryer was used for fractionation and biological assay work.

2.3. Analytical methods

HPLC analysis was carried out on a Phenomenex Kinetex 2.6 μ m C18 100A column (150 \times 2.10 mm), at 30 °C with injection volumes of 0.5 μ l using a Waters Acquity Ultra Performance LC system equipped with a photo diode array detector. The solvent system consisted of solvent A: 5% aqueous formic acid (analytical grade), and solvent B: MeOH (HPLC grade). Solvents were mixed using a linear gradient starting with 65% A, decreasing to 55% A at 10 minutes, held for 4 minutes, then decreasing to 20% A at 55 minutes, 5% A at 60 minutes, held at 5% for 5 minutes, then increased to 65% at 70 minutes. The column was then equilibrated for 10 minutes at 65% A before the next injection was made. A flow rate of 0.15 ml/min was used throughout. LCMS analysis was carried out as above for UHPLC analysis except that the Waters UPLC system used for the analysis was also equipped with a Waters SQD single quadrupole mass detector with solvent flow entering the detector after exiting the photodiode array detector. Both positive and negative electrospray ionisation modes were utilised and masses were recorded over a range of 50–1000 *m/z*.

2.4. Identification and synthesis of esters

The esters cinnamyl caffeate, 3-methyl-3-butenyl caffeate, benzyl caffeate, benzyl ferulate, and benzyl isoferulate were identified in propolis fractions by analysis of UV data, HPLC determination of products from alkaline hydrolysis and direct comparison with synthetically prepared esters. The general method for synthesis was as follows: 1.0 g of caffeic, ferulic or isoferulic acid, 1.2 mole equivalents of the alcohol and 50 mg of *p*-toluene sulphonic acid were added to 100 ml benzene and the mixture heated to reflux. The flask was fitted with a condenser and a side arm trap to capture any water generated during the reaction (Dean Stark apparatus). The heating was allowed to run for 3 days with 8 hours reflux each day. The reaction mixture was then purified by evaporation of the benzene in a rotary vacuum evaporator, the residue was suspended in water, passed through a C18 column which was washed with water and the crude ester eluted with methanol. The

esters were then further purified by preparative HPLC. The purity of the products was examined by HPLC and NMR.

2.5. Propolis fractionation

Initial fractionation was carried out on propolis resin using a glass column packed with Merck Lichroprep C18 reversed phase stationary phase (16 × 4 cm). Propolis resin (5.446 g) dissolved in EtOH (5 ml) was loaded on to the column and elution was carried out as a stepped gradient (250 ml) of reducing polarity consisting of 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% EtOH in water, followed by two 100% EtOH steps, then elution with 2-propanol (IPA), ethyl acetate (EtOAc), acetone, and CHCl₃. Following solvent removal, fraction masses were measured and samples prepared for bioassay. The two 100% EtOH fractions were combined as were the remaining four non-polar fractions (IPA, EtOH acetone, and CHCl₃) for bioassays due to their relatively low masses.

In the second stage fractionation the 20%, 30%, 60%, and 90% aqueous EtOH eluted fractions from the first stage were further fractionated by preparative HPLC using a Phenomenex Synergi 4 μ-RP Max 80A 250 × 30 mm C-12 column using a Gilson 321 preparative pump and Agilent 1100 series diode array detector with a flow rate of 20 ml/minute. Fractions were collected manually with online detection carried out at 268 nm (for flavonoids) and 327 nm (for caffeic acid derivatives). Analytical HPLC was carried out on all collected fractions and those showing a single compound or a highly purified compound were weighed, and prepared for biological assay. Fractions without a single or highly purified compound were combined to form 'non-single peak' fractions for biological assay. Two of the 60% EtOH sub-fractions, 60% EtOH F8 and 60% EtOH F9, required further chromatography using Sephadex LH20 (3 × 42 cm, 70% aqueous MeOH containing 0.1% TFA vol./vol.).

2.6. Anti-proliferative (cell viability) assays

DLD-1, HCT-116, KYSE-30, NCI-N87 gastro-intestinal cancer assays. Human gastrointestinal cancer cells DLD-1 (colorectal adenocarcinoma cells [ATCC CCI-221, obtained from ATCC]); HCT-116 (colon carcinoma cells [ECACC, obtained from Sigma Aldrich]); NCI-N87 (gastric carcinoma cells [ATCC CRL-5822, obtained from ATCC]), KYSE-30 (oesophageal squamous cell carcinoma [ECACC obtained from Sigma Aldrich]) were revived from cryostorage and cultured in the presence of the test and reference samples. The culture conditions for the cells were those described by the supplier of the cells and the assay methodology was based on previously reported procedures (see supplemental material). Briefly, working solutions of the test compounds and positive controls were prepared by dissolving the test fractions in 15% EtOH/HBSS to a concentration of 2 mg/ml solids. The final concentration of each sample in the test well was 200 μg/ml. The 5-fluorouracil (5-FU) positive control test well concentrations were 15.0 ng/ml and 7.5 ng/ml in the first stage of testing, 1.95 and 0.65 μg/ml in the second stage of testing, and 1.95, 0.65 and 0.195 μg/ml in the third stage of testing. After incubation a MTT cell viability assay was performed on the cultures to determine the effect of the samples on the inhibition of cell proliferation. Results were expressed as the percentage proliferation of cells cultured in the presence of the sample in comparison to the cells only control. For test samples generally 6 replicates were used and either 9 or 12 replicates for control samples.

2.7. Anti-inflammatory assays

Anti-inflammatory assays were carried out in two stages. In stage 1, neutrophils from rat blood were cultured in the presence of the test sample or positive control before being activated into an inflammatory response through the use of lipopolysaccharide (LPS). The incubation period before addition of LPS was 20 minutes. The cell culture

supernatants were then tested by ELISA for the presence of the cyclooxygenase COX-1 and COX-2 marker compounds thromboxane TXB₂ and prostaglandin PGE₂, respectively, and the cytokine TNF-α. The positive controls were chloroquine for the cytokine TNF-α; and indomethacin for the TXB₂ (COX-1) and PGE₂ (COX-2) inflammatory markers. The test well sample concentrations used were 50 μg and 200 μg of solids/ml. The incubation period for TXB₂ and PGE₂ was 3 hours, and for TNF-α was 24 hours. Fractions that showed 100% inhibition at both concentrations were retested at dilutions of 1 μg/ml, 3 μg/ml and 10 μg/ml for inhibition of TNF-α production, along with the eight standards chrysin, CAPE, cinnamic acid, chrysin-7-methyl ether (tectochrysin), galangin, pinocembrin, pinocembrin 7-methyl ether, and caffeic acid at concentrations of 1 μg/ml and 10 μg/ml.

3. Results and discussion

New Zealand propolis can be categorised as "European", i.e. obtained by honey bees mainly from the exudates of poplars. A comparison of the antioxidant activity, total phenol and flavonoid, and individual phenol and flavonoid composition for ethanol extracted propolis samples from 14 countries including New Zealand showed that New Zealand-sourced propolis is similar in composition to propolis from Bulgaria, Uzbekistan and Hungary; and to propolis from three South American countries – Chile, Uruguay and Argentina [34]. In our analysis of New Zealand propolis tinctures the levels of seventeen components are routinely measured and typical values (mg/g dry solids) are: caffeic acid 7, *p*-coumaric acid 3, cinnamic acid 9, pinobanksin 35, pinocembrin 119, 3-methyl-3-butenyl caffeate 9, benzyl caffeate 18, 1,1-dimethyl allyl caffeate 11, pinobanksin-3-*O*-acetate 71, CAPE 9, chrysin 33, galangin 51, cinnamyl caffeate 6, pinocembrin 7-methyl ether 13, benzyl ferulate and isoferulate 7, tectochrysin 4 and galangin 7-methyl ether 9. A typical chromatogram is shown in Fig. 1, with the phenolic compounds listed in the captions. Our analysis confirms earlier work [4] that showed New Zealand propolis has very high levels of the dihydroflavonoids pinocembrin and pinobanksin-3-*O*-acetate. The levels of the benzyl, dimethylallyl, and 3-methyl-3-butenyl caffeates were higher than CAPE.

3.1. Anti-proliferative activity

The *in vitro* anti-proliferative activity of propolis and propolis components was determined by MTT assay using human colorectal adenocarcinoma DLD-1 cells. The first stage involved samples obtained from the fractionation of propolis tincture dry solids using reversed phase column chromatography (ten fractions). 5-Fluorouracil was used as the positive control at a test concentration of 7.5 and 15 ng/ml. Each sample was tested in the assay at 200 μg/ml of solids. For this study, controls were performed with 9 replicates while assays were performed with 6 replicates. At this level of replication, statistically significant data can be generated. Crude propolis tincture dry solids inhibited proliferation of DLD-1 cells by 33%. The fractions that had the highest anti-proliferative activity in the DLD-1 cell assay were Fraction 1 (20% EtOH fraction, 68% inhibition), Fraction 2 (30% EtOH fraction, 73% inhibition), Fraction 5 (60% EtOH fraction, 41% inhibition) and Fraction 8 (90% EtOH fraction, 72% inhibition). Several other fractions demonstrated lower levels of inhibition of proliferation (Fig. 2). Fractions 1 and 2 were mainly phenolic acids. Caffeic acid was the major phenolic compound in Fraction 1 (20% ethanol) and was confirmed to be the bioactive compound present in the second stage of testing for both fraction 1 and 2. 3,4-dimethoxycinnamic acid was the major phenolic compound in Fraction 3 (40% EtOH fraction, weak activity) and so has low anti-proliferative activity. It was found to be inactive *in vitro* against murine colon cancer cell line 26-L5 [15,25]. Fraction 4 (50% EtOH fraction) had low levels of phenolics, with the largest contributors being cinnamic acid and pinobanksin. Fraction 5 (60% EtOH fraction) contained the bulk of the flavonoids and caffeate esters. Fractions 6 – 10 contained low levels of

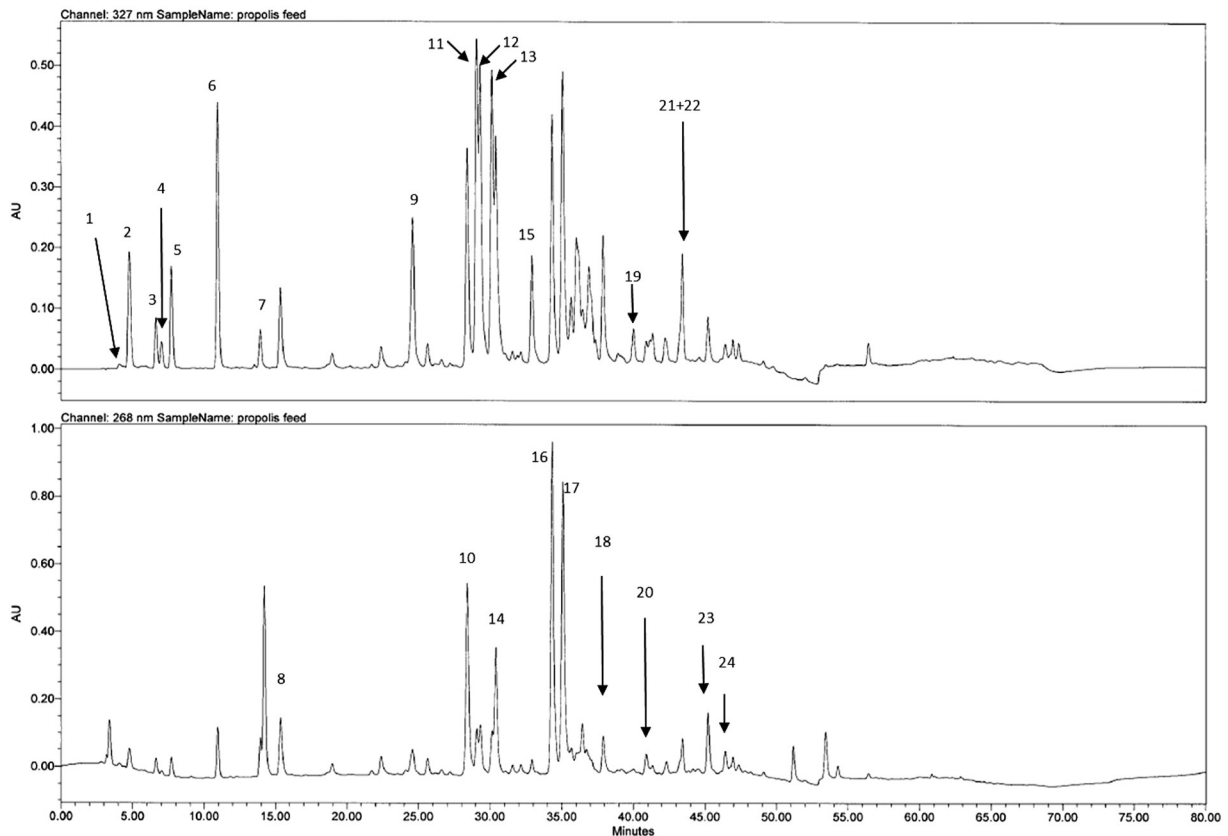


Fig. 1. New Zealand propolis tincture chromatogram recorded at 327 nm (top chromatogram) and 268 nm (lower chromatogram). Peak assignments; (1) 3,4-dihydroxy benzaldehyde, (2) caffeic acid, (3) *p*-coumaric acid, (4) isoferulic acid, (5) ferulic acid, (6) 3,4-dimethoxycinnamic acid, (7) cinnamic acid, (8) pinobanksin, (9) 5-phenylpenta-2,4-dienoic acid, (10) pinocembrin, (11) 3-methyl-3-butenyl caffeate, (12) benzyl caffeate, (13) 1,1-dimethylallyl caffeate, (14) pinobanksin-3-*O*-acetate, (15) CAPE, (16) chrysin, (17) galangin, (18) cinnamyl caffeate, (19) pinostrobin chalcone, (20) pinocembrin-7 methyl ether, (21) and (22) benzyl ferulate and benzyl isoferulate, (23) chrysin-7 methyl ether (tecto-chrysin), (24) galangin-7 methyl ether (izalpinin).

phenolics. Fraction 6 had the bulk of the ferulate esters. Fraction 7 contained the bulk of the flavonoid 7-methyl ethers. Fraction 8 was not investigated further in this work as it contained no phenolics of note.

Subsequently, the most active fractions; 1 (20%), 2 (30%), and 5 (60%), were submitted to further chromatographic separation resulting in 27 new fractions for further testing (fractions described as original fraction% followed by subsequent fraction numbers e.g. 60% F8-5). This procedure, guided by HPLC analysis of fractions, resulted in more highly purified fractions containing mainly single compounds, while some fractions still contained a number of compounds. The DLD-1 cells were exposed to these various fractions using the same test well concentration of 200 $\mu\text{g}/\text{ml}$. Additionally, the known propolis compounds chrysin, CAPE,

cinnamic acid, chrysin 7-methyl ether (tecto-chrysin), galangin, pinocembrin, pinocembrin 7-methyl ether, and caffeic acid were also tested at 200 $\mu\text{g}/\text{ml}$. The positive control test well concentrations were increased to 0.65 and 1.95 $\mu\text{g}/\text{ml}$ to ensure a stronger anti-proliferative response. A number of the fractions and standards showed significant inhibition of proliferation of the DLD-1 cells. The most active fractions afforded inhibition of proliferation of 90–100% and were considered cytotoxic at this concentration. The standards for which the anti-proliferative activity was demonstrated from strongest to weakest were: caffeic acid = pinocembrin > CAPE > chrysin = galangin >> tecto-chrysin = cinnamic acid >> pinocembrin 7-methyl ether (no activity).

The most active fractions tested were then further analysed by LCMS. The phenolic compounds identified in the most active fractions are given

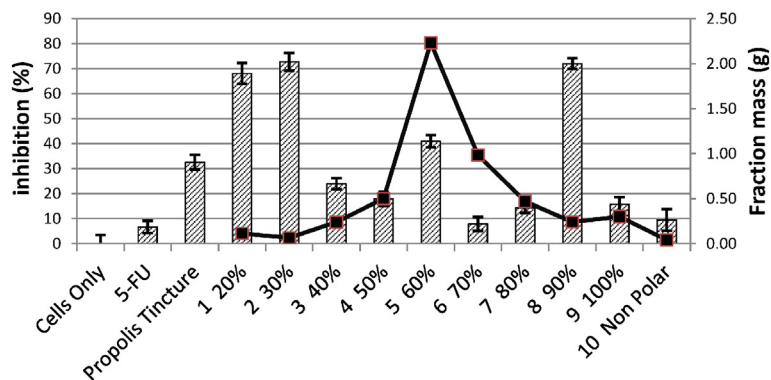


Fig. 2. Inhibition of DLD-1 cell proliferation for blank, positive control (5-FU), propolis tincture and fractions from first stage fractionation of propolis tincture using RP chromatography. Columns are % inhibition of cell growth at a given ethanol percentage in the eluent (error bars are \pm SD). Black squares are fraction masses (g).

Table 1
Anti-proliferative activity vs DLD-1 cells for second stage fractions. *See text.

Sample ID <i>Original RP fraction % and minor fraction number</i>	Compounds identified in sample	% inhibition sample (±SD)	% inhibition standard (±SD)
20% F8	Caffeic acid	95.2 ± 0.68	96.5 ± 0.68
30% F8	Caffeic acid	96.1 ± 0.19	96.5 ± 0.68
60% F7	5-Phenylpenta-2,4-dienoic acid	59.9 ± 1.91	43
60% F8-1	1,1-Dimethylallylcaffeate	95.0 ± 0.65	93
60% F8-2	3-Methyl-3-butenyl caffeate (major) 1,1-Dimethylallylcaffeate (minor)	61.9 ± 3.64	92
60% F8-3	Pinobanksin-3-O-acetate (minor)	58.9 ± 3.70	75
60% F8-4	Caffeic acid phenethyl ester (CAPE, major) Pinobanksin-3-O-acetate (minor)	90.4 ± 0.31	76.1 ± 2.19
60% F8-5	Pinocembrin (minor) Pinocembrin (major)	90.4 ± 0.65	94.0 ± 0.55, 92
60% F8-6	Benzyl caffeate (minor) Pinocembrin (minor)	85.3 ± 0.90	
60% F9-1	Benzyl caffeate (major)		
60% F9-2	Cinnamyl caffeate	57.7 ± 2.50	
60% F9-3	Chrysin	43.2 ± 2.50	61.9 ± 3.27
60% F9-4	Benzyl ferulate and benzyl isoferulate	68.7 ± 1.11	86, 91
60% F9-5	Pinostrobin chalcone	85.0 ± 1.98	83
60% F9-5	Galangin	62.8 ± 2.72	56.4 ± 1.17
60% F10	Tectochrysin	77.9 ± 1.73	29.4 ± 4.94*, 93

Notes: Data in italics from stage 3 testing, *determined using ethanol as solvent in stage 2.

below (Table 1). Caffeic acid was identified as the compound responsible for activity in fraction 20% F8 (i.e. fraction 8 from second stage fractionation of 20% fraction from first stage) and 30% F8. Fractions enriched in *p*-coumaric acid, or partially enriched in isoferulic acid and ferulic acid (less active fractions from 30% F8) had low anti-proliferative activity. However, the concentration of ferulic and isoferulic acid in these fractions was likely to be too low to give rise to activity, as ferulic acid along with caffeic acid has previously been demonstrated to have *in vivo* anti-clonogenic colon cancer activity [35]. The pure caffeic acid standard was also found to be highly anti-proliferative, at > 95% inhibition. Inhibition above 80% in the MTT bioassay can be considered cytotoxic. In contrast, caffeic acid was found to have low *in vitro* anti-proliferative activity in a previous study using the murine colon cancer cell line 26-L5 [15].

CAPE was found to be the main compound present in fraction 60% F8. The pure standard, at 76% inhibition, was also found to be highly active. Pinocembrin was the major compound in 60% F8-5, and the very high level of inhibition matched the standard. Chrysin and galangin were the major compounds in fractions 60% F9-2 and 60% F9-5, and had similar levels of activity to the corresponding standards at around 60% inhibition. In the studies reported using 26-L5 cells, pinocembrin had moderate *in vitro* activity, chrysin had both medium [25] and high [15] anti-proliferative activity whilst CAPE and galangin had high activity [25]. Tectochrysin was the major compound in 60% F10, but its activity at 78% was substantially higher than that of the pure standard at 29%. This discrepancy in activity was most likely due to the poor solubility of the tectochrysin standard in ethanol. It had low activity reported against 26-L5 [25]. In stage 3 of this work, the solvent for this compound was changed to triethylene glycol monomethyl ether which resulted in much higher anti-proliferative activity results. Cinnamic acid and pinocembrin-7-methyl ether standards were found to have low levels of activity, and fractions containing these compounds only had low activity. Cinnamic acid was also found to have no activity against 26-L5 [15].

This stage of bioassay guided fractionation identified eight compounds that were further investigated against 4 gastro-intestinal cancer cell lines in the third stage of testing. The compounds were 5-phenylpenta-2,4-dienoic acid, 1,1-dimethylallylcaffeate, 3-methyl-3-butenyl caffeate, pinobanksin-3-O-acetate, pinostrobin chalcone, benzyl ferulate, benzyl isoferulate and tectochrysin. Caffeic acid, CAPE, chrysin and galangin were not investigated further because either they were well known compounds present in many other raw materials, or their observed activity here matched already reported bioactivity.

The third stage of this investigation was to evaluate the relative activity of the identified phenolic compounds against a range of cell lines related to gastrointestinal cancers. Three of the compounds of interest were available commercially (5-phenylpenta-2,4-dienoic acid; 1,1-dimethylallylcaffeate; and tectochrysin), a fourth was from our laboratory collection (pinobanksin-3-O-acetate) while three more were caffeic or ferulic acid esters (3-methyl-3-butenyl caffeate; benzyl ferulate; and benzyl isoferulate) and were prepared synthetically from readily available precursors. Pinostrobin chalcone was purified from a larger sample of propolis resin.

The relative in % anti-proliferative bioactivities of the less well known or studied compounds identified from the bioassay guided fractionation using the DLD-1 cell line were determined for four human gastrointestinal cancer cell lines by MTT assay. These were DLD-1 colon adenocarcinoma, HCT-116 colon carcinoma, KYSE-30 oesophageal squamous cancer, and NCI-N87 gastric carcinoma. The eight compounds identified by bioassay-guided fractionation were tested against all four cell lines, along with pinocembrin and *p*-coumaric acid. Pinocembrin was included as a positive propolis control shown to be highly active in the first two stages and present in New Zealand propolis in high concentrations, but also because its anti-proliferative activity had not been widely reported [23]. *p*-Coumaric acid was included as a negative propolis control expected to have low activity. *p*-Coumaric acid is present in New Zealand propolis in low levels but in high levels in Eastern European propolis. 5-Fluorouracil (5-FU), a known anticancer pharmaceutical was used as the positive control and cells only as the negative control. All compounds except 5-FU were tested at 200 µg/ml. Pinocembrin, 1,1-dimethylallylcaffeate, 3-methyl-3-butenyl caffeate, and benzyl ferulate were also retested against KYSE-30 at 100, 100, 100 and 50 µg/ml respectively.

The results for testing of the compounds at 200 µg/ml (Fig. 3) show clearly that 1,1-dimethylallyl caffeate (DMAC), 3-methyl-3-butenyl caffeate, pinocembrin, benzyl ferulate, benzyl isoferulate and tectochrysin have good to excellent anti-proliferative activity against all human gastrointestinal cancer cell lines tested at greater than 80% inhibition of proliferation; pinobanksin-3-O-acetate, 5-phenyl-penta-2,4-dienoic acid and pinostrobin chalcone are moderately to strongly active, and *p*-coumaric acid was weakly active to almost inactive against all cell lines except against KYSE-30. Pinostrobin chalcone had no activity at all against NCI-N87, but a medium level of anti-proliferative activity against the other cell lines. The most resistant human cancer cell line to all compounds

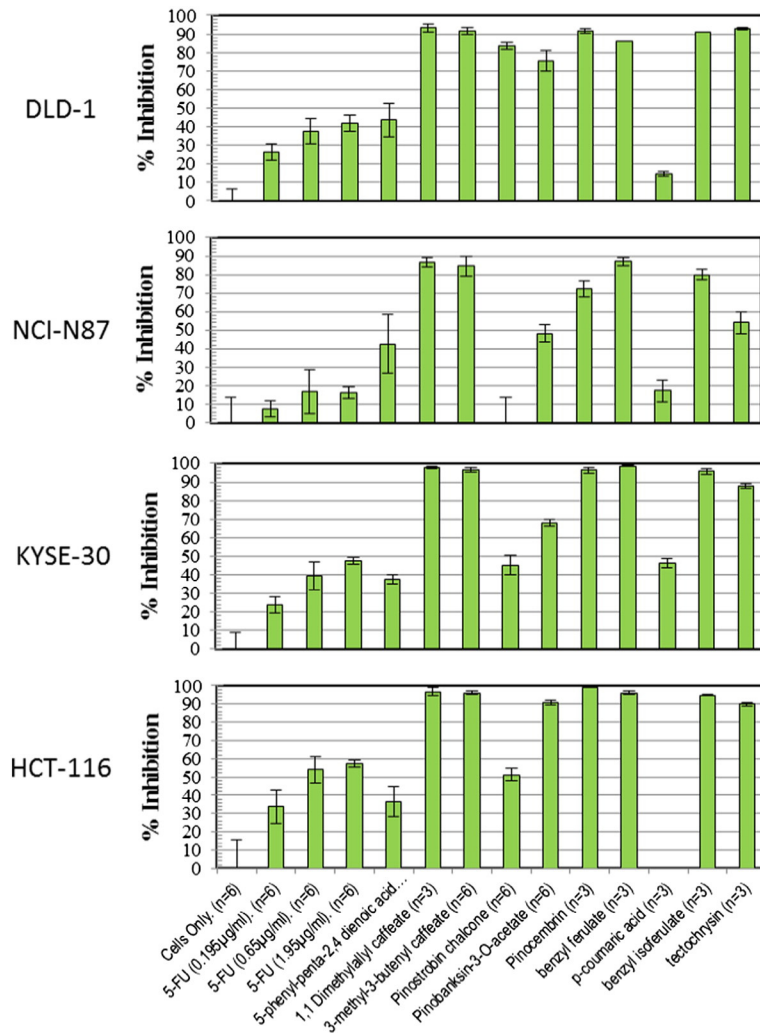


Fig. 3. Anti-proliferative activity against gastrointestinal cancer cell lines (zero = no, or stimulatory activity at a test concentration of 200 µg/ml; greater than 90% = cytotoxic, error bars are ± SD).

including 5-FU was NCI-N87. In general the most consistent activity is seen for the caffeate and ferulate type esters; 1,1-dimethylallyl caffeate, 3-methyl-3-butenyl caffeate, benzyl ferulate, and benzyl isoferulate. These esters show anti-proliferative activity against all four cell lines in the same order of magnitude as pinocembrin. This is consistent with other studies which show CAPE, benzyl caffeate and cinnamyl caffeate [15], to have good activity against a number of cancer cell lines. The activity of the two benzyl ferulate esters is not surprising as the equivalent caffeate ester, benzyl caffeate, also shows strong anti-proliferative activity against murine colon cancer cell line 26-L5 [15]. 1,1-dimethylallyl caffeate is more often cited for its allergenic activity although 3-methyl-3-butenyl caffeate is more allergenic [3]. These anti-proliferative bioactivity findings are consistent with other work demonstrating enhanced cytotoxicity of lipophilic ester derivatives of caffeic and/or ferulic acid [36–39]. Reducing the test concentration against KYSE-30 to 100 µg/ml for 1,1-dimethylallyl caffeate and 3-methyl-3-butenyl caffeate reduced the anti-proliferative activity to 89% and 40% respectively, whereas reducing the concentration of benzyl ferulate to 50 µg/ml only marginally reduced the activity to 88%. This indicates that the 3-methyl-3-butenyl ester is less bioactive than benzyl and dimethylallyl esters. Reducing the test concentration of pinocembrin against KYSE-30 to 100 µg/ml reduced its anti-proliferative activity to 50% inhibition. Pinostrobin chalcone shows moderate activity in contrast to its flavanone equivalent pinocembrin-7-methyl ether which is inactive. Pinostrobin chalcone from *Alpinia mutica* showed *in vitro* cytotoxic activity against the tested human cancer cells, such as

KB, MCF7 and Caski cells [40]. Tectochrysin showed good antiproliferative activity against HCT-116 [41].

3.2. Anti-inflammatory activity

Tumor necrosis factor- α (TNF- α) appears to play a key role in tumor cell proliferation and invasion for certain types of tumors [42]. The amount of TNF- α in the tumor microenvironment and tumor cell growth rate have been shown to be linked [43]. We therefore decided to examine the effect of the propolis fractions on inflammatory mediators and in particular TNF- α . The samples from the first stage fractionation were subjected to a rat blood neutrophil assay for inhibition of LPS induced inflammatory response factors at test concentrations of 50 and 200 µg/ml. All the samples showed inhibition of TNF- α production with most showing very high activity, many up to 100% inhibition (Table 2). In contrast, no inhibitory activity was observed for either TXB₂ or PGE₂ as proxies for inhibition of COX-1 and COX-2 enzymes respectively: in fact, all samples except the positive control indomethacin were stimulatory for TXB₂ and similarly all fractions except fraction 4 and indomethacin were stimulatory for PGE₂. The observed suppression of TNF- α production in this non-tumor cell model is a possible indicator of one of the modes of action giving rise to the anti-proliferative action of propolis fractions. However, in terms of general anti-inflammatory activity whilst strong activity was observed for the one of the first cytokines in the inflammatory cascade, TNF- α , it either

Table 2Effect of preparations on the inhibition of production of TNF- α by activated neutrophil cells.

Sample	% Inhibition (\pm SD)				
	200 μ g/ml	50 μ g/ml	10 μ g/ml	3 μ g/ml	1 μ g/ml
Propolis tincture	100	100	28.4 \pm 6	30 \pm 16	*
Propolis fraction #1	100	61.5 \pm 3	*	*	
Propolis fraction #2	98.4 \pm 0.2	100	*	6 \pm 5	
Propolis fraction #3	100	97.2 \pm 0.3	31.6 \pm 2	*	
Propolis fraction #4	100	100	40 \pm 12	3.9 \pm 13	
Propolis fraction #5	100	100	*	14.6 \pm 18	
Propolis fraction #6	100	100	*	*	
Propolis fraction #7	100	100	*	10.7 \pm 5	
Propolis fraction #8	100	100	*	*	
Caffeic acid phenyl ester (CAPE)			96.43 \pm 0.3		*
Pinocembrin			18.37 \pm 4		*
Caffeic acid			*		*

* Stimulation.

does not extend downwards through an arachidonic (lipid mediated) pathway; or that the almost complete suppression of TNF- α production results in stimulation of production of TXB₂ and PGE₂.

Selected fractions and pure compound standards were retested at 1, 3 and 10 μ g/ml (Table 2) for inhibition of TNF- α production. No inhibitory activity was observed for any samples or standards at 1 μ g/ml, which suggests that this concentration is too low. At 10 μ g/ml, CAPE showed strong activity and pinocembrin moderate activity. Propolis tincture and fractions 3 and 4 also showed moderate activity. The IC₅₀ for most compounds and fractions is therefore somewhere between 10 and 50 μ g/ml. Many samples showed anomalous slight stimulatory activity at 3 μ g/ml and 1 μ g/ml, in strong contrast to the results from the first stage trials. No further anti-inflammatory assays were performed. Other work on inhibition of NO by a range of compounds isolated from Netherlands propolis supports the data in the present study [29]. Similarly the lack of effect on COX enzymes is also confirmed by other studies [9,38,36]. Neutrophils are an important constituent of the human immune system. Propolis, propolis fractions and pure compounds did not appear to affect the viability of the activated neutrophils, and thus have the potential for modulation of the immune system.

These results give a strong first indication of the beneficial gastrointestinal health potential of New Zealand propolis resin as a chemopreventative agent. The anti-proliferative activity of New Zealand “poplar” type propolis against selected human gastrointestinal cancer cell lines can be ascribed to more than a dozen compounds. Apart from the well-known phenolic bioactives such as chrysin and CAPE this work has identified a number of flavonoids and caffeic type esters with strong activity. The major contribution to the total anti-proliferative activity of the propolis tincture can be attributed to the main active dihydroflavonoid compounds, pinocembrin and pinobanksin-3-O-acetate, as these compounds are the most dominant phenolic compounds in New Zealand propolis. Chrysin and galangin also contribute to the anti-proliferative activity, but are less active than the above dihydroflavonoids. Caffeic acid and its esters also collectively contribute to the activity of propolis. 1,1-dimethylallyl caffeate, benzyl caffeate and 3-methyl-3-butenyl caffeate all have strong activity and are present in New Zealand propolis in higher concentration than the known bioactive compound CAPE. The fractions from propolis also showed generally high levels of inhibition of the production of TNF- α , a cytokine which appears to play a key role in tumor cell proliferation and invasion for certain types of tumors.

Knowledge of the phenolic compounds responsible for the activity will assist in the development of new propolis resin-based product formulations enriched in bioactive compounds. Further work is planned to compare the New Zealand propolis with other poplar type propolis from Northern Hemisphere sources.

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Appendix A. Supplementary data

The supporting information provides more details on assay methods and tables of results for assays. Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fitote.2015.09.004>.

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