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Antioxidant activity of minor components of tree nut oils

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ABSTRACT

The antioxidative components of tree nut oils were extracted using a solvent stripping process. Tree nut oil extracts contained phospholipids, sphingolipids, sterols and tocopherols. The chloroform/methanol extracted oils had higher amounts of phenolic compounds than their hexane extracted counterparts. The antioxidant activity of tree nut oil minor component extracts were assessed using the 2,2-azino-bis (3-ethylbenzthiazoline sulphonate) (ABTS) radical scavenging activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity, β-carotene bleaching test, oxygen radical absorbance capacity (ORAC) and photochemiluminescence inhibition assays. Results of these studies demonstrated that extracts of chloroform/methanol extracted oils possessed higher antioxidant activities than extracts of their hexane extracted counterparts. Meanwhile the extract of chloroform/methanol extracted pecan oil possessed the highest antioxidant activity.

1. Introduction

Tree nuts and their oils are known to contain several bioactive and health-promoting components. However, little information is currently available on the nature and antioxidant activity of the bioactive constituents present in tree nut oils. Tree nuts have long been considered an important component of the Mediterranean diet (Hu & Stampfer, 1999). In July 2003 the US Food and Drug Administration (FDA) approved a qualified health claim stating that consumption 42 g (1.5 ounces) per day of most tree nuts may reduce the risk of heart disease. Epidemiological evidence indicates that the consumption of tree nuts may exert several cardioprotective effects, which are speculated to arise from their lipid component that includes unsaturated fatty acids, phytosterols and tocols (Hu & Stampfer, 1999). Recent investigations have also shown that dietary consumption of tree nut oils may exert even more beneficial health effects than consumption of whole tree nuts, possibly due to the replacement of dietary carbohydrates with unsaturated lipids and/or other components present in the oil extracts (Hu & Stampfer, 1999).

Natural antioxidants function as free radical scavengers, reducing agents, chelators of pro-oxidant metals, or as quenchers of singlet oxygen. They are widely used in the food industry to enhance the sensory, health-promoting, or keeping quality of foods (Koski et al., 2002). Natural antioxidants are also present in many health-promoting supplements (Lee, Kim, Kim, & Chang, 2002). Unrefined oils contain a number of minor components that are partially removed during the refining, bleaching and deodorization that most commercial vegetable oils undergo (Tasioula-Margari & Okogeri, 2001). Some minor constituents can act as pro-oxidants such as free fatty acids and hydroperoxides, or as antioxidants including tocopherols, phenols, and possibly phospholipids together with other components (Lee et al., 2002). Nutritionally important antioxidants such as tocopherols and carotenoids generally improve oil stability and thus oils naturally rich in these constituents are preferred (Warner & Frankel, 1987). Phytosterols and phytostanols present in vegetable oils have hypocholesterolemic effects and may also exhibit antioxidant activity (Wang, Hicks, & Moreau, 2002). Non-tocopherol phenolics influence the sensory and nutritional characteristics of the oil and possibly improve its stability (Alasalvar et al., 2003). Consumption of foods rich in natural antioxidants has been reported as being protective against certain types of cancer and may also reduce the risk of cardiovascular and cerebrovascular events. These actions of antioxidants have been attributed to their ability to scavenge free radicals, thereby reducing oxidative damage of cellular biomolecules such as lipids, proteins, and nucleic acids (Aruoma, 1998).

Much of the existing literature attributes the beneficial health effects of tree nuts and tree nut oils to their high oleic acid content; however, very little research has been conducted on the compositions and activities of their minor components. Therefore, this study aimed to analyze the lipid composition of minor component

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extracts of tree nut oils, including analysis of lipid classes, tocopherols, sterols and stanols, as well as to examine the antioxidant activity of minor components of tree nut oils using a number of *in vitro* assays. A further objective of this work was to compare the effects of two oil extraction solvents, namely hexane and chloroform–methanol, on minor components present.

2. Materials and methods

2.1. Materials

Commercially available shelled and unsalted almonds, Brazil nuts, hazelnuts (filberts), pecans, pine nuts, pistachios and walnuts were purchased fresh from the local wholesale market in St. John's, NL, Canada, or acquired from the International Treenut Council (Reus, Spain) or its affiliates. Samples were stored at −20 °C until use. All chemicals were obtained from Sigma–Aldrich Canada (Oakville, ON, Canada) or Fisher Scientific (Ottawa, ON, Canada), unless otherwise stated. All solvents were of American Chemical Society grade, or better, unless otherwise specified.

2.2. Fat extraction

2.2.1. Hexane extraction

Forty grams of each tree nut sample were first ground into a fine powder and combined with 400 ml of hexanes, followed by homogenization at 8000 rpm using a polytron (Polytron model PT 3000, Kinematica, Littau, Switzerland) for 3 min as detailed elsewhere (Miraliakbari & Shahidi, 2008). The resulting mixture was filtered through a Whatman #4 filter paper with suction using a Büchner funnel. The residue was re-extracted twice; the filtrates from the three extractions were combined and a portion of the solvent was removed from the extract using a rotary evaporator (Rotavapor model 461, Büchi, Flawil, Switzerland) at 40 °C. The hexane–oil mixture was then passed through a layer of anhydrous sodium sulphate placed over a filter paper in a funnel and the remaining solvent was removed using a rotary evaporator at 40 °C. The resulting oil was weighed and transferred into 10 ml sample vials, capped with nitrogen and stored at −80 °C until use.

2.2.2. Peroxide value

The peroxide value was measured using the official method of the (AOCS, 1990) using 1.0–2.0 ± 0.01 g of oil.

2.2.3. Anisidine value

The anisidine value was determined at 350 nm in a 1.0 cm cell of a solution containing 1.0 g of oil in 100 mL of isooctane according to the method CD 18–90 of the AOCS (1990).

2.3. Extraction of minor components

Minor components were extracted from tree nut oils as described by Ramadan, Kroh, and Morsel (2003), with minor modifications. Twenty grams of oil were combined with 200 ml of hexane in a 500 ml separatory funnel. One hundred millilitres of methanol were added to the mixture, and the separatory funnel was sealed and agitated for 5 min with periodic venting. The separatory funnel was then sealed with nitrogen and stored at 4 °C for 1 h. The methanol fraction was decanted into a 1 l round bottom flask. The methanol extraction process was repeated four times and the pooled extracts were evaporated down to 20 ml, resulting in an extract with a final concentration of 1 g oil equivalent per millilitre. The extracts were stored in methanol at −80 °C until used within a maximum period of one week.

2.4. Lipid analysis

2.4.1. Lipid class analysis

The lipid class composition of the tree nut oil extracts was analyzed using an automated thin layer chromatography-flame ionization detection (TLC-FID) apparatus, as described elsewhere (Alasalvar et al., 2003). The samples were chromatographed on silica gel-coated Chromarods S III and then analyzed using an Iatroscan MK-5 (Iatroscan Laboratories Inc., Tokyo, Japan) analyzer equipped with a flame ionization detector connected to a computer loaded with TSCAN software (Scientific Products and Equipment, Concord, ON, Canada) for data handling. The identity of each peak was determined by comparison with a chromatogram of standards acquired concurrently with the samples. The percentages of individual lipid classes (by weight) were determined using the calibration curves procured for each authentic standard.

2.4.2. Tocopherol analysis

The tocopherols in tree nut oil extracts were analyzed using an Agilent model 1100 HPLC/UV-DAD/MS system (Agilent Technologies, Palo Alto, CA, USA) as described by Chatzimichalakis, Samanidou, and Papadoyannis (2004), with minor modifications. Separation of tocopherol homologs was achieved using a Phenomenex fused silica analytical column (250 mm × 4.6 mm; Phenomenex, Torrance, CA, USA) packed with 5 μm particles. Forty microliters of the tocopherol extract were loaded onto the column and then eluted isocratically using hexane/2-propanol (98:2, v/v) with a flow rate of 1 ml/min at ambient temperature. Tocopherol homologs were quantified using an ultraviolet-diode array detector (UV-DAD) at a wavelength of 290 nm. Quantification of tocopherol homologs was achieved by comparison of each sample peak response to that of the corresponding authentic standard. In order to confirm the identity of each tocopherol peak, the HPLC/UV-DAD effluent was channeled into an Agilent 1100 APCI-MS (Palo Alto, CA, USA) operating in the negative mode. The MS conditions were as follows: auxiliary gas flow, 10 units; sheath gas pressure, 70 psig; capillary temperature, 150 °C; vaporizer temperature, 400 °C; corona current, 5 μA; scan time, 1 s; and scan range, 40–600 *m/z*. Analysis of chromatographic and spectral data was performed using Agilent Chemstation software (Palo Alto, CA, Canada).

2.5. Antioxidant activity of tree nut Oil extracts

2.5.1. Total phenolics content (TPC) of tree nut oil extracts

The total phenolics content of oil extracts was assessed by employing the method described by Singleton and Rossi (1965) using. Folin and Ciocalteu's reagent. Results were expressed as milligrams of gallic acid or α-tocopherol equivalents per gram of oil.

2.5.2. The 2,2-azino-bis (3-ethylbenzthiazoline sulphonate) radical scavenging activity of tree nut oil extracts

The free radical scavenging capacity of extracts was determined as described by van den Berg, Haenen, van den Berg, and Bast (1999) using 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonate) solution (ABTS) and 2,2′-azo-bis (2-methylpropionamidine) dihydrochloride (AAPH), for production of the ABTS radical ($ABTS^{\bullet-}$). The TEAC value of an extract represents the concentration of a Trolox solution that has the same antioxidant capacity as the extract.

2.5.3. DPPH scavenging capacity of tree nut oil extracts

Scavenging activities of tree nut oil extracts towards the DPPH radical was assessed as previously described, with some modifications (Banda, Sherry, & Blois, 1977). There was a linear absorbance range for DPPH concentration between 0.01 and 0.3 mM. Tree nut oil extracts were assayed undiluted (1 g oil equivalent/mL extract) and diluted 2, 4, 8 and 10 times; the data were used to construct a

% DPPH scavenged versus extract concentration expression. This expression was then used to calculate the extract concentration required to scavenge 50% of the DPPH in the assay medium, referred to as the IC_{50}. The DPPH scavenging capacity of α-tocopherol was also assayed at concentrations of 6.25, 12.5, 25.0, 50.0 and 100 μM to develop a DPPH scavenging capacity standard curve for α-tocopherol.

2.5.4. β-carotene bleaching test with tree nut oil extracts

The β-carotene bleaching test was performed as described by Miller (1971), with minor modifications. A solution of 5 mg/10 ml of β-carotene was prepared in chloroform and 3 mL of this solution were pipetted into a 100 ml round bottom flask. Chloroform was removed under vacuum using a rotary evaporator at 40 °C, then 40 mg linoleic acid, 400 mg Tween 40 emulsifier and 100 ml of aerated distilled water were added to the flask with vigorous shaking. Aliquots of 4.8 ml of this emulsion were transferred into a series of test tubes containing 200 μl of the tree nut oil extracts or methanol (control). Alpha-tocopherol was used as the reference antioxidant (10 μM). Immediately after the addition of the emulsion to each tube, the zero time absorbance was measured at 470 nm. Subsequent absorbance readings were recorded over a two-hour period at 20 min intervals by keeping the reaction tubes in a water bath set to 50 °C. Blank samples devoid of β-carotene were prepared for background subtraction. The capacity of the extracts to protect against oxidation of β-carotene was determined as follows:

$$((A_{t=0\ \text{Sample}} - A_{t=0\,\text{Blank}}) - (A_{t=120\ \text{min Sample}} - A_{t=120\text{min Blank}}))$$
$$/((A_{t=0\,\text{Control}}) - (A_{t=120\text{min Control}})) = \mathbf{C}$$
$$\beta\text{-Carotene retention }(\%) = 100\% - (\mathbf{C}) * 100\%$$

where A is the absorbance at a particular time; and **C**, carotene depletion factor.

2.5.5. Photochemiluminescence inhibition capacity of tree nut oil extracts

The photochemiluminescence (PCL) inhibition capacity of tree nut oil extracts was assessed as described by Popov and Lewin (1996) using an automated PCL inhibition capacity analyzer system (Analytik Jena Photochem, Analytik Jena USA, Woodlands, TX, USA) in the antioxidative capacity of lipids (ACL) mode and expressing results as α-tocopherol equivalents.

2.5.6. Oxygen radical absorbance capacity (ORAC) of tree nut oil extracts

The ORAC of tree nut oil extracts was tested as previously described (Dalavlos, Gomez-Cordoves, & Bartolome, 2004), with modifications using a FLOUstar Optima fluorescence microplate reader equipped with two reagent injectors (BMG Labtechnologies, Durham, NC, USA). Non-transparent 96-well microplates (Costar model 3095, Corning Life Sciences, Corning, NY, USA) were used in the ORAC assay. Fluorescence filters with excitation and emission wavelengths of 485 and 520 nm, respectively, were used; these conditions correspond to the fluorescence properties of fluorescein. All solutions were prepared in 75 mM phosphate buffer (pH 7.4). Samples were diluted by a factor of 30 in 5% randomly methylated cyclodextrin solution (Cyclodextrin Technologies Inc., High Springs, FL, USA) before being assayed. Twenty microliters of diluted tree nut oil extracts were manually pipetted into sample wells of the microplate which was then placed in the microplate reader and incubated for 15 min at 37 °C. During the first cycle 120 μl of fluorescein solution (disodium salt, 20 μM) were injected into each well using the first automated reagent injector, each injection was followed by a 1 s mixing cycle. During the second cycle 60 μL of AAPH (45 mM) were injected into each well using the second automated reagent injector, followed by a 1 s mixing cycle. Following mixing, the initial fluorescence was read. Fluorescence readings were then taken every 30 s, with a total assay time of 30 min. A standard curve was prepared with α-tocopherol using a concentration range of 12.5–150 μM. The relative fluorescence versus time graph of each sample was recorded using the FLOUstar Optima computer software (BMG Labtechnologies, Durham, NC, USA), from which the area under curve (AUC) of each sample was calculated. The AUC of each sample was used along with the standard curve to calculate the ORAC of each extract, expressed as μmol α-tocopherol equivalents/g oil equivalent.

2.6. Statistical analysis

All experiments were performed in triplicate; mean values and standard deviations were calculated for each case. Analysis of variance (ANOVA) followed by Tukey's studentized range test were performed at the $p \leqslant 0.05$ level using Minitab statistical software version 14 (Minitab Inc., State College, PA, USA) to evaluate the significance of differences among different mean values (Snedecor & Cochran, 1980).

3. Results and discussion

3.1. Oil yield, chemical characteristics and stripping of tree nut Oils

The oils used in this study were extracted from fresh raw tree nuts. Comparison of the two fat extraction processes, namely hexane and chloroform/methanol, showed that the latter solvent system afforded a higher oil yield for all tree nut varieties studied, whereas hexane provided an oil with higher clarity; implying that hexane extraction renders a more refined oil extract compared to the chloroform/methanol solvent system. Pine nuts yielded the highest amount of oil; 73.9% with hexane and 75.4% with chloroform/methanol, whereas almonds had the lowest oil yield, 51.2% with hexane and 53.5% with chloroform/methanol (Table 1). The oil yields of several tree nut varieties have previously been reported and show the oil contents (w/w) of 50.6% for almond, 66.4% for Brazil nut, 72.0% for pecan, 68.4% for pine nut, 46.4% for pistachio and 65.2% for walnut (USDA, 2005; Shahidi & Miraliakbari, 2005). Results from this study are therefore in good agreement with those reported previously.

The oxidative qualities of the extracted oils were examined using the conjugated dienes, peroxide value and anisidine value

Table 1
Oil yields and chemical characteristics of hexane extracted and chloroform/methanol extracted tree nut oils[A]

Sample	Oil yield (% ww)	PV (meq/kg oil)	*p*-AV
Hexane-extracted			
Almond	51.2 ± 0.5 ^{h}	0.040 ± 0.002^{a}	0.120 ± 0.003^{m}
Brazil nut	67.4 ± 0.2^{c}	0.047 ± 0.003^{a}	0.189 ± 0.006^{l}
Hazelnut	60.4 ± 0.4^{e}	0.031 ± 0.001^{a}	0.591 ± 0.008^{c}
Pecan	71.5 ± 0.4^{a}	0.030 ± 0.003^{a}	0.433 ± 0.007^{h}
Pine nut	73.9 ± 0.5^{a}	0.030 ± 0.002^{a}	0.267 ± 0.002^{j}
Pistachio	52.3 ± 0.2^{g}	0.023 ± 0.002^{a}	0.545 ± 0.006^{e}
Walnut	70.6 ± 0.4^{a}	0.030 ± 0.004^{a}	0.230 ± 0.005^{k}
Chloroform/methanol-extracted			
Almond	53.5 ± 0.2^{f}	0.030 ± 0.002^{a}	0.561 ± 0.004^{d}
Brazil nut	68.9 ± 0.3^{b}	0.030 ± 0.003^{a}	0.821 ± 0.004^{a}
Hazelnut	61.9 ± 0.2^{d}	0.058 ± 0.005^{a}	0.288 ± 0.003^{i}
Pecan	73.4 ± 0.3^{a}	0.023 ± 0.002^{a}	0.294 ± 0.005^{i}
Pine nut	75.1 ± 0.2^{a}	0.015 ± 0.002^{a}	0.735 ± 0.002^{b}
Pistachio	54.1 ± 0.4^{f}	0.015 ± 0.002^{a}	0.735 ± 0.002^{b}
Walnut	72.5 ± 0.3^{a}	0.015 ± 0.002^{a}	0.462 ± 0.005^{g}

[A] Values in the same column bearing different superscripts are significantly ($p \leqslant 0.05$) different.

tests, all of which were well below the recommended values for oil acceptability (Table 1) (Dobarganes & Ruiz, 1998). No headspace aldehydes were detected in fresh tree nut oils (data not shown). These findings demonstrate that the fat extraction processes employed here were gentle enough to preserve the oxidative integrity of the oils, as expected for fresh products.

A liquid–liquid phase partitioning system was used to extract minor components from tree nut oil samples (solvent stripping process). This method was chosen over solid phase stripping processes (Khan & Shahidi, 2002) because of the relative ease of the solvent stripping process, and to reduce oxidative deterioration of the minor components; thereby preserving their antioxidant activity. A similar solvent extraction process was employed by Ramadan et al. (2003) to extract antioxidative components from black cumin, coriander and Niger oils, reporting DPPH radical scavenging activity for oil extracts examined. However, the nature of the active compounds involved was not investigated (Ramadan et al., 2003).

3.2. Lipid compositions of tree nut oil extracts

The lipid class compositions of tree nut oil minor component extracts, analyzed using TLC-FID, revealed the presence of sterols, phosphatidylserine, phosphatidylinositol, phosphatidylcholine and sphingolipids. Tocopherol compositions were analyzed using reversed-phase HPLC. Lipid classes and tocopherols were expressed as parts per million of the final methanolic extract (ppm; mg compound/kg oil equivalents). The minor component extract of chloroform/methanol extracted hazelnut oil contained the highest total tocopherol content (321 mg/kg oil equivalents), followed by chloroform/methanol extracted pine nut oil (309 mg/kg oil equivalents) (Table 2). The minor component extract of hexane extracted Brazil nut oil contained the lowest total tocopherol content (135 mg/kg oil equivalents). The relative percentages of individual tocopherols in tree nut oil extracts did not significantly differ ($p > 0.05$) from the relative percentages in their non-stripped counterparts.

The minor component extracts of chloroform/methanol extracted tree nut oils contained higher amounts of phospholipids and sphingolipids than their hexane extracted counterparts. The minor component extract of chloroform/methanol extracted pistachio oil possessed the highest amount of phospholipids and sphingolipids among all samples tested (10.3 g/kg oil equivalents), followed by chloroform/methanol extracted walnut oil (9.3 g/kg oil equivalents) and then chloroform/methanol extracted pecan oil (8.6 g/kg oil equivalents). The minor component extract of hexane extracted hazelnut oil possessed the lowest amount of phospholipids and sphingolipids (2.6 g/kg oil equivalents). Phosphatidic acid was detected only in the extracts of hazelnut oil.

3.3. Total phenolics contents (TPC) of tree nut oil extracts

The TPC of tree nut oil extracts, determined by the method of Singleton and Rossi (1965), were expressed as equivalents of gallic acid/kg oil equivalents (Table 3) and as equivalents of α-tocopherol/kg oil equivalents (Table 3). These two reference standards were used because of their different solubility characteristics; gallic acid is a water soluble phenolic while α-tocopherol is a lipid soluble phenolic. Minor component extracts of chloroform/methanol extracted oils had higher TPC than their hexane extracted counterparts, expressed as gallic acid equivalents or α-tocopherol equivalents. This strongly suggests that chloroform/methanol was more effective than hexane for extraction of phenolic compounds. Since several antioxidative phenolic compounds occur in tree nuts (Shahidi & Miraliakbari, 2005), inclusion of these compounds in tree nut oils is expected to enhance their antioxidant activity as well as their content of minor components.

Among the oil extracts studied, chloroform/methanol extracted pecan oil had the highest TPC (711 mg/kg gallic acid equivalents or 783 mg/kg α-tocopherol equivalents) followed by chloroform/methanol extracted walnut oil (689 mg/kg gallic acid equivalents or 759 mg/kg α-tocopherol equivalents) and then chloroform/methanol extracted Brazil nut oil (381 mg/kg gallic acid equivalents or 429 mg/kg α-tocopherol equivalents). Hexane extracted almond oil extracts had the lowest TPC (40 mg/kg gallic acid equivalents or 124 mg/kg α-tocopherol equivalents). The α-tocopherol equivalence values of chloroform/methanol extracted pecan and walnut oil extracts were almost twofold greater than their total tocopherol contents as determined using HPLC, which implies that phenolic compounds other than tocopherols that are present in these extracts enhance their antioxidant activities. No obvious correlations existed between α-tocopherol equivalence values (TPC) and total tocopherol contents (HPLC), with 8 out of 14 extracts having lower α-tocopherol equivalence values than total tocopherol contents (HPLC). The difference between α-tocopherol equivalence values and total tocopherol contents did not exceed

Table 2
Lipid class contents (mg/kg oil equivalents) and compositions of tree nut oil extracts[A,B]

Lipid class	Sterols[C]	Phosphatidylserine[C]	Phosphatidylinositol[C]	Phosphatidylcholine[C]	Phosphatidic acid[C]	Sphingolipids[C]	Tocopherols[D]
A–H	800 ± 152^{a}	500 ± 99^{c}	700 ± 73^{c}	800 ± 92^{d}	ND	2100 ± 142^{a}	170.6 ± 0.3^{j}
A–BD	900 ± 108^{a}	1100 ± 137 ^{b}	600 ± 52^{c}	2000 ± 149^{c}	ND	2400 ± 166^{a}	179.3 ± 0.2^{i}
BN–H	900 ± 99^{a}	1200 ± 186^{b}	200 ± 35^{f}	1000 ± 107^{d}	ND	3200 ± 185^{a}	106.8 ± 0.6^{m}
BN–BD	800 ± 125^{a}	1200 ± 205^{b}	200 ± 48^{f}	3900 ± 237^{a}	ND	2900 ± 182^{a}	126.5 ± 0.4^{l}
HN–H	1100 ± 184^{a}	1100 ± 163^{b}	300 ± 37^{e}	1100 ± 175^{d}	200 ± 39^{b}	100 ± 26^{b}	288.5 ± 0.6^{c}
HN–BD	1100 ± 193^{a}	1300 ± 138^{b}	300 ± 28^{e}	2100 ± 226^{c}	500 ± 63^{a}	200 ± 46^{b}	321.9 ± 0.5^{a}
P–H	1200 ± 157^{a}	1500 ± 263^{b}	400 ± 34^{d}	1000 ± 78^{d}	ND	2100 ± 238^{a}	244.4 ± 0.3^{f}
P–BD	1600 ± 179^{a}	2400 ± 206^{a}	700 ± 75^{c}	2300 ± 287^{c}	ND	3200 ± 280^{a}	269.6 ± 0.5^{d}
PN–H	500 ± 68^{a}	1000 ± 149^{b}	600 ± 47^{c}	1000 ± 95^{d}	ND	2700 ± 261^{a}	263.1 ± 0.8^{e}
PN–BD	700 ± 128^{a}	1500 ± 242^{b}	700 ± 19^{c}	1400 ± 125^{d}	ND	2800 ± 235^{a}	309.4 ± 0.3^{b}
PO–H	1100 ± 142^{a}	2100 ± 172^{a}	900 ± 64^{b}	2400 ± 277^{c}	ND	2900 ± 263^{a}	218.5 ± 0.9^{h}
PO–BD	1100 ± 123^{a}	2800 ± 238^{a}	1200 ± 36^{a}	3000 ± 284^{b}	ND	3300 ± 213^{a}	236.1 ± 0.4^{g}
W–H	1100 ± 118^{a}	1700 ± 127^{b}	1200 ± 149^{a}	1200 ± 115^{d}	ND	2500 ± 173^{a}	149.1 ± 0.6^{k}
W–BD	1500 ± 134^{a}	2700 ± 236^{a}	1300 ± 233^{a}	2400 ± 203^{c}	ND	2900 ± 218^{a}	267.2 ± 0.9^{d}

[A] Abbreviations used: A–H, almond oil–hexane extract; A–CM, almond oil–chloroform–methanol extract; BN–H, Brazil nut oil – hexane extract; BN-CM, Brazil nut oil–chloroform-methanol extract; HN–H, hazelnut oil–hexane extract; HN–CM, hazelnut oil–chloroform–methanol extract; P–H, pecan oil–Hexane extract; P–CM, pecan oil–chloroform–methanol extract; PN–H, pine nut oil–hexane extract; PN–CM, pine nut oil–chloroform–methanol extract; PO–H, pistachio oil–hexane extract; PO–CM, pistachio oil–chloroform–methanol extract; W–H, walnut oil–hexane extract; W–CM, walnut oil–chloroform–methanol extract; ND, not detected.
[B] Values in the same column with different superscripts are significantly ($p \leqslant 0.05$) different. Experiments performed in triplicate.
[C] Analyzed using TLC-FID.
[D] Analyzed using HPLC.

Table 3
Total phenolics contents (mg/kg) of extracts from tree nut oils[A]

Nut	Gallic acid equivalents		α-Tocopherol equivalents	
	Hexane extracted	Chloroform/methanol extracted	Hexane extracted	Chloroform/methanol extracted
Almond	124 ± 11^{d}	168 ± 15^{d}	40 ± 5^{d}	73 ± 7^{d}
Brazil nut	153 ± 12^{d}	429 ± 19^{b}	48 ± 4^{d}	381 ± 11^{b}
Hazelnut	159 ± 13^{d}	338 ± 14^{c}	91 ± 12^{d}	163 ± 17^{c}
Pecan	196 ± 15^{d}	783 ± 18^{a}	54 ± 8^{d}	711 ± 32^{a}
Pine nut	148 ± 12^{d}	423 ± 16^{c}	53 ± 11^{d}	157 ± 15^{c}
Pistachio	158 ± 12^{d}	379 ± 15^{c}	59 ± 8^{d}	173 ± 18^{c}
Walnut	210 ± 16^{d}	759 ± 22^{a}	63 ± 7^{d}	689 ± 23^{a}

[A] Values with different superscripts are significantly ($p \leqslant 0.05$) different.

twofold for any of the samples tested. These differences could be due to the presence of non-tocopherol phenolics or other compounds in some samples that reacted with the Folin and Ciocalteu's reagent, and thus increased their TPC beyond what would be expected from their total tocopherol contents alone.

There are no previous reports on TPC of tree nut oils. The total phenolics contents of minor component extracts of black cumin (*Nigella sativa* L.) oil, coriander (*Coriandrum sativum* L.) oil, and Niger (*Guizotia abyssinica* Cass.) oil have been reported as 24, 11 and 5 mg caffeic acid equivalents per kg oil equivalents (Ramadan et al., 2003). The TPC of olive oil has been studied by a number of research groups who have reported values ranging from 11 to 76 mg syringic acid per kg oil equivalents (Gomez-Alonso, Salvador, & Fregapane, 2002). However, in both of these reports the TPC were considerably lower than the total tocopherol contents of the oils studied, implying that the TPC assay employed in these studies did not adequately assess the total amounts of phenolic compounds present in the oil samples.

3.4. Trolox equivalent antioxidant capacity (TEAC) of tree nut oil extracts

The Trolox equivalent antioxidant capacity measures the ability of antioxidants to scavenge the 2.5 mM 2,2′-azo-bis (2-methylpropionamidine) dihydrochloride (AAPH)/2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonate) ($ABTS^{\cdot-}$) radical pair; the antioxidant activities are expressed as equivalents of Trolox, a water soluble vitamin E analogue. The TEAC value is defined as the molar concentration of Trolox solution having the antioxidant capacity equivalent to the sample solution being tested. Trolox equivalent antioxidant capacity values of tree nut oil extracts were calculated using a Trolox standard curve and were expressed as μM Trolox equivalents/g oil equivalents (μM Trolox/g oil). Results showed that minor component extracts of chloroform/methanol extracted nut oils possessed higher TEAC values compared to their hexane extracted counterparts (Table 4). All minor component extracts exhibited antioxidant activity; chloroform/methanol extracted pecan oil had the greatest TEAC value (2047.3 μM Trolox/g oil), followed by chloroform/methanol extracted Brazil nut oil (1217 μM Trolox/g oil), and then chloroform/methanol extracted walnut oil (958.9 μM Trolox/g oil) ($p \leqslant 0.05$). The minor component extracts of hexane extracted almond oil and hexane extracted Brazil nut oil had the lowest TEAC values (67.6 and 81.8 μM Trolox/g oil, respectively). The high TEAC value of chloroform/methanol extracted pecan oil may stem from its tocopherol composition that is very rich in γ-tocopherol. However, the presence of other antioxidative components acting alone or synergistically with γ-tocopherol is likely involved since the TEAC value of chloroform/methanol extracted pecan oil greatly exceeds that of its hexane extracted counterpart.

These results indicate that the chloroform/methanol extraction system affords oils with higher amounts of antioxidative components, which are expected to improve oil stability and potentially exert beneficial health effects.

3.5. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity of tree nut oil extracts

The DPPH radical scavenging capacity assay was used to examine the antioxidant activity of tree nut oil extracts. Tree nut oil extracts were assayed over a range of dilutions to establish the concentration of each extract required to scavenge 50% of the DPPH radical present in the assay medium, referred to as the IC_{50}. Under the assay conditions employed here, the IC_{50} of pure α-tocopherol was 23.6 μg. The minor component extracts of chloroform/methanol extracted oils possessed greater DPPH radical scavenging activity than their hexane extracted counterparts. Among the chloroform/methanol extracted oils, pecan oil had the greatest DPPH radical scavenging capacity with an IC_{50} of 0.03 g oil equivalents which equates to 786.7 μg α-tocopherol equivalents per gram oil equivalents, followed by walnut and Brazil nut oils (337.1 and 295.0 α-tocopherol equivalents per gram oil equivalents, respectively), then pistachio and pine nut oils (214.5 and 181.6 α-tocopherol equivalents per gram oil equivalents, respectively), followed by hazelnut oil (112.4 α-tocopherol equivalents per gram oil equivalents) and finally, almond oil (62.1 α-tocopherol equivalents per gram oil equivalents) ($p \leqslant 0.05$) (Table 4). Among the hexane extracted oils, pecan and walnut oils exhibited the greatest DPPH scavenging activity (107.3 and 98.3 α-tocopherol equivalents per gram oil equivalents, respectively), followed by pistachio, hazelnut and pine nut oils (87.4, 84.3 and 76.1 α-tocopherol equivalents per gram oil equivalents, respectively), then Brazil nut oil (65.5 α-tocopherol equivalents per gram oil equivalents), and finally almond oil (46.3 α-tocopherol equivalents per gram oil equivalents) ($p \leqslant 0.05$).

Table 4
Antioxidant activity of minor components of tree nut oil extracts

Nut	ABTS		DPPH		β-Carotene		ORAC		PCL	
	Hexane	Chloroform–methanol	Hexane	Chloroform–methanol	Hexane	Chloroform–methanol	Hexane	Chloroform–methanol	Hexane	Chloroform–methanol
Almond	67.6 ± 19.5^{g}	345.3 ± 11.6^{e}	46.3 ± 7.4^{g}	62.1 ± 9.9^{f}	7.4 ± 0.1^{i}	30.1 ± 0.9^{h}	0.97 ± 0.03^{h}	2.12 ± 0.05^{e}	1.30 ± 0.14^{e}	2.11 ± 0.22^{d}
Brazil Nut	81.8 ± 9.8^{g}	1216.9 ± 12.6^{b}	65.5 ± 10.5^{e}	295.0 ± 23.6^{c}	12.3 ± 0.5^{j}	62.5 ± 1.1^{c}	1.34 ± 0.05^{b}	1.96 ± 0.04^{f}	1.22 ± 0.18^{e}	1.81 ± 0.12^{d}
Hazelnut	216.9 ± 22.6^{f}	412.8 ± 15.4^{e}	84.3 ± 13.5^{d}	112.4 ± 18.0^{d}	24.2 ± 0.4^{i}	45.2 ± 1.3^{e}	1.48 ± 0.04^{g}	2.93 ± 0.09^{c}	1.92 ± 0.19^{d}	2.12 ± 0.11^{d}
Pecan	329.1 ± 12.5^{f}	2047.3 ± 27.6^{a}	107.3 ± 17.2^{d}	786.7 ± 31.5^{a}	35.6 ± 0.8^{g}	79.2 ± 1.2^{a}	0.75 ± 0.07^{i}	4.04 ± 0.08^{a}	1.85 ± 0.17^{d}	8.63 ± 0.25^{a}
Pine Nut	254.7 ± 18.6^{f}	689.9 ± 17.5^{d}	76.1 ± 12.2^{e}	181.6 ± 29.0^{c}	10.2 ± 0.2^{k}	38.6 ± 0.8^{f}	1.39 ± 0.02^{g}	1.99 ± 0.04^{f}	1.33 ± 0.12^{e}	2.47 ± 0.20^{d}
Pistachio	258.7 ± 13.7^{f}	585.9 ± 18.7^{d}	87.4 ± 14.0^{e}	214.5 ± 34.3^{c}	15.0 ± 0.4^{i}	48.5 ± 0.7^{d}	1.08 ± 0.07^{h}	2.24 ± 0.06^{d}	2.57 ± 0.19^{d}	3.89 ± 0.21^{c}
Walnut	298.0 ± 16.2^{f}	958.9 ± 20.7^{c}	98.3 ± 15.7^{d}	337.1 ± 27.0^{b}	33.6 ± 0.8^{g}	70.0 ± 1.4^{b}	1.53 ± 0.07^{g}	3.41 ± 0.03^{b}	2.96 ± 0.26^{d}	6.88 ± 0.24^{b}

Values bearing different superscripts for each set of data for hexane and chloroform-methanol extracts are significantly ($p \leqslant 0.05$) different.
Symbols are: ABTS radical anion; DPPH, diphenyl-2-picrylhydrazyl; ORAC, oxygen radical absorbance capacity and PCL, phtochemilumenescence inhibition capacity.

The relatively strong antioxidant activities of pecan and walnut oil extracts may be due to their high content of tocopherols that is rich in γ-tocopherol. However, the α-tocopherol equivalence values of tree nut oil extracts obtained using the DPPH radical scavenging assay do not strictly correlate with their actual tocopherol contents since the chloroform/methanol extracts exhibited significantly higher antioxidant activities than their hexane extracted counterparts, although both contained similar tocopherol contents. This indicates that non-tocopherol components present in the chloroform/methanol extracts also contributed to the total antioxidant capacity of the extracts. Besides tocopherols, other antioxidative minor components of tree nut oils include phospholipids (Hildebrand, Terao, & Kito, 1984), phytosterols and phytosterol conjugates (Wang et al., 2002), and possibly non-tocopherol phenolics, among others (Jung, Yoon, & Min, 1989). Synergistic antioxidant activities have been reported between tocopherols and nitrogen-containing phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (Segwa, Kamata, & Totani, 1995), all of which are present in tree nut oils and their minor component extracts and may help explain the high antioxidant activities observed in this work, particularly for chloroform/methanol extracted oil extracts.

The DPPH radical scavenging capacity of tree nut oil extracts have not previously been reported. The DPPH radical scavenging activity of black cumin (*N. sativa* L.), coriander (*C. sativum* L.), and Niger (*G. abyssinica* Cass.) oil extracts have been studied, showing that coriander oil exhibited the greatest DPPH radical scavenging activity, followed by black cumin oil and then Niger oil (Ramadan et al., 2003). The antioxidant activities were attributed to both phenolic and non-phenolic compounds such as phospholipids present in the minor component extracts.

3.6. Inhibition of β-carotene bleaching of tree nut oil extracts

The β-carotene bleaching test is a convenient test used to measure the ability of a compound or a mixture to inhibit the oxidation of β-carotene. Results showed that the extract of chloroform/methanol extracted pecan oil exhibited the highest antioxidant activity, with 79% of β-carotene remaining after 120 min of assay (Table 4). The chloroform/methanol extracted walnut oil possessed the second highest activity (70% of β-carotene remaining after 120 min assay), followed by chloroform/methanol extracted Brazil nut oil (62.5% of β-carotene remaining after 120 min assay). Hexane extracted almond oil exhibited the lowest antioxidant activity (7.4% of β-carotene remaining after 120 min assay). The β-carotene bleaching test is similar to an oil-in-water emulsion system; differences in the solubilities of antioxidant compounds influence their activity in this assay. Hydrophobic antioxidants are reported to perform more efficiently than hydrophilic antioxidants in the β-carotene bleaching test by orienting themselves in the lipid phase and the lipid–water interface, thus directly combating lipid radical formation and β-carotene oxidation (Frankel & Meyer, 2000). The strong activity of chloroform/methanol extracted oil minor components may be due to their higher level of hydrophobic antioxidants such as tocopherols.

3.7. Oxygen radical absorbance capacity (ORAC) of tree nut oil extracts

The ORAC assay is a free radical scavenging assay; it is based on the time resolved fluorescence of an oxidizable compound, namely fluorescein. In the presence of antioxidants, the oxidation and subsequent loss of fluorescence by fluorescein is inhibited and the extent of this inhibition is directly related to antioxidant activity and/or antioxidant concentration, usually expressed in Trolox or α-tocopherol equivalents. The ORAC values of samples are derived by calculating the area under curve (AUC) of the time resolved fluorescence graph for the assay containing the sample, which is then used along with a standard curve to calculate the ORAC value as equivalents of a pure antioxidant. Results showed that extracts of chloroform/methanol extracted oils possessed higher ORAC values than their hexane extracted counterparts (Table 4) ($p \leqslant 0.05$). Among chloroform/methanol extracted oils, pecan oil extract possessed the highest ORAC value (4.04 μmol α-tocopherol equivalents/g oil equivalents), followed by walnut oil (3.41 μmol α-tocopherol equivalents/g oil equivalents), hazelnut oil (2.93 μmol α-tocopherol equivalents/g oil equivalents), pistachio oil (2.24 μmol α-tocopherol equivalents/g oil equivalents), almond oil (2.12 μmol α-tocopherol equivalents/g oil equivalents), and finally, pine nut oil and Brazil nut oil (1.99 and 1.96 μmol α-tocopherol equivalents/g oil equivalents, respectively) ($p \leqslant 0.05$). Among the hexane extracted oils, those of walnut, hazelnut, pine nut and Brazil nut had similar ORAC values (1.53, 1.48, 1.39 and 1.34 μmol α-tocopherol equivalents/g oil equivalents, respectively), followed by pistachio and almond oils (1.08 and 0.97 μmol α-tocopherol equivalents/g oil equivalents, respectively) and finally, pecan oil (0.75 μmol α-tocopherol equivalents/g oil equivalents) ($p \leqslant 0.05$).

The ORAC of hexane/dichloromethane (1:1, v/v) extracts of tree nuts have previously been reported by Wu et al. (2004), showing that lipidic extract of Brazil nut possessed the highest antioxidant activity (5.6 μmol of Trolox equivalents/g), followed by walnut, cashew, pecan, hazelnut, pine nut and almond (4.8, 4.7, 4.2, 3.7, 2.8 and 1.7 μmol of Trolox equivalents/g, respectively). Since Wu et al. (2004) used a different antioxidant extraction procedure and ORAC standard, therefore direct comparison of ORAC values obtained here with their results is impossible; however, when comparing the overall trends among samples, some striking similarities exist. These include relatively high antioxidant activities for lipidic extracts of Brazil nuts, walnuts and pecans and lowest activities for almond extracts. In addition, the observed differences between this work and that of Wu et al. (2004) may also be partly attributable to cultivar for nut varieties studied and/or cultivation and climatic conditions. For example, an investigation of lipid compositions of various walnut oil cultivars revealed that the amount of total tocopherols can vary by up to 40% (269–436 mg tocopherols/kg oil) (Savage, Dutta, & McNeil, 1999).

3.8. Photochemiluminescence (PCL) inhibition assay for evaluation of antioxidant activity of tree nut oil extracts

The PCL inhibition assay measures the superoxide scavenging capacity of tree nut oil extracts. In the early PCL inhibition methods, superoxide generation was mediated by the xanthine oxidase system (Ricci, Fraternale, Giamperi, Epifano, & Curini, 2005); however, problems associated with enzyme activity and method reproducibility led to the development of PCL inhibition methods using photogenerated superoxide (Popov & Lewin, 1996). In this work, an automated version of the PCL inhibition assay was used with luminol acting as both the photosensitizer and the superoxide radical detection agent.

Among oils tested, extracts of chloroform/methanol extracted oils possessed a higher antioxidant activity in the PCL inhibition assay compared to hexane extracted oils (Table 4). Among chloroform/methanol extracted oils, pecan oil extract exhibited the highest PCL inhibition activity (8.63 μmol α-tocopherol/g oil), followed by extracts of walnut, pistachio, hazelnut and almond oils (6.88, 3.89, 2.47, 2.12 and 2.11 μmol α-tocopherol/g oil, respectively), and finally, Brazil nut oil extract (1.81 μmol α-tocopherol/g oil). Among hexane extracted oils, walnut oil extract had the highest PCL inhibition activity (2.96 μmol α-tocopherol/g oil), followed by extracts of pistachio (2.57), then hazelnut (1.92), pecan

(1.85), pine nut (1.33), almond (1.30), and finally, Brazil nut oils at 1.23 μmol α-tocopherol/g oil, respectively.

The observed differences in PCL inhibition activity between the minor component extracts of chloroform/methanol extracted oils and their hexane extracted counterparts are likely due to compositional differences between the minor component extracts. Since chloroform/methanol extracted oils possessed higher amounts of antioxidative minor components such as tocopherols and phospholipids, their enhanced antioxidant activities can easily be attributed to this difference.

4. Conclusions

The tree nut oil minor component extracts were tested for their total phenolic content and their antioxidant activity was examined using the TEAC assay, DPPH radical scavenging assay, β-carotene bleaching test, PCL inhibition assay and ORAC assay. Similar antioxidant activity trends were observed in all tests employed in this study, with extracts of chloroform/methanol extracted oils exhibiting higher activities than extracts of hexane extracted oils. Minor component extracts of chloroform/methanol extracted pecan and walnut oils possessed the highest antioxidant activities among samples tested, which is likely due to their high amounts of tocopherols and other antioxidative minor components such as phospholipids and possibly other unidentified phenolic and/or non-phenolic components present in the extracts. Minor component extracts of almond oils were the least active among samples examined in this study. Isolating the active components of tree nut oil minor component extracts and identifying their individual contribution to the overall antioxidant activity would be of paramount importance in understanding the reason(s) for the observed high activities of certain nut oil extracts examined.

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