Journal of Apicultural Research

ISSN: 0021-8839 (Print) 2078-6913 (Online) Journal homepage: http://www.tandfonline.com/loi/tjar20

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To cite this article: Yahya Al Naggar, Jianxian Sun, Albert Robertson, John P. Giesy & Steve Wiseman (2016) Chemical characterization and antioxidant properties of Canadian propolis, Journal of Apicultural Research, 55:4, 305-314, DOI: 10.1080/00218839.2016.1233700

To link to this article: http://dx.doi.org/10.1080/00218839.2016.1233700

Journal of Apicultural Research, 2016
Vol. 55, No. 4, 305–314, http://dx.doi.org/10.1080/00218839.2016.1233700

ORIGINAL RESEARCH ARTICLE

Chemical characterization and antioxidant properties of Canadian propolis

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(*Received 23 May 2016; accepted 25 August 2016*)

Propolis is a multifunctional material collected and used by honey bees in the construction and maintenance of their hives. It has been used in folk medicine for centuries. Concentrations of major constituents and antioxidant characteristics of ethanolic extracts of three samples of propolis (EEPs) collected from different geographical locations in Canada (Saskatchewan, Ontario and British Columbia) were determined. Twenty-one compounds were identified in each EEP, of which 18 were polyphenols. Semi-quantitative measurements showed that benzyl caffeate, pinocembrin, sakuranetin and pinobanksin-3-acetate were most abundant in propolis from Ontario. Total phenolic content of EEPs were quantified by using the Folin–Ciocalteu reagent which ranged between 410.81 and 429.61 mg GAE/g EEP. Free radical scavenging activities of propolis were confirmed by use of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and by using Nrf2 Luciferase reporter cell lines. The three EEPs exhibited strong scavenging of free radicals, and protective activity against oxidative stress caused by exposure to H_2O_2 in this *in vitro* system. These results support the use of propolis from these regions of Canada as a source of natural antioxidants.

Caracterización química y propiedades antioxidantes del propóleos canadiense

El propóleos es un material multifuncional recogido y utilizado por las abejas melíferas en la construcción y el mantenimiento de sus colmenas. Se ha utilizado en la medicina popular durante siglos. Se determinaron las concentraciones de los constituyentes principales y las características antioxidantes de extractos etanólicos de tres muestras de propóleos (EEP) recogidos en diferentes localizaciones geográficas de Canadá (Saskatchewan, Ontario y Columbia Británica). Veintiún compuestos fueron identificados en cada EEP, de los cuales 18 eran polifenoles. Las mediciones semicuantitativas mostraron que el cafeato bencilo, la pinocembrina, sakuranetina y pinobanksina-3-acetato fueron más abundantes en propóleos de Ontario. Se cuantificó el contenido de fenoles totales (CFT) de los EEPs utilizando el reactivo de Folin-Ciocalteu, el cual osciló entre 410.81 y 429.61 mg GAE/ g EEP. Las actividades de captación de radicales libres de los propóleos se confirmaron mediante el uso del ensayo de 2,2-difenil-1-picrilhidrazil (DPPH) y mediante el uso de líneas celulares indicadoras de luciferasa Nrf2. Los tres EEPs exhibieron una fuerte actividad de barrido de radicales libres, y actividad protectora contra el estrés oxidativo causado por la exposición a H2O2 en este sistema in vitro. Estos resultados apoyan el uso de propóleos de estas regiones de Canadá como una fuente de antioxidantes naturales.

Keywords: natural antioxidants; bees; total polyphenol; Nrf2 luciferase reporters; free radicals

Introduction

Propolis or bee glue, a resinous hive product collected by honey bees from parts of plants, buds, and exudates is used to seal holes in honey combs, smooth internal walls and protect the hive entrance against intruders (Burdock, 1998). Various biological activities, such as anticancer, anti-oxidant, anti-inflammatory, antibiotic and antifungal effects have been reported for propolis and its constituents (Kosalec et al., 2007; Marcucci, 1995; Oršolić, 2010). The medical applications of propolis extract have led to an increased interest in its chemical composition, their botanical origins, and their medicinal properties. Polyphenolic compounds have been identified in propolis collected by *Apis mellifera*. Flavonoids, the main polyphenols in propolis, have been found to be quantitatively or qualitatively variable, depending on species of plants in the vicinity of the hive (Marcucci, 1995; Park, Alencar, & Aguiar, 2002; Park, Paredes-Guzman, Aguiar, Alencar, & Fujiwara, 2004).

Very little is known about the composition of chemicals and biological properties of propolis form Canada. Propolis from Sydenham, Ontario (Canada), has been analyzed and was found to originate from the bud exudates of poplars from Section *Aigeiros*: *Populus deltoides* Marsh., *Populus fremontii* Wats. or *Populus maximoviczi* Henry (Garcia-Viguera, Ferreres, & Tomas-Barberan, 1993). Chemical compositions and radical scavenging capacities of propolis from two climatic and vegetation regions of Canada - the Boreal forest located northeast of Montreal, and the Pacific coastal forest of British

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Columbia, where poplars of Section *Aigeiros* are not present have been studied (Christov, Trusheva, Popova, Bankova, & Bertrand, 2006) and it was demonstrated that honey bees of northern regions of North America were, in the absence of their most preferred source *P. nigra* L., able to find alternative suitable plant sources of propolis. Therefore, propolis collected from different geographical regions of Canada was considered to be a promising source of biologically active substances deserving of further investigation.

Free radicals and reactive oxygen species (ROS) are generated by normal cellular processes. Antioxidant defense mechanisms protect against adverse effects of free radicals and ROS and this defense mechanism can be upregulated when levels of these chemicals increase. Oxidative stress is a state of redox imbalance that occurs when concentrations of radicals and ROS are greater than can be dealt with antioxidant defense mechanisms (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). A variety of environmental factors, including metals and organic chemicals, and UV irradiation can cause oxidative stress. By reacting with DNA, carbohydrates, proteins, and lipids, ROS can cause cellular and tissue injury and might result in cell death. Oxidative stress can impair immune functions leading to the development and progression of oxidative DNA damage and might result in formation of tumors (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). Oxidative/nitro-oxidative stress is linked to several pathogenesis of cardiovascular dysfunction, including hypertension, cerebrovascular accidents, and heart failure (Aviram, 2000); reproductive dysfunction (Santos et al., 2006); cancer (Asaduzzaman Khan, Tania, Zhang, & Chen, 2010); and neurodegenerative diseases (Wood-Kaczmar, Gandhi, & Wood, 2006).

Epidemiological and clinical studies suggest that natural products can combat oxidative stress and reduce the morbidity and mortality associated with chronic diseases. Many natural compounds such as flavonoids are potential antioxidants that protect against ROS or reactive nitrogen species (RNS) induced damage and ameliorate oxidative stress-related diseases (Chen, Jia, Pan, & Anandh Babu, 2015). Although various natural products have been shown to possess potential protective effects against chronic diseases that are linked to oxidative stress (Asaduzzaman Khan et al., 2010), bioactivities of a great number of natural compounds remain unknown. Therefore, understanding and validating the bioactivities of natural compounds and the molecular mechanisms involved would provide solid scientific foundation to use the natural compounds for the prevention and treatment of oxidative stress-related diseases (Chen et al., 2015).

The antioxidant capacity of propolis may be related to some of its biological effects, including chemoprevention. The flavonoids in propolis are powerful antioxidants, capable of scavenging free radicals and thereby protecting the cell membrane against lipid peroxidation (Kolankaya, Selmanoğlu, Sorkun, & Salih, 2002). Therefore, the objective of this work was to characterize and compare the chemical composition and anti-oxidant properties of samples of propolis collected from different geographical regions in Canada - Saskatchewan (SK), Ontario (ON) and British Columbia (BC). It was hypothesized that propolis collected from various climatic regions dominated by different communities of plants will differ in their chemical composition and their biological activities.

Materials and methods

Chemicals and reagents

Standards (>98% purity) of caffeic acid, quercetin, *p*-coumaric acid, naringenin and gallaic acid which were some of the compounds identified in extracts of propolis, were purchased from Sigma Aldrich (Oakville, ON, Canada). All solvents (methanol, ethanol, DCM, DMSO and MeCN) used for preparation of samples and chromatographic separation were of HPLC grade and were purchased from Fisher Scientific (Ottawa, ON, Canada). Folin–Ciocalteu's phenol reagent and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma–Aldrich) were used for spectrophotometric determination of total phenolics and free radical scavenging activity, respectively.

Collection and extraction of propolis

Propolis was collected in 2015 from three different geographical regions of Canada; the town of Cawston in the south Similkameen valley of British Columbia (BC), near the city of Saskatoon, Saskatchewan (SK), and from apiaries east of Lake Huron, Ontario (ON). Crude samples of propolis was extracted according to procedures described previously (Kalogeropoulos, Konteles, Troullidou, Mourtzinos, & Karathanos, 2009). Briefly 10 g of pulverized, crude propolis were extracted for 3 days by stirring with a 10-fold volume of 70% ethanol in tightly closed bottles at ambient temperature in the dark. Next, to remove waxes and less soluble substances, suspensions were frozen at −20 °C for 24 h, then filtered through Whatman No. 1 paper. The freezing-filtration cycle was repeated three times. Final filtrates representing balsam tincture of propolis are referred to as EEP (ethanolic extract of propolis). Solutions were evaporated to near dryness on a rotary evaporator under reduced pressure at 40 °C, freeze-dried, and resulting powders were dissolved in 80:20 ethanol: water in order to get 5% w/v stock solutions of EEP. Clean up of EEPs was carried out by use of solid-phase extraction cartridges according to a method described previously (Petelinc, Polak, Demšar, & Jamnik, 2013).

Qualitative and semi-quantitative analyses of EEPs by liquid chromatography–mass spectrometry

The EEPs were dissolved to a concentration of 10 mg ml^{-1} in DMSO: water (2:1 v/v). Next, samples were diluted 1:200 (v/v) with acetonitrile: Water (1:4 v/v) prior to analysis by use of high performance liquid chromatography–electrospray mass spectrometry (HPLC–ESI/MS). Qualitative and semi-quantitative analyses were done with an LTQ Orbitrap XL mass spectrometer coupled to a 1,200 series capillary LC pump and auto-sampler (Agilent Technologies, Santa Clara, CA, USA). The ion source was adsorption electrospray ionization Omni Spray (Prosolia, Indianapolis, IN, USA), which was operated in nanoelectrospray mode for negative and positive ions. The HPLC separation was achieved with a Zorbax SB-C18 column (100 × 0.5 mm; 5 μm particle size), using an elution mixture composed of solvent A (0.05% acetic acid in water) and solvent B (acetonitrile). Injection volume was 1 μl and the flow rate was 10 μl min. The elution gradient was from 20 to 100% of solvent B in 40 min; hold at 100% for 6 min and re-equilibration for 8 min at 20% of solvent B. Samples were directly injected into the HPLC column, which was directly coupled to the ion source spray capillary by a liquid junction (Sulaiman et al., 2011).

Identification of compounds

A clear separation of chromatographic peaks of substances was achieved by combining retention times and high resolution XIC of the corresponding deprotonated molecular ions. Each peak was characterized by a measured accurate mass (with an error equal or less than 5 ppm) and by its unique acquired MS/MS spectrum. For other peaks, measured accurate masses and the most probable chemical formula were compared with reference compounds available online at different chemical database websites (www.chemspider.com, www.chemfinder.com and pubchem.ncbi.nlm.nih.gov) or information that corresponded to the observed mass spectra. Some components remained unidentified because of the lack of library spectra of corresponding compounds.

Determination of total polyphenol content

Crude extracts of propolis were redissolved in 95% ethanol at a concentration of 50 mg/ml. Ethanolic extracts (0.1 ml) were diluted with 95% ethanol (0.9 ml) and mixed with 5 ml of 10-fold diluted solution of 2 N Folin–Ciocalteu reagent (Sigma-Aldrich). Four milliliters of saturated sodium carbonate solution were added to the mixtures and then shaken. Reaction mixtures were allowed to stand for 2 h at room temperature in the dark and then absorbance's of reaction mixtures were measured at 765 nm (Spanos & Wrolstad, 1990). Total phenolic content (TPC) was quantified and reported as Caffeic acid equivalents (CAF) in mg of Caffeic acid per gram of sample by comparing with a calibration curve constructed for different concentrations of a standard of Caffeic acid. For standard curves of TPC, 1 ml aliquots of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml Caffeic acid solution in methanol were mixed with 5 mL of Folin-Ciocalteu reagent (diluted 10-fold) and 4 ml of sodium carbonate (Miliauskas, Venskutonis, & van Beek, 2004).

Scavenging of free radicals

Several methods have been proposed to determine capacities of antioxidants to scavenge free radicals (Aksoy, Kolay, Ağılönü, Aslan, & Kargıoğlu, 2013). In the current study, capacities of extracts of propolis to scavenge free radicals were measured by use of DPPH (Kumar, KK, Dang, & Husain, 2008). The DPPH method that was used in this study is fast, easy and reliable and does not require specialized equipment. DPPH is a stable, synthetic radical that does not disintegrate in water, methanol, or ethanol. Due to its unpaired electron, the radical of DPPH has a deep violet color, but as radicals are quenched a pale yellow non-radical form is produced. Capacity to scavenge free radicals can be followed spectrophotometrically by decreases in absorbance at 517 nm. For this assay, various concentrations of EEPs (25, 50 and 100 μg mL^{-1}) were diluted in 80% ethanol (1.5 ml) before being added to 0.5 ml of 60 μM DPPH solution in 96% ethanol. After 30 min in the dark, absorbances were measured at 517 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) with ethanol as a blank. Quercetin and caffeic acid at concentrations of 25, 50 or 100 μg ml^{-1} were used as positive controls. Absorbance of the control DPPH radical without sample was measured. As a measure of capacities of extracts of propolis to scavenge free radicals, decreases in absorbance, expressed as a percent of the original absorbances of samples, were calculated (Equation 1):

$$\%\ \text{Decrease in color} = (1 - \text{Abs} - \text{sample}/\text{Abs} - \text{control}) \times 100 \quad (1)$$

Nrf2 transactivation bioassay

A stable transactivation cell line containing luciferase as a reporter gene under control of Nuclear factor erythroid 2-related factor 2 (Nrf2) (Signosis, Santa Clara, CA, USA) was used to assess whether EEPs can activate a cellular response to oxidative stress and whether EEPs can attenuate oxidative stress caused by hydrogen peroxide (H_2O_2). Nrf2 is a transcription factor that is activated by cellular stresses, such as oxidative stimuli (Chan, Lu, Chang, & Kan, 1996; Dinkova-Kostova et al., 2002). When activated, Nrf2 translocates into the nucleus and binds to promoters of genes containing the antioxidant response element (ARE), regulating their

transcription (Itoh et al., 1997; Kensler, Wakabayashi, & Biswal, 2007; Venugopal & Jaiswal, 1996). Thus, activation of the Nrf2-mediated pathway is increasingly proposed as a way to prevent or treat diseases that involve oxidative stress, including cancer (Kwak & Kensler, 2010), cardiovascular (Mann, Bonacasa, Ishii, & Siow, 2009), obesity (Pi et al., 2010) and by inhibiting oxidative mechanisms that lead to neurodegenerative disease (Calkins et al., 2009).

Nrf2 Luciferase reporter cells were maintained in Dulbecco's Modified Medium (DMEM) (Sigma-Aldrich) supplemented with 10% defined fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 75 mg/L G418 (Life Technologies). Cells were incubated at 37 °C in a 5% humidified CO_2 incubator. Cytotoxicity was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetr azolium bromide (MTT) assay (Biotium, Hayward, CA, USA). Briefly, cells were plated and seeded to 96-well white, flat bottom microplates with 5×10^4 cells in 100 µl of medium per well. After incubation for 24 h, EEP were serially diluted and spiked to exposure media (final ethanol 0.1%, v/v). Solvent control wells were spiked with 0.1% ethanol. After 16 h of exposure to EEP at 37 °C in a 5% humidified CO_2 incubator, 10 µl of MTT was added per well and plates were incubated for 4 h at 37 °C. Next, 200 µl of DMSO was added to each well for 30 min to dissolve crystals of formazan. OD_{570} of the supernatant was measured and corrected for background absorbance at 690 nm by use of a POLARStar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany). If viability of cells was < 80% of the solvent control, the corresponding exposure dose was considered to be cytotoxic and that concentration of propolis was not tested in the Nrf2 assay.

To quantify activation of Nrf2 signaling, cells were prepared in assay medium (DMEM high glucose containing 0.1% FBS), and seeded to 96-well white, flat bottom microplates with 5×10^4 in 100 µl per well. After incubation for 24 h, EEP were serially diluted and spiked to exposure media (final concentration of ethanol was 0.1%, v/v). Solvent control wells were spiked with 0.1% ethanol. All exposures were conducted with four replicates. Cells exposed to H_2O_2were used as a positive control, and cells were co-exposed to 1 mM H_2O_2 and different concentrations of EEPs to text the anti-oxidant potential. The amount of luciferase produced is directed proportional to the activation of the Nrf2 signaling pathway and thus also to the response to oxidative stress. After 16 h of exposure, production of light by luminescence of luciferase was detected by use of the Steadylite Plus Kit according to protocols provided by the manufacturer (PerkinElmer, MA, USA).

Data analyses

Data analyses were performed with MINITAB Software (Minitab, 2013). Normality of data was assessed by use of the Kolomogrov–Smirnov test, and homogeneity of variance was determined with a Levene's test. If necessary to meet assumptions of parametric tests, data were $\log_{10}$ transformed to ensure normality and homogeneity of variance. Activation of Nrf2 reporter genes of luciferase reporter cells in response to the three EEPs was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test. An alpha level of 0.05 was used for all tests.

Results

Chemical composition of EEP

There were differences in chemical composition of each EEP analyzed by use of HPLC–ESI/MS (Table 1; Figure 1). The sum of all peak areas (total peak area, TPA) of the MS total ion current (TIC) varied among the three samples. The greatest TPA was observed in EEP collected from ON (8.77×10^6), followed by EEP collected from BC (8.17×10^6) and from SK (6.93×10^6). Eleven phenolic acids and esters were identified in each sample of EEP, and generally, amounts were greater in EEP from SK, but differences among samples were very small. However, abundance of benzyl caffeate was 4.55-fold greater in the EEP from ON compared to BC or SK. Seven flavonoids were detected in EEPs and abundances were greater in EEP from ON compared to SK and BC. However, hesperetin was identified only in EEP from BC with TIC of 4.7. The most abundant flavonoid in each sample was pinocembrin, and TIC was 32.24, 21.05 and 19.37 in EEP from ON, SK and BC, respectively. EEPs from each location contained the long-chain aliphatic acids azelaic acid, oleic acid, stearic acid and palmitic acid. Of these four compounds, abundance of azelaic acid was smallest. Abundances of oleic acid, stearic acid and palmitic acid were greater than that of any phenolic compounds and flavonoids that were detected.

Total polyphenolic content

The total polyphenol content (TPC) of samples of propolis from Canada reported here ranged from 410.81 to 429.61 mg GAE/g EEP (Table 2). Caffeic acid was employed as a standard compound for estimation of TPC because it is one of the most abundant phenolic acids in propolis. The magnitude of TPC in EEPs was ranked as ON > SK > BC.

Free radical scavenging activity

Antioxidant capacities of the EEPs from the three regions of Canada were compared with those of quercetin and caffeic acid, which are synthetic antioxidants that were used as positive controls (Table 3). All EEPs were able to scavenge radicals at all concentrations tested (25–100 µg/ml). EEPs from Canada exhibited concentration-dependent scavenging of free radicals.

Table 1. Compounds identified by use of HPLC–ESI/MS in ethanolic extracts of propolis (EEPs) collected from three different geographical regions of Canada.

Compound	Molecular formula	M.M.	$[M\text{-}H]^-$	% of the total ion current		
				BC	SK	ON
Phenolic acids and esters						
Vanillin	C8 H8 O3	152.0469	151.039	2.36	3.04	2.69
Coumaric acid	C9 H8 O3	164.0468	163.0389	3.95	5.47	5.19
Gallaic acid	C7 H6 O5	170.0314	169.0235	2.13	2.54	2.25
Caffeic acid	C9 H8 O4	180.0418	179.0339	4.42	5.65	5.00
Ferulic acid	C10 H10 O4	194.0575	193.0496	4.46	5.99	5.26
Ferulic acid methyl ester	C11 H12 O4	208.0730	207.0651	4.98	6.25	5.63
Isoprenyl coumarate	C14 H16 O3	232.1091	231.1012	2.23	2.62	2.39
Prenyl caffeate	C14 H16 O4	248.1035	247.0956	4.04	4.45	4.62
Isopentyl caffeate	C14 H18 O4	250.1197	249.1118	4.06	4.98	4.25
Isoprenyl ferulate	C15 H18 O4	262.1200	261.1121	4.45	3.81	3.11
Benzyl caffeate	C16 H14 O4	270.0887	269.0808	1.84	1.84	8.38
Flavonoids						
Chrysin	C15 H10 O4	254.0574	253.0495	4.49	2.61	2.89
Pinocembrin	C15 H12 O4	256.0731	255.0652	19.37	21.05	32.24
Naringenin	C15 H12 O5	272.0680	271.0601	13.67	3.36	13.25
Sakuranetin	C16 H14 O5	286.0836	285.0757	17.36	5.00	19.86
Hesperetin	C16 H14 O6	302.0786	301.0707	4.71	0.00	0.00
Pinobanksin-3-acetate	C17 H14 O6	314.0786	313.0707	5.05	7.97	35.33
Pinobanksin-3-propionate	C18 H16 O6	328.0942	327.0863	2.53	4.05	3.65
Aliphatic acids						
Azelaic acid	C9 H16 O4	188.1044	187.0965	6.42	7.98	7.12
Oleic acid	C18 H34 O2	282.2647	281.2568	32.76	40.24	35.83
Stearic acid	C18 H36 O2	284.2760	283.2681	77.59	100.00	82.04
Palmitic acid	C16 H32 O2	256.2398	255.2319	98.24	94.19	100.00
Total peak area (TPA) $\times 10^6$				8.17	6.93	8.77

Notes: $[M\text{-}H]^-$: Pseudo-molecular ion performed in negative ion mode. BC, SK and ON: Propolis from British Columbia, Saskatchewan and Ontario, respectively.

Similarities were observed at equivalent concentrations of EEPs from ON and SK, while that of the EEP from BC exhibited the least capacity to quench free radicals.

Activation of Nrf2

Concentrations of EEPs greater than 25 μg/ml were cytotoxic. Activation of Nrf2 signaling by H_2O_2, which is an oxidizing agent, was partially attenuated in a dose-dependent manner by each sample of EEP but effects were significant only at 25 μg/ml (Figure 2). Relative to cells exposed only to H_2O_2, amounts of luciferase produced in cells co-exposed to 25 μg/ml of EEP from SK, ON and BC was lesser by 2.33, 3.20 and 22.85%, respectively (Figure 2). These results are in agreement with the observation that each sample of EEP scavenges free radicals (Figure 3). In addition to attenuating activation of Nrf2 signaling by H_2O_2, each sample of EEP activated Nrf2 signaling (Figure 3). Activation of Nrf2 signaling in cells exposed to 6.25 and 25 μg/ml of the three EEPs were significantly greater (fold-change ranged from 1.58–2.63 at 6.25 μg/ml of EEP, and from 3.02 to 4.71 for 25 μg/ml of EEP) compared to that of the solvent control (Figure 3).

Discussion

Propolis from the three geographical regions of Canada investigated had different physical properties. Propolis from BC was gummy, waxy and dark brown in color, while that from SK was dark brown in color and solid and that from ON was waxy and dark green in color. These differences are probably a result of differences in vegetation at the site of collection and phenology of plants (Bankova, 2005; Chen et al., 2008).

There were differences in abundances of compounds analyzed in this study compared to propolis from other regions of Canada. TIC % of coumaric acid, ferulic acid, caffeic acid, benzyl caffeate, pinocembrin, sakuranetin, pinobanksin-3-acetate, oleic acid, stearic acid and palmitic acid were greater than those detected in propolis collected from other locations in Canada, including Victoria, BC and Richmond, Quebec (Christov et al., 2006). Additionally, some of the compounds detected in this study (gallaic acid, chrysin, naringenin, hesperetin and pinobanksin-3-propionate) were not detected in extracts of propolis from other locations in Canada including Sydenham, ON (Garcia-Viguera et al., 1993), Victoria, BC and Richmond, Quebec (Christov et al., 2006). However, kaempferol, apigenine, quercetin, methyl

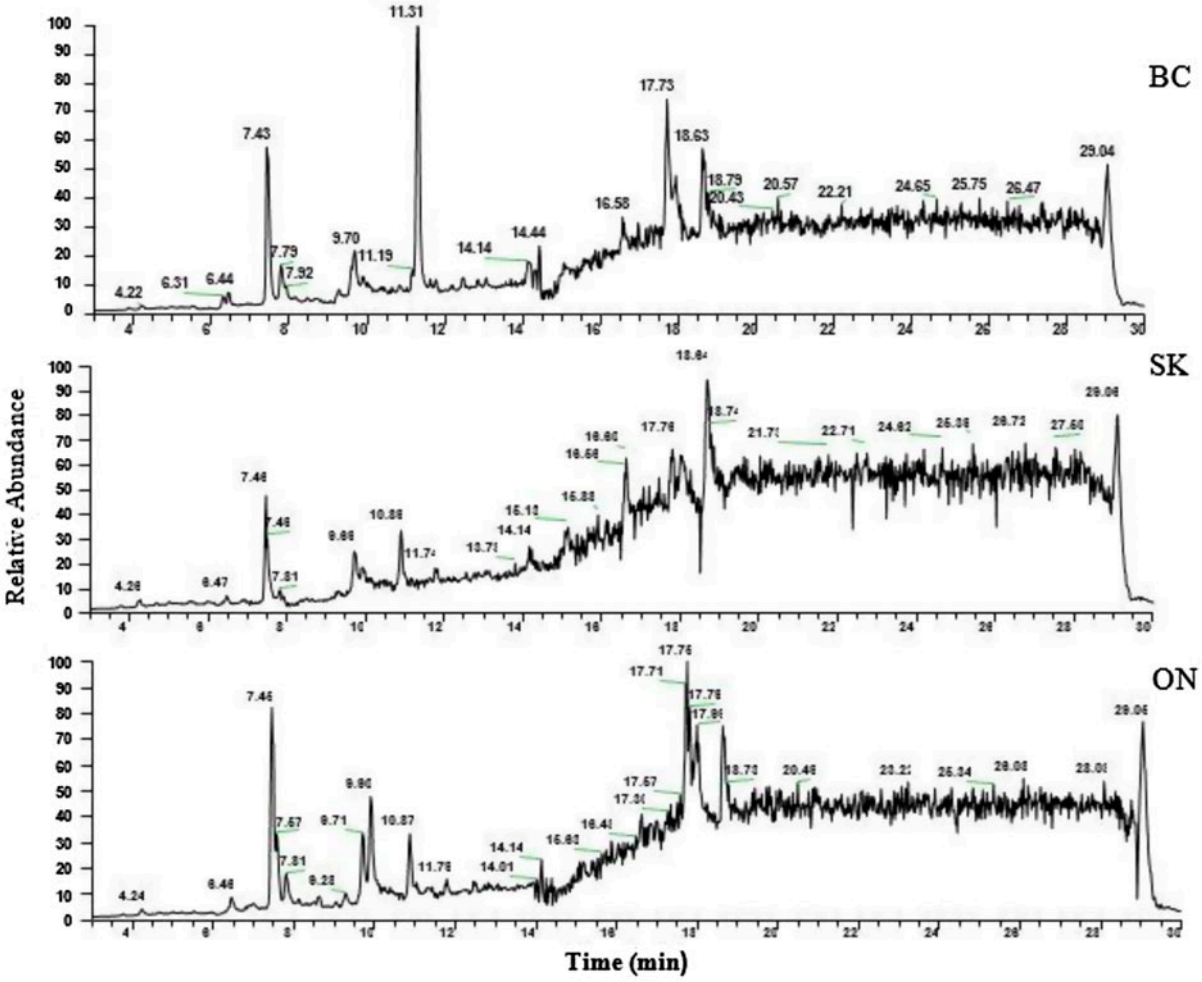

Figure 1. HPLC–MS total ion chromatograms (TICs) of ethanolic extracts of propolis (EEPs) collected from three geographical regions in Canada. TICs were obtained by use of a LTQ-Orbitrap XL instrument operated in negative ion mode. Full scan spectra (m/z = 150–1,000). BC, SK and ON: Propolis from British Columbia, Saskatchewan and Ontario, respectively.

Table 2. Total polyphenol content in ethanolic extracts of propolis (EEPs) collected from three different geographical regions of Canada.

	Mean ± SD (n = 3)		
Compound	BC	SK	ON
Total phenolic compound concentration (mg CAE[a]/g EEP)	410.81 ± 14.62	425.32 ± 0.23	429.61 ± 3.71

Notes: BC, SK and ON: Propolis samples from British Columbia, Saskatchewan and Ontario, respectively. CAE[a] = Caffeic equivalent.

quercetin, cinnamic acid, luteolin that have been identified in extracts of propolis from other regions of Canada and from other countries, were not detected in EEPs of the present study (Christov et al., 2006; Garcia-Viguera et al., 1993; Kalogeropoulos et al., 2009; Sulaiman et al., 2011). These results confirm that due differences in constituents of propolis differ among geographical regions likely because of difference in communities of plants among these regions (Bankova, 2005; Chen et al., 2008).

In general, it is accepted that propolis from temperate climatic zones, like Europe, North America and the non-tropical regions of Asia, originate mainly from exudates of buds of species of the genus Populus and their hybrids, which are rich in flavonoids, phenolic acids and their esters (Bankova, de Castro, & Marcucci, 2000; Bankova, Popova, Bogdanov, & Sabatini, 2002). Of the 22 compounds identified in EEP (Table 1; Figure 1), several were flavonoids, phenolic acids and their esters, and are known to have antioxidant, anti-inflammatory and antimicrobial activities (Kosalec et al., 2007; Oršolić et al., 2010). Alternatively, propolis from tropical regions, where no poplars and birches exist, are rich in prenylated benzophenons, diterpenes and flavonoids

Table 3. Capacities of ethanolic extracts of propolis (EEPs) from three geographical regions of Canada to scavenge free radicals of DPPH.

	Mean ± S.D. (n = 3)					
	25 μg ml^{-1}		50 μg ml^{-1}		100 μg ml^{-1}	
Samples and compounds	Absorbance[a]	% Discoloration (FRSA)[b]	Absorbance[a]	% Discoloration (FRSA)	Absorbance[a]	% Discoloration (FRSA)
BC	0.018 ± 0.0004	64.64	0.009 ± 0.0006	81.93	0.005 ± 0.0004	91.19
SK	0.006 ± 0.0010	88.15	0.004 ± 0.0003	92.10	0.003 ± 0.0004	93.33
ON	0.010 ± 0.0004	80.25	0.004 ± 0.0009	92.03	0.003 ± 0.0004	93.72
Quercetin	0.003 ± 0.0007	93.78	0.004 ± 0.0002	92.29	0.003 ± 0.0001	93.85
Caffeic acid	0.003 ± 0.0014	94.62	0.002 ± 0.002	97.02	0.002 ± 0.0004	96.37

Notes: BC, SK and ON: Propolis samples from British Columbia, Saskatchewan and Ontario, respectively.
[a]Control absorbance: 0.051 ± 0.0011.
[b]FSRA: Free radical scavenging activity.

(Ahn et al., 2007; Bankova, 2005; Banskota, Tezuka, & Kadota, 2001). Greater proportions of pinocembrin, chrysin, naringenin, sakuranetin, pinobanksin-3-acetate and pinobanksin-3-propionate in propolis from Canada originate from American species of Populus section Aigeiros rather than the European poplar (Populus nigra) (Garcia-Viguera et al., 1993; Greenaway, English, & Whatley, 1990).

Phenols are compounds that are important for providing protection against free radicals. These compounds give up hydrogen atoms from their hydroxyl groups to radicals and form stable phenoxyl radicals; hence, they play an important role in antioxidant activity. Therefore, determination of the quantity of phenolic compounds is important for predicting antioxidant capacities of extracts from plants (Das & Pereira, 1990; Hatano, Edamatsu, Hiramatsu, Mori, & Fujita, 1989; Saint-Cricq de Gaulejac, Glories, & Vivas, 1999). The propolis samples studied have TPC values greater than those reported for propolis from several other regions of the world (Kumazawa, Hamasaka, & Nakayama, 2004).

Each of the three samples of propolis were capable of scavenging free radicals. Scavenging of free radicals by extracts depends on abilities of antioxidants to lose hydrogen, which is determined in part by structural conformations of these components (Fukumoto & Mazza, 2000; Shimada, Fujikawa, Yahara, & Nakamura, 1992). At a concentration of 100 μg/ml, EEPs had a capacity to scavenge free radicals consistent with equivalent concentrations of quercetin and caffeic acid. These results are consistent with those of previous studies of antioxidative capacities of propolis from various geographic origins (Christov et al., 2006; Kumazawa et al., 2004; Lu, Chen, & Chou, 2003; Scheller et al., 1990; Sulaiman et al., 2011). Capacity to scavenge free radicals by EEPs studies was consistent with the TPC and was as fellow ON > SK > BC. This might be attributed to the presence of phenolic compounds, which, other than flavonoids, are reported to be among the most abundant and most effective antioxidant compounds (Isla, Nieva Moreno, Sampietro, & Vattuone, 2001; Kumazawa et al., 2004; Moreno, Isla, Sampietro, & Vattuone, 2000).

The response to oxidative stress protects cells against damage caused by oxidative stimuli. An important regulator of the response to oxidative stress is the Nrf2 transcription factor that is activated by oxidants or electrophiles and regulates expression of genes that protect against oxidative stress (Chan et al., 1996; Dinkova-Kostova et al., 2002; Lee et al., 2010). Obtained results support other studies that have demonstrated that constituents of propolis, such as phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydrogen peroxide (H_2O_2) or lipid peroxyl and thus inhibit oxidative mechanisms that lead to degenerative diseases (Calkins et al., 2009).

Previously, it has been demonstrated that caffeic acid phenethyl ester (CAPE) that is an active component of propolis, and that had antioxidant activity in the free radical scavenging assay in the current study (Table 3), activates the Nrf2 pathway (Lee et al., 2010). The mechanism of this effect was attributed to functional groups on CAPE that might directly activate Nrf2 by generating ROS or by reacting with Keap1 protein, the cytosolic repressor of Nrf2. Activation of Nrf2 signaling by each of the EEPs in the current study might occur by either of these mechanisms. Because samples of EEP activated Nrf2 signaling, it is not clear why greater expression of luciferase was not detected in cells co-exposed to EEP and H_2O_2. Even if effects of H_2O_2 are attenuated by EEP, it would be expected that any chemicals in EEP, such as CAPE, still would activate Nrf2. Additional studies are required to determine the mechanism of this effect.

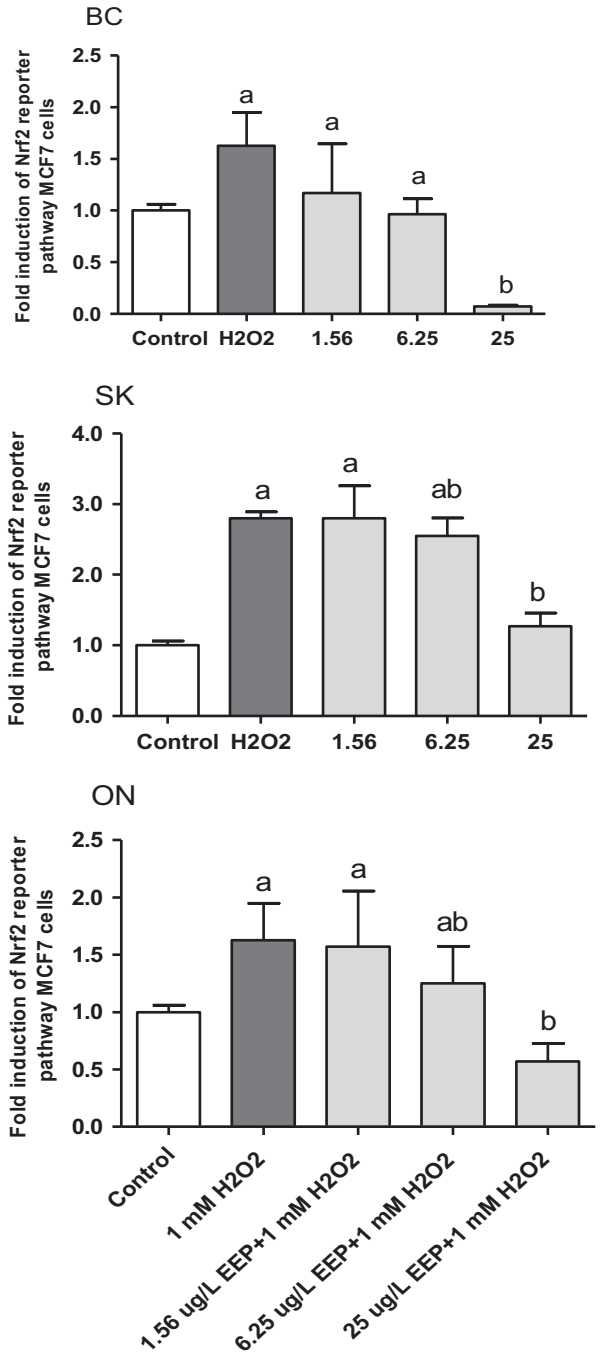

Figure 2. Effect of propolis on activation of Nrf2 signaling pathway by hydrogen peroxide. Cells were exposed either to the solvent control (0.1% v/v of ethanol) or 1 mM H_2O_2, or co-exposed to different concentrations of ethanolic extracts of propolis (EEPs) (final ethanol 0.1%, v/v) with 1 mM H_2O_2 for 16 h. Activation of Nrf2 signaling was measured as luciferase fluorescence and data is expressed as mean ± standard deviation of four exposures. Different letters denote significant differences compared to H_2O_2 (one way-ANOVA with Dunnett's test, $\rho < 0.05$). BC, SK and ON: Propolis from British Columbia, Saskatchewan and Ontario, respectively.

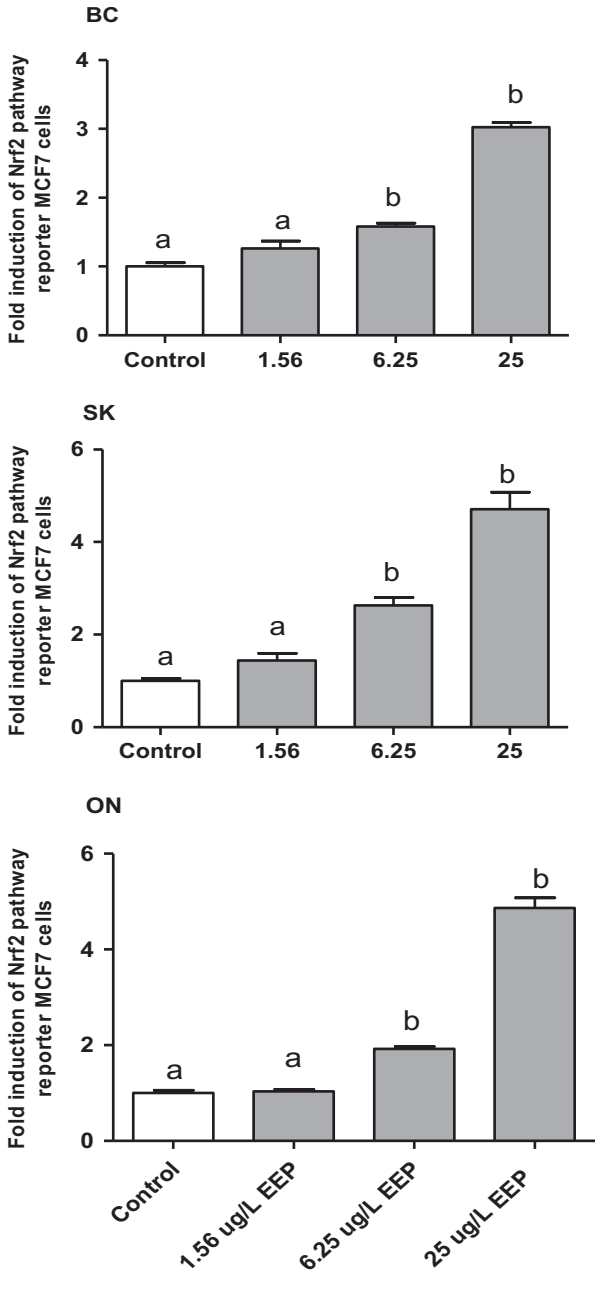

Figure 3. Effect of propolis on activation of the Nrf2 signaling pathway. Cells were exposed to EEPs or the solvent control (0.1% v/v of ethanol) for 16 h. Activation of Nrf2 reporter activity was measured as luciferase fluorescence and data is expressed as mean ± standard deviation of 4 exposures. Different letters denote significant differences among treatments (one way-ANOVA with Dunnett's test, $\rho < 0.05$). BC, SK and ON: Propolis from British Columbia, Saskatchewan and Ontario, respectively.

In the present study, propolis from the three regions of Canada was characterized by identifying and semi-quantifying major chemical components. Despite differences in chemical composition of each sample of propoli, each exhibited relatively similar antioxidant capacities. Capacity to scavenge free radicals by EEPs studies was found to be consistent with TPC. The current study is the first of its kind to evaluate and report the antioxidant capability of EEP *in vitro* using Nrf2 luciferase reporter gene assay. Because compounds present in EEP can protect against oxidative stress, results of

this study support use of propolis as a possible natural antioxidant for incorporation into some food products and supplements to prevent many free radical-mediated diseases and improve the health benefit of consumers.

Funding

This work was supported by Canada Excellence Research Chairs, Government of Canada [grant number #GDT20143200016], Natural Sciences and Engineering Research Council of Canada [grant number Project #326415–07] and Western Economic Diversification Canada [grant number Project #6578 and 6807].

Acknowledgements

The authors wish to acknowledge the support of grants Engineering Research Council of Canada (Project #326415-07), Western Economic Diversification Canada (Project #6578 and 6807), and an instrumentation grant from the Canada Foundation for Infrastructure. Prof. Giesy was supported by the Canada Research Chair program, the 2012 "High Level Foreign Experts" (#GDT20143200016) program, funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences. The authors also acknowledge the help of Glen Ackroyd, Ontario, Tyson Vesper, BC, Rudi Laprell and Meadow Ridge Enterprises LTD, Saskatoon, Saskatchewan for propolis collection.

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References

Ahn, M. R., Kumazawa, S., Usui, Y., Nakamura, J., Matsuka, M., Zhu, F., & Nakayama, T. (2007). Antioxidant activity and constituents of propolis collected in various areas of China. *Food Chemistry, 101*, 1383–1392.

Aksoy, L., Kolay, E., Ağılönü, Y., Aslan, Z., & Kargıoğlu, M. (2013). Free radical scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic *Thermopsis turcica*. *Saudi Journal of Biological Sciences, 20*, 235–239.

Asaduzzaman Khan, M., Tania, M., Zhang, D. Z., & Chen, H. C. (2010). Antioxidant enzymes and cancer. *Chinese Journal of Cancer Research, 22*, 87–92.

Aviram, M. (2000). Review of human studies on oxidative damage and antioxidant protection related to cardiovascular diseases. *Free Radical Research, 33*, S85–97.

Bankova, V. (2005). Recent trends and important developments in propolis research. *Evidence-Based Complementary and Alternative Medicine, 2*, 29–32.

Bankova, V., de Castro, S., & Marcucci, M. (2000). Propolis: Recent advances in chemistry and plant origin. *Apidologie, 31*, 3–15.

Bankova, V., Popova, M., Bogdanov, S., & Sabatini, A. G. (2002). Chemical composition of European propolis: Expected and unexpected results. *Zeitschrift für Naturforschung C, 57*, 530–533.

Banskota, A. H., Tezuka, Y., & Kadota, S. (2001). Recent progress in pharmacological research of propolis. *Phytotherapy Research, 15*, 561–571.

Bhattacharyya, A., Chattopadhyay, R., Mitra, S., & Crowe, S. E. (2014). Oxidative stress: An essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological Reviews, 94*, 329–354. doi:10.1152/physrev.00040.2012

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). Oxidative stress and antioxidant defense. *World Allergy Organization Journal, 5*, 9–19. doi:10.1097/WOX.0b013e3182439613

Burdock, G. A. (1998). Review of the biological properties and toxicity of bee propolis (propolis). *Food and Chemical Toxicology, 36*, 347–363.

Calkins, M. J., Johnson, D. A., Townsend, J. A., Vargas, M. R., Dowell, J. A., Williamson, T. P., ... Johnson, J. A. (2009). The Nrf2/ARE pathway as a potential therapeutic target in neurodegenerative disease. *Antioxidants & Redox Signaling, 11*, 497–508.

Chan, K., Lu, R., Chang, J. C., & Kan, Y. W. (1996). NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proceedings of the National Academy of Sciences, 93*, 13943–13948.

Chen, W., Jia, Z., Pan, M. H., & Anandh Babu, P. V. (2015). Natural products for the prevention of oxidative stress-related diseases: Mechanisms and strategies. *Oxidative Medicine and Cellular Longevity* 4628502. doi:10.1155/2016/4628502

Chen, Y. W., Wu, S. W., Ho, K. K., Lin, S. B., Huang, C. Y., & Chen, C. N. (2008). Characterisation of Taiwanese propolis collected from different locations and seasons. *Journal of the Science of Food and Agriculture, 88*, 412–419.

Christov, R., Trusheva, B., Popova, M., Bankova, V., & Bertrand, M. (2006). Chemical composition of propolis from Canada, its antiradical activity and plant origin. *Natural Product Research, 20*, 531–536.

Das, N. P., & Pereira, T. A. (1990). Effects of flavonoids on thermal autoxidation of palm oil: Structure-activity relationships. *Journal of the American Oil Chemists' Society, 67*, 255–258.

Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., ... Talalay, P. (2002). Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proceedings of the National Academy of Sciences, 99*, 11908–11913.

Fukumoto, L. R., & Mazza, G. (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. *Journal of Agricultural and Food Chemistry, 48*, 3597–3604.

Garcia-Viguera, C., Ferreres, F., & Tomas-Barberan, F. A. (1993). Study of Canadian propolis by GC-MS and HPLC. *Zeitschrift für Naturforschung C, 48*, 731–735.

Greenaway, W., English, S., & Whatley, F. R. (1990). Variation in bud exudate composition of *Populus nigra* assessed by gas chromatography-mass spectrometry. *Zeitschrift für Naturforschung C, 45*, 931–936.

Hatano, T., Edamatsu, R., Hiramatsu, M., Mori, A., & Fujita, Y. (1989). Effects of the interaction of tannins with Co-existing substances. VI. Effects of tannins and related polyphenols on superoxide anion radical, and on 1,1-diphenyl-2-picrylhydrazyl radical. *Chemical & Pharmaceutical Bulletin, 37*, 2016–2021.

Isla, M. I., Nieva Moreno, M. N., Sampietro, A. R., & Vattuone, M. A. (2001). Antioxidant activity of Argentine propolis extracts. *Journal of Ethnopharmacology, 76*, 165–170.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., ... Yamamoto, M. (1997). An Nrf2/small maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and Biophysical Research Communications, 236*, 313–322.

Kalogeropoulos, N., Konteles, S. J., Troullidou, E., Mourtzinos, I., & Karathanos, V. T. (2009). Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. *Food Chemistry, 116*, 452–461.

Kensler, T. W., Wakabayashi, N., & Biswal, S. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annual Review of Pharmacology and Toxicology, 47*, 89–116.

Kolankaya, D., Selmanoğlu, G., Sorkun, K., & Salih, B. (2002). Protective effects of Turkish propolis on alcohol-induced serum lipid changes and liver injury in male rats. *Food Chemistry, 78*, 213–217.

Kosalec, I., Sankovic, K., Zovko, M., Orsolic, N., Bakmaz, M., Kalogjera, Z., & Pepeljnjak, S. (2007). Antimicrobial and antioxidant activity of propolis from Croatia and Brazil: A comparative study. In *55th International Congress and Annual Meeting of the Society for Medicinal Plant Research*, 73, 165.

Kumar, N., KK, M. A., Dang, R., & Husain, A. (2008). Antioxidant and antimicrobial activity of propolis from Tamil Nadu zone. *Journal of Medicinal Plants Research, 2*, 361–364.

Kumazawa, S., Hamasaka, T., & Nakayama, T. (2004). Antioxidant activity of propolis of various geographic origins. *Food Chemistry, 84*, 329–339.

Kwak, M. K., & Kensler, T. W. (2010). Targeting NRF2 signaling for cancer chemoprevention. *Toxicology and Applied Pharmacology, 244*, 66–76.

Lee, Y., Shin, D. H., Kim, J. H., Hong, S., Choi, D., Kim, Y. J., ... Jung, Y. (2010). Caffeic acid phenethyl ester-mediated Nrf2 activation and IκB kinase inhibition are involved in NFκB inhibitory effect: Structural analysis for NFκB inhibition. *European Journal of Pharmacology, 643*, 21–28. doi:10.1016/j.ejphar.2010.06.016

Lu, L. C., Chen, Y. W., & Chou, C. C. (2003). Antibacterial and DPPH free radical-scavenging activities of the ethanol extract of propolis collected in Taiwan. *Journal of Food and Drug Analysis, 11*, 277–282.

Mann, G. E., Bonacasa, B., Ishii, T., & Siow, R. C. (2009). Targeting the redox sensitive Nrf2–Keap1 defense pathway in cardiovascular disease: Protection afforded by dietary isoflavones. *Current Opinion in Pharmacology, 9*, 139–145.

Marcucci, M. C. (1995). Propolis: Chemical composition, biological properties and therapeutic activity. *Apidologie, 26*, 83–99.

Miliauskas, G., Venskutonis, P. R., & van Beek, T. A. (2004). Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry, 85*, 231–237.

Minitab. (2013). *Minitab Version 17.1.0*. Pennsylvania: Penn State University.

Moreno, M. I. N., Isla, M. I., Sampietro, A. R., & Vattuone, M. A. (2000). Comparison of the free radical-scavenging activity of propolis from several regions of Argentina. *Journal of Ethnopharmacology, 71*, 109–114.

Oršolić, N. (2010). A review of propolis antitumor action *in vivo* and *in vitro*. *Journal of ApiProduct and ApiMedical Science, 2*, 1–20. doi:10.3896/IBRA.4.02.1.01

Oršolić, N., Benković, V., Lisičić, D., Dikić, D., Erhardt, J., & Knežević, A. H. (2010). Protective effects of propolis and related polyphenolic/flavonoid compounds against toxicity induced by irinotecan. *Medical oncology, 27*, 1346–1358.

Park, Y. K., Alencar, S. M., & Aguiar, C. L. (2002). Botanical origin and chemical composition of Brazilian propolis. *Journal of Agricultural and Food Chemistry, 50*, 2502–2506.

Park, Y. K., Paredes-Guzman, J. F., Aguiar, C. L., Alencar, S. M., & Fujiwara, F. Y. (2004). Chemical constituents in baccharis dracunculifolia as the main botanical origin of Southeastern Brazilian propolis. *Journal of Agricultural and Food Chemistry, 52*, 1100–1103.

Petelinc, T., Polak, T., Demšar, L., & Jamnik, P. (2013). Fractionation of phenolic compounds extracted from propolis and their activity in the yeast saccharomyces cerevisiae. *PLoS ONE, 8*, e56104.

Pi, J., Leung, L., Xue, P., Wang, W., Hou, Y., Liu, D., & Yehuda-Shnaidman, E. (2010). Deficiency in the nuclear Factor E2-related Factor-2 transcription factor results in impaired adipogenesis and protects against diet-induced obesity. *Journal of Biological Chemistry, 285*, 9292–9300.

Saint-Cricq de Gaulejac, N. S. C., Glories, Y., & Vivas, N. (1999). Free radical scavenging effect of anthocyanins in red wines. *Food Research International, 32*, 327–333.

Santos, F. W., Graça, D. L., Zeni, G., Rocha, J. B., Weis, S. N., Favero, A. M., & Nogueira, C. W. (2006). Sub-chronic administration of diphenyl diselenide potentiates cadmium-induced testicular damage in mice. *Reproductive Toxicology, 22*, 546–550.

Scheller, S., Wilczok, T., Imielski, S., Krol, W., Gabrys, J., & Shani, J. (1990). Free radical scavenging by ethanol extract of propolis. *International Journal of Radiation Biology, 57*, 461–465.

Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry, 40*, 945–948.

Spanos, G. A., & Wrolstad, R. E. (1990). Influence of processing and storage on the phenolic composition of Thompson Seedless grape juice. *Journal of Agricultural and Food Chemistry, 38*, 1565–1571.

Sulaiman, G. M., Sammarrae, K. W., Ad'hiah, A. H., Zucchetti, M., Frapolli, R., Bello, E., ... Bagnati, R. (2011). Chemical characterization of Iraqi propolis samples and assessing their antioxidant potentials. *Food and Chemical Toxicology, 49*, 2415–2421.

Venugopal, R., & Jaiswal, A. K. (1996). Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H: quinone oxidoreductase1 gene. *Proceedings of the National Academy of Sciences, 93*, 14960–14965.

Wood-Kaczmar, A., Gandhi, S., & Wood, N. W. (2006). Understanding the molecular causes of Parkinson's disease. *Trends in Molecular Medicine, 12*, 521–528.