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# Anti-gastrointestinal cancer activity of cyclodextrin-encapsulated propolis

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ABSTRACT

New Zealand propolis resin and tinctures are rich in compounds that have *in vitro* anti-gastrointestinal cancer activity. However, propolis tincture has restricted acceptability for human consumption due to pungency in taste and aroma. We have formulated New Zealand propolis into cyclodextrin complexes (CD) that resolve these acceptability issues, and carried out *in vitro* bioactivity testing of these complexes using human anti-gastrointestinal cancer, anti-inflammatory and anti-oxidant assays. Demonstration of encapsulation was performed by physical, enzymic and chemical measurements. The New Zealand propolis γCD, αCD, and βCD complexes inhibited the proliferation of 4 human gastro-intestinal cancer cell lines, with the extent of inhibition increasing with increasing exposure time. The complexes were also strongly anti-inflammatory *in vitro* with respect to the cytokine TNF-α, and showed strong lipid anti-oxidant activity. The bioassay results give a strong first indication of beneficial gastro-intestinal health potential of New Zealand propolis – cyclodextrin complexes.

## 1. Introduction

Propolis is a heterogenous material consisting of resin collected by honey bees from the leaf, buds and bark of certain tree species, which then undergoes enzymatic digestion by bees' salivary enzymes and admixture with beeswax produced from the wax glands of the worker bees. While the wax composition appears to depend only upon the bee (Aichholz & Lorbeer, 1999; Vyssotski, Lagutin, & Catchpole, 2017), the resin composition depends upon the proximity of tree species with exudates that can be collected by the bee. New Zealand propolis is classified as 'European' type, which is collected mainly from poplar species (Catchpole, Mitchell, Bloor, Davis, & Suddes, 2015). The composition of propolis collected in tropical regions is much more diverse. The most well-known type is Brazilian green propolis which contains resin collected from the *Baccharis dracunculifolia* tree (De Oliveira et al., 2014). Propolis is used by bees to defend the hive against invaders, and to reduce air flow into the hive to retain heat. Propolis has been used as a folk remedy since antiquity for its beneficial health properties, which are attributable to the compounds found in the resin. In poplar-type propolis, these compounds are aglycone flavonoids and hydroxycinnamic acids and esters, with the most actively researched being caffeic acid phenethyl ester (CAPE) and chrysin (Sawicka, Car, Borawska, & Nikliński, 2012; Watanabe, Amarante, Conti, & Sforcin, 2011). In Brazilian green propolis, the active compounds are mainly prenylated coumaric acid derivatives, particularly artepillin-c (De Oliveira et al., 2014). Poplar-type propolis is almost completely insoluble in water, has a very strong taste which is unpleasant for many consumers, and a very strong aroma. Typical propolis products include ethanol/water tinctures, liquids (propolis dispersed into propylene glycol), tablets, capsules, toothpastes, throat sprays, syrups for oral health and immune regulation. The tinctures and liquids have a very strong taste and aroma that are unpleasant for many consumers. In addition, the use of ethanol or propylene glycol restricts its acceptability in many countries. There is a need for a new way to formulate propolis into powder that minimises the taste and smell which additionally provides a controlled release of the bioactives to the desired site in the body.

Cyclodextrins are cyclic oligomers of glucose that are commercially produced by enzymatic reaction from starch (Das et al., 2013; Stella & He, 2008; Szente & Szejtli, 2004; Uekaji, Jo, & Terao, 2013). Naturally synthesized cyclodextrins are alpha, beta and gamma cyclodextrin (αCD, βCD, γCD), consisting of 6, 7 and 8 glucose monomers respectively. These cyclodextrins are approved for food use. The cyclodextrins have a toroidal, cone-like shape, with a hydrophobic interior and hydrophilic exterior. This shape encourages molecular encapsulation of hydrophobic compounds into the interior, with water solubility/dispersibility conferred by the hydrophilic exterior. The water solubility of αCD, βCD, and γCD at 25 °C is 14.5, 1.85 and 23.2 g/100 ml of water respectively (Das et al., 2013).

The encapsulation of propolis, and compounds found in propolis

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using cyclodextrins has been previously reported (Coneac et al., 2008; Kalogeropoulos et al., 2009; Nafady et al., 2003; Rocha et al., 2012; Szente & Szejtli, 1987). The formation of propolis-βCD complexes was first reported in 1987 (Szente & Szejtli, 1987). The separation of poorly water-soluble components including artepillin-C from Brazilian green propolis using βCD/water mixture followed by filtration, evaporation of the supernatant and then ethanol extraction of the dry complex was then reported (Nafady et al., 2003). The yield of isolated compounds was very low. The extraction of 3 types of Romanian propolis using 3 different ethanol/water concentrations to make tinctures, followed by βCD complexation was then reported (Coneac et al., 2008). Encapsulation of Greek propolis in βCD was also carried out (Kalogeropoulos et al., 2009) using a modification to the method of Nafady et al. (2003). The Greek propolis used was very rich in abietic acid, a pine resin acid. A hydroxypropyl-βCD – Brazilian green propolis complex was recently made and thermally characterized (Rocha et al., 2012). CycloChem Bio (Japan) has shown that γCD is superior to both α- and βCD for dispersibility of the complex in water, reduction of pungency and retention of key propolis bioactives (Anon, 2011a). They also report an improvement in CAPE stability through prevention of hydrolysis to caffeic acid and phenethyl alcohol in water by the formation of an inclusion complex with γCD (Anon, 2011b).

There are very few reports on the bioactivity of propolis or compounds present in propolis that are encapsulated in cyclodextrins. Chinese propolis was encapsulated in βCD and then tested for anti-diabetes activity by measuring blood glycaemic control, lipid metabolism and insulin resistance in type 2 diabetes mellitus rats (Li, Chen, Xuan, & Hu, 2012). They found good control of these parameters when encapsulated propolis was incorporated into the feed pellets and fed to the rats. CycloChem Bio demonstrated that orally administered CAPE encapsulated in γCD was more effective in retarding the growth of fibrosarcoma cancers induced in rats that CAPE alone (Anon, 2011c). Manuka Health has begun development of a new product consisting of NZ propolis encapsulated with γCD (herein referred to as Propolis-γCD complex). The *in vitro* anti-proliferative activity of Propolis-γCD complex alone (Drago, Bordonaro, Lee, Atamna, & Lazarova, 2013) and with a number of other compounds (Bordonaro, Drago, Atamna, & Lazarova, 2014) was determined against the colon cancer cell lines HCT116 and HCT116-R (resistant to butyrate). The compounds and mixtures selected blocked one or more of the pathways to developing resistance. Propolis-γCD complex was found to be effective alone at inducing apoptosis, but efficacy improved in the presence of butyrate (Drago et al., 2013) and butyrate and roasted coffee extract (Bordonaro et al., 2014). Intestinal microflora produce short chain fatty acids such as butyric acid from the anaerobic fermentation of prebiotic soluble fibre (Cummings, Macfarlane, & Englyst, 2001). αCD and βCD (Szente & Szejtli, 2004) and some γCD complexes (Uekaji et al., 2013) are known to reach the colon before degradation and digestion by fermentation. Propolis- γCD complex was compared to γCD alone for ameliorating the growth rate or preventing pre-cancerous colon lesions in a C57BL/6J mouse model using a Western (high fat) diet or normal diet. Propolis-γCD complex was more effective in minimising the lesion size and degree of dysplasia in the control diet, and preventing obesity in the Western diet (Cho et al., 2016). In this work, we further investigate the anti-gastrointestinal cancer activity of NZ propolis encapsulated by cyclodextrin, including the Propolis-γCD complex.

## 2. Materials and methods

### 2.1. Materials

Premium grade wax free propolis tinctures containing either 25 or 40% dissolved solids, were supplied by Manuka Health. The tinctures were commercially manufactured from crude propolis selected on the basis of having at least 300 mg/g on a wax-free basis of the compounds pinocembrin, pinobanksin, pinobanksin-3-O-acetate, galangin, chrysin, tectochrysin, caffeic acid, 1,1-dimethylallyl caffeate, 3-methyl-3-butenyl caffeate, benzyl caffeate, cinnamyl caffeate, cinnamic acid, *p*-coumaric acid, and CAPE. The tinctures were produced for Manuka Health by ethanol/water extraction of at least 500 kg of crude high grade propolis as previously reported (Catchpole et al., 2015). The extraction process results in a wax-free propolis resin dissolved in an ethanol/water solvent mixture, which is standardised to a 40% propolis resin solids concentration. 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 Brazilian green Alecrim (*Baccharis dracunculifolia*) propolis used was obtained commercially from Polenectar (Brazil) as a 40% solution in propylene glycol, which is the standard solvent for this type of propolis. αCD and γCD were supplied by Wacker AG (Germany) as CAVAMAX W6 Food and CAVAMAX W8 Food respectively. βCD was supplied by Sigma-Aldrich (Missouri, USA). CAPE and trans β-carotene and pinocembrin were supplied by Sigma-Aldrich (Missouri, USA). Linoleic acid was purchased from Acros Organics (New Jersey, USA). Artepillin-C was from Hayashibara Biochemical Laboratory (Okayama, Japan). Human leucocyte elastase and peptide substrate were supplied by Elastin Products Company, Inc. (Missouri, USA). Chymotrypsin was supplied by Worthington Biochemical corporation (Lakeview, New Jersey, USA). Butylated hydroxy toluene (BHT) was from MP Biomedicals (Santa Ana, California, USA).

### 2.2. Formation of propolis-cyclodextrin complexes

New Zealand propolis–γCD complexes CD1 and CD2 were manufactured by CycloChem Bio (Japan) by mixing a propolis tincture containing 25% propolis solids with dry γCD powder until a homogenous sloppy paste was obtained. Water was then mixed in with the paste to make a dispersion that was then spray dried. For CD2, additional CAPE was added to the propolis tincture to approximately double the concentration of CAPE. The complexes contained 27% by mass propolis solids as determined by comparison of the concentration of key flavonoid bioactives in the complex with that in the parent propolis tincture. The production method was then modified to ensure both maximum retention of the bioactivity of potentially thermally labile compounds, and to give a process that could be industrially performed in New Zealand by replacing spray drying with freeze-drying. New Zealand propolis–γCD complex CD3 was made by mixing a propolis tincture containing 40% propolis solids with γCD until a paste was obtained and then water was added with vigorous stirring. The resultant suspension was then freeze-dried. New Zealand propolis –αCD and –βCD complexes CD4 and CD5 were made at a laboratory scale by mixing the same 40% propolis tincture with dry αCD powder (CD4) or dry βCD powder (CD5) in a mortar and pestle until a homogenous paste or powder was obtained. Water was then admixed with the paste or powder in a number of steps until well mixed, and then the mixtures were added to glass round-bottom flasks. Pure compound complexes were made in a similar fashion, but with the pure compound dissolved in ethanol or ethanol/water mixtures. The flasks were rotated in an acetone/dry ice bath until frozen, and then freeze-dried. Brazilian green propolis – γCD complex CD6 was made by first dissolving γCD powder into water. A 40% solution of Brazilian green propolis in propylene glycol was then added dropwise to the γCD solution with vigorous stirring until the desired ratio had been added. The final dispersion was frozen and freeze-dried as per complexes CD4 and CD5.

### 2.3. Analytical methods

Cyclodextrin propolis complexes CD1-CD6 were analysed by HPLC by accurately weighing approximately 0.8 g of material into a 25 ml volumetric flask, dissolving in approximately 20 ml dimethylsulphoxide (DMSO) by ultra-sonication then diluting to 25.0 ml with DMSO. Resultant solutions were filtered through a 0.2 μm regenerated cellulose