



Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Production, composition and toxicology studies of Enzogenol[®] *Pinus radiata* bark extract

Mathias A.E. Frevel^{a,*}, Andrew Pipingas^b, Warren J. Grigsby^c, Chris M. Frampton^d, Nigel L. Gilchrist^e

^a ENZO Nutraceuticals Limited, P.O. Box 303503, North Harbour, Auckland 0751, New Zealand

^b Centre for Human Psychopharmacology, Swinburne University of Technology, Mail H24, P.O. Box 218, Hawthorn, VIC 3122, Australia

^c Biopolymer Network Limited, Scion, Private Bag 3020, Rotorua 3046, New Zealand

^d Department of Medicine, University of Otago, P.O. Box 4345, Christchurch 8140, New Zealand

^e CGM Research Trust, P.O. Box 731, c/o The Princess Margaret Hospital, Cashmere Road, Christchurch, New Zealand

ARTICLE INFO

Article history:

Received 6 May 2012

Accepted 28 August 2012

Available online 12 September 2012

Keywords:

Pine bark extract

Proanthocyanidins

Toxicology

Food safety

ABSTRACT

Enzogenol[®] pine bark extract is a dietary supplement and food ingredient produced by water extraction of *Pinus radiata*. We present production method, composition, and safety data from rat and dog toxicological and human clinical studies. The dry powder contains proanthocyanidins (>80%), taxifolin (1–2%), other flavonoids and phenolic acids (up to 8%), and carbohydrates (5–10%). Reverse mutation assays showed lack of mutagenic activity. Single and 14-day repeat dosing in rats and dogs had no influence on body weight, feed consumption, blood chemistry, and haematology at any dose level. There were no treatment related findings on gross and detailed necropsy, organ weights, organ weight ratios and histology. The only adverse events were emesis and diarrhoea in dogs occurring mainly in un-fed condition and at the highest dose level in a total of 18% of applications. The MTD and NOAEL in the present rat and dog studies were 2500 and 750 mg/kg/day, respectively. Consumption of 480 mg/day for 6 months and 960 mg/day for 5 weeks in two human studies showed Enzogenol[®] had no adverse influence on liver and kidney function, haematology, and did not cause any adverse events. Our studies indicate lack of toxicity of Enzogenol[®] and support safe use as a food ingredient.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Pine bark extracts (PBE) from different pine species have been used commercially in food and dietary supplement products for several years as active ingredients for their antioxidant and anti-inflammatory properties attributed to the bioflavonoid composition of proanthocyanidins (PA), monomeric flavonoids and phenolic acids. PA, the most abundant flavonoid component of PBE, are flavan-3-ols composed most commonly of 2–10 units of catechin and epicatechin connected by different inter-flavan linkages resulting in varying oligo- and polymeric structures.

Abbreviations: BUN, blood urea nitrogen; CV, column volume; ESI MS, electron spray ionisation mass spectrometry; ¹H NMR, proton nuclear magnetic resonance; MTD, maximum tolerated dose; NOAEL, no observed adverse effect level; PA, proanthocyanidins; PBE, pine bark extract; QOL, quality of life; RP-HPLC, reverse phase-high performance liquid chromatography; SD, standard deviation; SF-36, short form 36 health survey; WHO, World Health Organization.

* Corresponding author. Mobile: +64 21 581933; fax: +64 9 9293409.

E-mail addresses: mattfrevel@enzo.co.nz (M.A.E. Frevel), Apipingas@groupwise.swin.edu.au (A. Pipingas), Warren.Grigsby@scionresearch.com (W.J. Grigsby), chris.frampton@chmeds.ac.nz (C.M. Frampton), nigel.gilchrist@gm-research.org.nz (N.L. Gilchrist).

Antioxidant and anti-inflammatory properties of PBE and PA have been documented in numerous studies (Cho et al., 2001; Guri et al., 2006; Packer et al., 1999; Wood et al., 2002). Various physiological effects in humans have been reported in clinical studies that point towards a number of potential benefits for cardiovascular and brain health associated with the consumption of PBE (Chayarisobhon, 2006; Pipingas et al., 2008; Senthilmohan et al., 2003; Shand et al., 2003; Young et al., 2006).

Despite the widespread use of PBE, there is very little original research available on the safety of these extracts. Here we report on the composition and safety of one particular extract from *Pinus radiata*, produced under the proprietary name Enzogenol[®].

Extractives of *P. radiata* bark were first characterised by Markham and Porter (1973) and Porter (1974) who found approximately 20% of high molecular weight PA, and several low molecular weight procyanidins B-1, B-3, B-6 and C-2; and approximately 2% by weight of taxifolin, the second most abundant phenolic in the bark. Other phenolics found in lower amounts included quercetin, myricetin, catechin, and astringenin with or without glycosylation. More recently, PA from hot-water extracts of *P. radiata* bark were investigated by Ku and Mun (2007). NMR spectroscopy showed that the PA comprised procyanidins to 94% with a

small amount of prodelphinidin (6%). The average molar mass (Mw, Mn), and degree of polymerisation (DP) of the PA were 3800 (Mw), 1200 (Mn), and 13 (DP), respectively.

In addition to toxicological studies on PBE, a comparison of its composition to the flavonoid content of common food sources may contribute to evaluating its safety for human consumption. Flavonoids are ubiquitous secondary plant metabolites that are naturally part of our diet when we consume plant based foods, including fruits and vegetables, juices, tea, red wine, chocolate or nuts. PA represent the most common type of flavonoids in our diet (Aron and Kennedy, 2008). Data of PA content is available for some foods, and some studies have attempted to give estimates of PA intake ranging from several tens to several hundreds of milligrams per day (Santos-Buelga and Scalbert, 2000). Prior and Gu (2005) calculated 53.6 mg/day/person as an average PA intake over all ages. Wang et al. (2011) estimated total PA intake at 95 mg/day/adult based on food consumption data for 8809 US adults. Gu et al. (2004) estimated daily PA intake in the US population at 57.7 mg/person. These estimates appear low, and likely reflect consumption of few plant based foods, as a diet with PA-rich foods would result in substantially higher PA intakes as we will show in our discussion.

Here we present the method of production for the *P. radiata* bark extract Enzogenol® with compositional analyses, and safety data from toxicological and human studies. We discuss the compositional data in comparison to dietary intake of PA and other flavonoids, offer an evaluation of the safety data for Enzogenol® with reference to the safety data available elsewhere for pine bark and other PA-rich extracts.

2. Materials and methods

2.1. Industrial scale production of Enzogenol®

The source raw material for Enzogenol® is the bark of 15–30 year old *P. radiata* trees grown for the purpose of timber production in New Zealand pine plantations. Bark obtained from felled timber logs is cleaned and visually inspected before it enters the extraction process. During inspection including metal detection any foreign matter, like wood chips or stones, and any bark that appears dirty or contaminated with mould, moss or other growth is removed. After the inspection the bark enters the closed-system, continuous flow, extract production that includes the following steps: (1) grinding, (2) washing, (3) extraction with de-ionised hot water (95–99 °C for 30 min), (4) removing extracted solids, (5) cooling of the raw liquor by heat exchanger, (6) concentrating raw liquor by reverse osmosis to approximately 25% dissolve solids, (7) continuous removal of undissolved solids, (8) freeze drying, (9) grinding, (10) blending, (11) quality control steps. The entire process uses only the screened pine bark and deionised water. There are no chemicals involved in the production process. Town drinking water that has passed through a deionisation unit resulting in conductivity of less than 1 mS/m is used for extraction. The process results in a very reproducible compound profile. After production the extract enters Quality Control undergoing physical, chemical, microbiological, heavy metal, herbicide and pesticide analyses to ensure conformity with its product specifications.

2.2. Compositional analyses

C18-reverse phase high performance liquid chromatography (C18-RP-HPLC) was carried out as described by Peng et al. (2001) to quantify PA, taxifolin, catechin and astrigenin in Enzogenol®. Instrumentation was a Waters 2690 Separation Module with 996 Photodiode Array Detector. The column was Phenomenex, Jupiter C18 5 µ (250 × 4.6 mm). Solvents and gradient as previously described adding 8 min re-equilibration. Enzogenol® samples were prepared at 1 mg/ml by dissolution of the dry powder in water at 37 °C in a sonicating water bath with sonication at maximum setting for 10 min. All samples were centrifuged at high speed and transferred to HPLC vials. Molecular standards at 0.01 mg/ml were (+)-catechin-hydrate (Sigma C-1251, 98% pure) in water, taxifolin = (+)-dihydroquercetin (Terra Biochem, 98% pure) in 20% acetonitrile, astrigenin = trans-3,3',4,5'-tetrahydroxystilbene (Cayman chemical, 98% pure) in 20% acetonitrile. The molecular standard for PA was prepared in house (95% pure), and used at 0.1 mg/ml in water. PA elute as one single peak in this HPLC (Fig. 1).

PA-standard was prepared using preparative chromatography with 30 ml column volume (CV) of Sephacryl HR-100 gel (Sigma: S100HR) equilibrated with 4 × CV distilled water. Enzogenol® (4 ml of a 10% solution in 10% acetone) was carefully

loaded. Starting flow of approximately 0.5 ml/min allowed the sample to enter the gel surface. Elution steps were under flow of 1 ml/min: first 4 × CV of distilled water, second 4 × CV of 20% acetone, third 4 × CV of 30% acetone. From the third elution we discarded the first 30 ml of flow through, and collected the following 90 ml, followed by freeze drying this fraction to obtain the PA standard. The resulting powder contains mainly tetramer and hexamer PA of *P. radiata* bark at a purity of greater than 95% as determined by HPLC and mass spectrometry.

Proton nuclear magnetic resonance (¹H NMR) analysis was conducted on a Bruker 400 MHz Avance instrument using a standard pulse sequence and acquisition time. The samples were dissolved in d₆-acetone/D₂O mixture (2 mg/mL) ensuring complete dissolution of the sample. Estimation of sample carbohydrate content used the integrated peak areas between 7.56–6.36 ppm and 4.26–3.10 ppm for aryl and carbohydrate peaks, respectively. The integrated areas were converted to the component mass ratios using the area of the 4.26–3.1 ppm integral and protons present on the sugar repeating units. This was then converted to % carbohydrate.

Electrospray ionisation mass spectrometry (ESI MS) samples were dissolved in methanol at 0.2–0.4 mg/5 mL, and injected directly into the electrospray mass spectrometer (Thermo Finnigan LCQ Deca XP ion trap mass spectrometer). Molecular ion patterns were attained with the instrument operating in negative ion mode. Definitive identification of peaks was assisted by reference.

2.3. Bacterial reverse mutation test (Ames test)

Enzogenol® was examined for its mutagenic potential in histidine auxotrophic *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 (from NCTC, Central Public Health Laboratory, HPA, London, UK), and in the tryptophan auxotrophic *Escherichia coli* strain WP2 uvrA (pKM101) (from NCIMB, Aberdeen, Scotland, UK) using the treat and plate method (Ames et al., 1975; Maron and Ames, 1983). After preliminary testing established a maximum dose of 5000 µg/plate to be feasible two assay series with different test substance concentrations were carried out. In the first assay tester bacteria were exposed in triplicate to 50, 158, 500, 1581, and 5000 µg/plate of Enzogenol® with and without S-9 mixture. In the second assay concentrations were 100, 266, 701, 1880, and 5000 µg/plate. The following controls were run simultaneously: positive for all *S. typhimurium* strains 2-aminoanthracene 4 µg/plate; positive for *E. coli* 2-aminoanthracene 30 µg/plate; negative for TA98 2-nitrofluorene 2 µg/plate, for TA100 and TA 1535 sodium azide 1 µg/plate, for TA1537 9-aminoacridine 50 µg/plate; negative for *E. coli* 4-nitroquinoline-1-oxide 4 µg/plate. Chemicals and standards were obtained from Sigma–Aldrich Chemie GmbH, Germany, and Sigma–Aldrich Inc, St. Luis, USA.

2.4. Oral toxicity and maximum tolerated dose study (MTD) in rats

This was a two-phase toxicity study in Sprague Dawley rats aged 9–10 weeks with the test substance being Enzogenol® solution prepared in sterile distilled water (5 mg/ml). Rats were bred and sourced from Department of Safety Assessment, Advinus Therapeutics Private Limited, Bangalore, India. Rats were housed under standard laboratory conditions, air conditioned (13.5 air changes/hour; 19–24 °C; 65–68% relative humidity) with 12 h light and 12 h dark cycle. Rats were housed individually or in pairs. Standard food pellets (Ssniff rats/mice pellet food, by Ssniff Spezialdiäten GmbH, Germany) and water was supplied *ad libitum*.

Phase I comprised 5 groups of 2 males and 2 females per group. Treatment was by single oral gavage administration at dose levels of 500 (G1a), 1000 (G2a), 1500 (G3a), 2000 (G4a) or 2500 (G5a) mg/kg body weight. Phase II comprised 4 main groups (G1b–G4b), each consisting of 10 male and 10 female rats, and 4 recovery groups (G1bR–G4bR), each consisting of 5 males and 5 females. In phase II, treatment was administered once daily at dose rates of 0 (G1b/G1bR), 1000 (G2b/G2bR), 2000 (G3b/G3bR) or 2500 (G4b/G4bR) mg/kg for 14 consecutive days. The dose of 2500 mg/kg body weight was the highest feasible dose based on solubility of Enzogenol® in water and a maximal practicable gavage volume of 20 ml/kg body weight. Animals in the recovery groups were observed for an additional 14 days without treatment following the initial 14 day treatment period.

In phase I, all rats were observed for mortality and clinical signs; body weights were recorded on days 1, 4, 8, and food intake calculated for days 1–4 and 4–8. Animals were subjected to detailed necropsy on day 8. In phase II, all rats were observed for mortality and clinical signs once daily. Body weights were recorded on days 1, 4, 7, 11, 14, and in the recovery group also on days 21 and 28. Food intake was calculated for days 1–4, 4–7, 7–11, 11–14, and in the recovery group also for days 14–21 and 21–28. Ophthalmological examination was performed before the start of treatment and prior to terminal sacrifice. Haematology, coagulation, clinical chemistry and urine analysis were performed in Phase II animals prior to sacrifice of main groups on day 15 and in recovery groups on day 29. Detailed necropsies were carried out on all rats after sacrifice. Organ weights for adrenal glands, brain, epididymides/uterus, heart, kidneys, liver, ovaries/testes, spleen and thymus were recorded and relative organ weights to the fasting body weights were calculated. Tissues were collected, preserved and examined for gross lesions in all animals. Histopathological examination was carried out on phase II control (G1b) and high dose (G4b) group animals. Gross lesions from all animals were examined microscopically. Tissues were processed for routine paraffin embedding and 5 micron sections were stained with Mayer's haematoxylin eosin stain. Details on tissues and methods are available in the online Supplementary data.

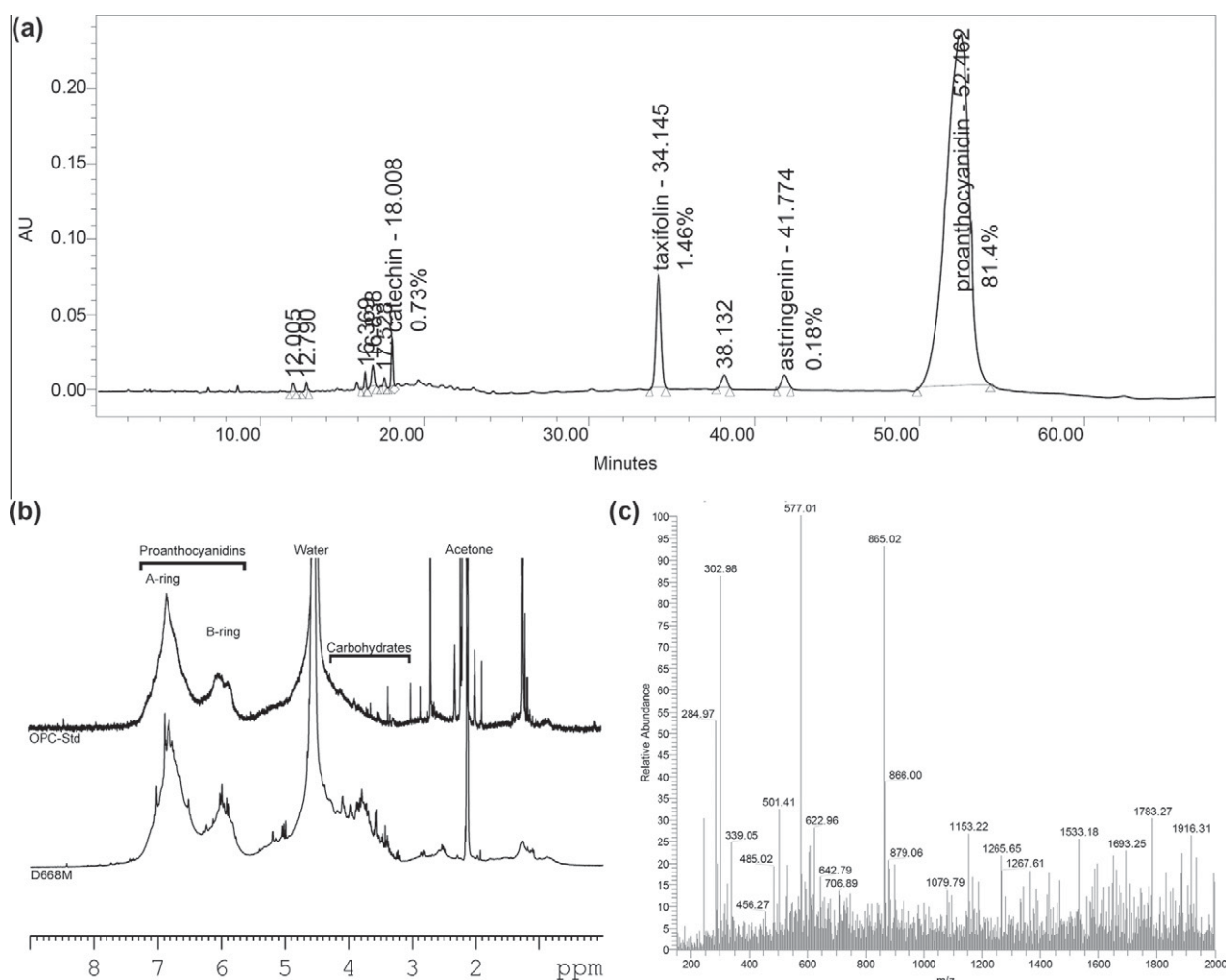


Fig. 1. C18-RP HPLC (a), ^1H NMR (b), and ESI MS analyses (c) of Enzogenol[®] pine bark extract batch# D668M.

Table 1
Blood urea nitrogen values in dogs in phase II.

Group		G1 and G1R	G2	G3	G4 and G4R
Dose (mg/kg/day)		0	300	750	1250
Males	Day 1	5.01 (± 1.29)	4.18 (± 0.81)	4.09 (± 0.15)	4.12 (± 0.61)
	Day 15	4.77 (± 0.79)	4.10 (± 0.83)	3.21* (± 0.28)	3.24* (± 0.47)
Females	Day 1	4.07 (± 0.56)	3.20 (± 0.64)	4.13 (± 0.60)	3.82 (± 0.50)
	Day 15	4.64 (± 0.89)	3.30* (± 0.54)	3.57 (± 0.54)	3.26* (± 0.54)

* Significantly lower than controls.

2.5. Oral toxicity and maximum tolerated dose study (MTD) in dogs

This was a two phase study in Beagle dogs. Phase I was to determine the MTD using dose escalation in one male and one female dog (age 9–10 months) using doses of 300, 750 and 1250 mg/kg body weight per day (10 ml/kg) under fasted condition, and at 300 and 1250 mg/kg/day under fed condition with a minimum 48-h washout period between doses.

Phase II was a 14-day repeated dose study to determine toxicity and kinetic profile, and estimate the No Observed Adverse Effect Level (NOAEL) in dogs. Thirty-two Beagle dogs (16 of each sex, age 10–12 months) were assigned to vehicle control (G1), low (G2), mid (G3) and high dose (G4) groups, 3 males and 3 females each, and two recovery groups (G1R and G4R) with 2 males and 2 females each. Dosages administered under fed conditions were 300 (G2), 750 (G3), and 1250 (G4 and G4R) mg/kg/day. All administration of Enzogenol[®] was as suspension in distilled water by oral gavage with 10 ml/kg body weight. Vehicle controls received 10 ml water per kg body weight.

Dogs were acclimatised for 7 or 5 days for phase I and II, respectively, and observed once daily for any abnormalities. Dogs were bred and sourced from Department of Safety Assessment, Advinus Therapeutics Private Limited, Bangalore, India. All dogs were declared suitable for the study by the study veterinarian. Housing was

in individual floor pens, at 21–24 °C, 66–67% RH, with 12 h light dark cycles. Dogs were fed standard dog chow (Pedigree, manufactured by Mars International). For treatments under fed condition, 300 g of food was offered to each dog starting 1 h before and was withdrawn at time of dosing. Food was offered to each dog 2 h after dosing and stayed available for a minimum duration of 3 h per day after which the food was withdrawn. Purified water was *ad libitum*. Dosing was in the morning hours at approximately 10 am \pm 1 h.

Assessments included clinical signs, changes in body weight, food consumption, electrocardiogram (ECG), haematology, clinical chemistry, urine analysis, toxicokinetics, gross pathology, organ weights and histopathological examination. Catechin and taxifolin standards were HPLC grade obtained from Sigma–Aldrich Production GmbH, Buchs, Switzerland. Further details of the assessment methods are available in the online Supplementary data.

2.6. Human clinical studies

2.6.1. Five-week brain function trial

This was a 5-week, randomised, double-blind parallel design study with the treatment group receiving 960 mg Enzogenol[®] + 120 mg vitamin C and the control group receiving 120 mg vitamin C only per day. There were 44 men aged 50–65

Table 2
Safety parameters in 5-week cognitive function clinical trial.^a

	Enzogenol® group (n = 22)		Control group (n = 20)		Normal range
	Mean ± SD		Mean ± SD		
	Baseline	5 weeks	Baseline	5 weeks	
<i>Urea and electrolytes</i>					
Sodium (mmol/L)	141.1 ± 2.3	141.1 ± 1.9	141.3 ± 1.98	140.3 ± 2.7	[135–145]
Potassium (mmol/L)	4.3 ± 0.3	4.3 ± 0.2	4.3 ± 0.2	4.3 ± 0.2	[3.5–5]
Chloride (mmol/L)	103.7 ± 2.4	104 ± 2.5	104.2 ± 1.8	103.3 ± 2.1	[98–106]
Bicarbonate (mmol/L)	27.3 ± 2.3	27.5 ± 1.9	27.5 ± 2.5	27.5 ± 3.3	[20–29]
Urea (mmol/L)	5.9 ± 1.1	5.6 ± 1.1	5.6 ± 1.3	5.7 ± 1.4	[2.1–8.5]
Creatinine (mmol/L)	0.09 ± 0.01	0.09 ± 0.11	0.084 ± 0.01	0.08 ± 0.01	[0.06–0.12]
Anion gap (µmol/L)	14.41 ± 3.2	13.9 ± 2.1	13.8 ± 2.1	13.7 ± 2.8	[8–16]
<i>Liver Function Tests</i>					
ALT (U/L)	37.4 ± 21.3	36.7 ± 18.3	31.5 ± 15.8	29.7 ± 10.6	[0–41]
AST (U/L)	28.8 ± 18.2	30.1 ± 22.1	24.6 ± 7.7	21.9 ± 6.2	[0–38]
Alk Phos (U/L)	73.9 ± 19.1	75.0 ± 19.7	70.5 ± 27.2	71.6 ± 22.0	[50–160]
GGT (U/L)	51.5 ± 71.6	48.6 ± 52.9	27.5 ± 22.8	27.6 ± 19.6	[5–80]
Bilirubin (µmol/L)	13 ± 4.8	12.7 ± 4.0	13.4 ± 4.4	12.8 ± 4.8	[0–22]
Albumin (g/L)	44.1 ± 2.8	44.7 ± 3.2	43.2 ± 2.2	43.4 ± 2.0	[35–50]
Globulin (g/L)	28.6 ± 4.4	27.4 ± 3.3	28.8 ± 4.4	28.7 ± 3.4	[23–35]
Total Protein (g/L)	72.7 ± 3.9	72.1 ± 3.8	72.2 ± 4.1	71.6 ± 3.4	[60–84]
<i>Full blood counts</i>					
Haemoglobin (g/L)	152.1 ± 9.9	152.6 ± 0.03	151.4 ± 6.7	151.2 ± 7.0	[130–180]
Haematocrit (%)	0.45 ± 0.3	0.45 ± 0.03	0.4 ± 0.02	0.44 ± 0.02	[0.37–0.55]
Red blood cells (x10 ¹² /L)	5.0 ± 0.4	4.98 ± 0.32	4.9 ± 0.30	4.93 ± 0.4	[4.2–6.9]
MCV (fL)	90.3 ± 2.6	90.7 ± 2.64	90.5 ± 4.2	90.1 ± 3.7	[76–100]
MCH (p/L)	30.7 ± 1.0	30.8 ± 1.1	30.8 ± 1.7	30.8 ± 1.6	[27–32]
MCHC (g/L)	339.2 ± 5.8	338.8 ± 6.6	340.7 ± 6.5	342.2 ± 7.9	[310–360]
RDW	13.4 ± 0.7	13.5 ± 0.7	13.4 ± 0.6	13.5 ± 0.72	[10–14.5]
Platelet count (x10 ⁹ /L)	241.6 ± 37.8	227.5 ± 64.2	221.9 ± 38.7	222.1 ± 39.9	[150–400]
White blood cells (x10 ⁹ /L)	6.4 ± 1.5	6.5 ± 1.4	6.2 ± 1.3	6.3 ± 1.3	[4–11]
Neutrophil (x10 ⁹ /L)	3.6 ± 1.1	3.8 ± 1.1	3.6 ± 0.9	3.7 ± 0.95	[2.5–7.5]
Lymphocytes (x10 ⁹ /L)	2.0 ± 0.5	1.98 ± 0.49	1.9 ± 0.5	1.87 ± 0.6	[1–4]
Monocytes (x10 ⁹ /L)	0.6 ± 0.15	0.55 ± 0.14	0.5 ± 0.1	0.5 ± 0.13	[0–1]
Eosinophils (x10 ⁹ /L)	0.16 ± 0.13	0.16 ± 0.13	0.2 ± 0.1	0.2 ± 0.15	[0–0.7]
Basophils (x10 ⁹ /L)	0.009 ± 0.03	0.03 ± 0.05	0.005 ± 0.02	0.01 ± 0.03	[0–0.03]
<i>Lipids</i>					
Cholesterol (mmol/L)	5.4 ± 0.8	5.45 ± 0.70	5.3 ± 0.9	5.3 ± 0.93	[<6]
HDL (mmol/L)	1.4 ± 0.34	1.42 ± 0.4	1.43 ± 0.32	1.42 ± 0.28	[>1]
LDL (mmol/L)	3.3 ± 0.75	3.3 ± 0.7	3.14 ± 0.82	3.15 ± 0.86	[<4.1]
LDL/HDL ratio	2.4 ± 0.7	2.41 ± 0.72	2.31 ± 0.85	2.31 ± 0.78	[<4]

^a ANOVA showed no significant difference in change from baseline to 5-weeks between groups on any of the safety parameters. Therefore, *p*-values are not shown here.

(58.3 ± 4.1 mean ± SD) with mean body weight of 92.8 kg ± 17.2 (SD), and a body mass index of greater than 25 in the study. The mean Enzogenol® dose in the treatment group was 10.3 mg/kg body weight per day. Relevant for safety are the full venous blood analyses shown in Table 2, and adverse event records. Detailed study design and methodology have been reported by Pipingas et al. (2008).

2.6.2. Six-month general health trial in elderly retirement village residents

This randomised, double-blind, controlled, parallel-design study was carried out by the Canterbury Geriatric Medical Research Trust under Dr. Nigel Gilchrist. Ethics approval was given by the Canterbury Ethics Committee, University of Canterbury, Christchurch, NZ.

The study enrolled 60 retirement village residents above age 70 that were cognitively intact and independently mobile. Excluded were individuals with heavy alcohol or tobacco use, taking any flavonoid or antioxidant preparations, having significant lung, renal, cardiac, hepatic, or psychiatric disease, or being unable to complete any of the assessments used in the study. Participants were randomly assigned to treatment and control groups.

Treatment was 4 capsules/day containing 120 mg Enzogenol® and 15 mg vitamin C each, taken with water as one dose in the morning; controls received 4 identically looking capsules containing 15 mg vitamin C each. 52 participants completed the study, 1 deceased, 7 withdrew (see details in Table 4).

The study investigated the following safety parameters: full blood count, electrolytes, liver and kidney function tests, measured at baseline (day before first dose of treatment) and after 6 months of treatment. Adverse events were recorded throughout the study. General health assessments at baseline, 3 and 6 months were SF-36 and WHO Quality Of Life questionnaires, Berg Balance, and Timed Up and Go test. Ophthalmic measurements were taken at baseline and 6 months. Participant's eyes were evaluated by checking the refraction for best-corrected visual acuity and near acuity along with general ocular examination, including dilating the pupils to check the lens and macula. Cataracts, if present, were assessed as capsular, cortical or nuclear and graded from 0 to 4. Any other eye abnormalities were recorded.

2.7. Statistical analyses

All continuous outcome measures from the animal studies were tested for between dose group differences using ANOVA. Where the ANOVA indicated significant differences between groups these were further explored using Dunnett's tests between each dose group and the control. In situations where the data was not normally distributed or there was evidence of heteroscedasticity then data were transformed prior to analysis. A *p*-value <0.05 was taken to indicate statistical significance.

The changes from baseline in both human clinical trials were compared between Enzogenol® and control groups using repeated measures ANOVA. Where variables were non-normally distributed the changes were compared using the non-parametric Mann-Whitney U test. Given the nature of this safety data, whereby elevated type I error rates would represent a conservative approach to the assessment of safety a two-tailed *p*-value <0.05 was taken to indicate statistical significance.

3. Results

3.1. Compositional analyses

HPLC of 21 consecutive production batches of Enzogenol® during 2009–2011 showed the mean content (±SD) of the three most abundant polyphenolic components of Enzogenol® are: PA 84.3% (±3.7), taxifolin 1.47% (±0.14), and catechin 0.67% (±0.08). Low standard deviations indicate a high consistency of extract composition between batches. Fig. 1 shows a representative HPLC for Enzogenol® batch D668M quantifying the above phenolics, and one

Table 3
Safety parameters in 6-month clinical trial in elderly retirement village residents.

	Enzogenol® group (n = 31)		Control group (n = 29)		p ^a
	Mean ± SD		Mean ± SD		
	Baseline	6 months	Baseline	6 months	
Age	82.6 ± 5.6		80.9 ± 5.4		
Gender	20F/11 M	20F/11 M	21F/8 M	18F/8 M	
Heart rate	71.9 ± 2.1	72.0 ± 1.9	69.7 ± 1.9	71.2 ± 1.7	0.47
Systolic BP	144.9 ± 4.0	137.7 ± 3.9	143.1 ± 4.0	135.0 ± 3.4	0.98
Diastolic BP	75.8 ± 2.0	73.3 ± 1.6	75.4 ± 1.7	72.7 ± 1.7	0.75
Body Mass Index	26.6 ± 0.8	26.5 ± 0.8	25.3 ± 1.0	26.5 ± 1.0	0.09
<i>Blood tests</i>					
Sodium (mmol/L)	141.1 ± 2.4	140.68 ± 2.48	139.93 ± 4.11	139.81 ± 3.2	0.65
Potassium (mmol/L)	3.91 ± 0.4	3.92 ± 0.4	3.78 ± 0.31	3.84 ± 0.26	0.55
Creatinine (mmol/L)	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.03	0.09 ± 0.03	0.16
ALT (U/L)	20.48 ± 7.99	12.55 ± 6.46	20.28 ± 7.02	13.65 ± 5.58	0.63
AST (U/L)	23.61 ± 5.11	24.84 ± 6.36	23.62 ± 4.62	24.23 ± 4.06	0.47
Alk Phos (U/L)	113.03 ± 114.3	114.87 ± 112.2	91.9 ± 57.12	100.5 ± 62.14	0.16
GGT (U/L)	26.87 ± 21.28	24.87 ± 19.31	24.86 ± 12.34	26.96 ± 18.72	0.25
Albumin (g/L)	38 ± 2.67	38.77 ± 3.72	38.21 ± 2.85	38.69 ± 2.81	0.73
Total protein (g/L)	73.26 ± 4.2	75.19 ± 4.85	72.76 ± 4.52	74.19 ± 4.24	0.57
Haemoglobin (g/L)	134.06 ± 13.52	133.23 ± 14.43	135.34 ± 9.51	133.62 ± 10.27	0.40
Haematocrit (%)	0.4 ± 0.04	0.4 ± 0.04	0.4 ± 0.03	0.4 ± 0.03	0.47
Red blood cells (x10 ¹² /L)	4.28 ± 0.5	4.31 ± 0.47	4.3 ± 0.36	4.32 ± 0.36	0.48
MCV (fL)	92.32 ± 3.69	91.9 ± 3.83	93.17 ± 4.7	92.19 ± 4.45	1.00
MCH (p/L)	31.42 ± 1.36	30.9 ± 1.49	31.69 ± 1.67	30.96 ± 1.84	0.94
MCHC (g/L)	340.1 ± 7.08	336.29 ± 6.5	338.9 ± 7.05	335.62 ± 7.26	0.82
Platelet count (x10 ⁹ /L)	275.29 ± 66.75	265.1 ± 65.16	293.97 ± 97.96	286.38 ± 77.06	0.90
White blood cells (x10 ⁹ /L)	6.32 ± 1.55	6.15 ± 1.65	6.51 ± 1.22	6.76 ± 1.69	0.11
Neutrophil (x10 ⁹ /L)	4 ± 1.2	3.92 ± 1.36	4.24 ± 1.17	4.53 ± 1.64	0.23 ^b
Lymphocytes (x10 ⁹ /L)	1.5 ± 0.62	1.44 ± 0.51	1.53 ± 0.45	1.45 ± 0.44	0.44 ^b
Monocytes (x10 ⁹ /L)	0.58 ± 0.2	0.55 ± 0.19	0.56 ± 0.18	0.57 ± 0.18	0.09 ^b
Eosinophils (x10 ⁹ /L)	0.22 ± 0.12	0.22 ± 0.1	0.18 ± 0.09	0.18 ± 0.09	0.67 ^b
Basophils (x10 ⁹ /L)	0.01 ± 0.03	0.01 ± 0.02	0.01 ± 0.04	0.01 ± 0.03	0.29 ^b

^a *p*-values for difference in change from baseline to 6-months between groups by ANOVA.

^b Mann–Whitney U-test when data distribution was not normal.

Table 4
Adverse events in 6-month clinical trial in elderly retirement village residents.

Type of event	Total number of events/Number of serious events (H = hospital)/ Number of temporary stop (TS) or withdrawal (W) from study	
	Enzogenol® group	Control group
Gastrointestinal	7/0/3 TS	8/0/2 TS, 1 W
Injury	3/0/0	2/0/1 TS
Cardiovascular	2/0/0	3/0/2 W
Back pain	1/0/0	0
Cerebrovascular accident	0	2/1 H, 1 Death/1 TS, 1 W
Psychiatric	0	4/1 H/0
Influenza/rhinitis	2/0/0	7/0/1 TS
Chest infection	9/3/0	0
Deep vein thrombosis	0	1/1 H/1 TS
Pain	4/0/0	2/0/0
Skin disorder	4/0/0	1/0/0
Anaemia	1/0/0	0
Oedema	1/0/0	0
Paraesthesia	1/0/0	0
Surgical procedure	2/1H/0	3/1 H/1 TS
Lethargy	0	2/0/1 W
Urinary disorder	0	3/0/0
Weight loss	1/0/0	0
Septicaemia	0	1/1 H/0
Venous occlusion	0	1/0/0
Eye/vision	0	2/0/1 TS
Dehydration	0	1/1 H/0
Total	38/3, 1H/0	43/6 H, 1 Death/8 TS, 5 W

additional compound, astringenin, a minor stilbene constituent of *P. radiata* bark. ¹H NMR analysis of batch D668M shows peaks between 5.6 and 7.5 ppm attributable to polyphenols, mainly PA, and peaks between 4.26 and 3.10 ppm due to the presence of carbohydrates with 8.6% content (Fig. 1b). ESI MS analysis of D668M

showed molecular ion peaks above the noise level at 285, 303, 577, and 865 consistent with the presence of monomeric catechin, taxifolin, PA-dimer and trimer, respectively. PA-tetramer peak (1153) was visible but already within the noise area. The spectrum did not sufficiently distinguish a molecular ion peak for PAs with

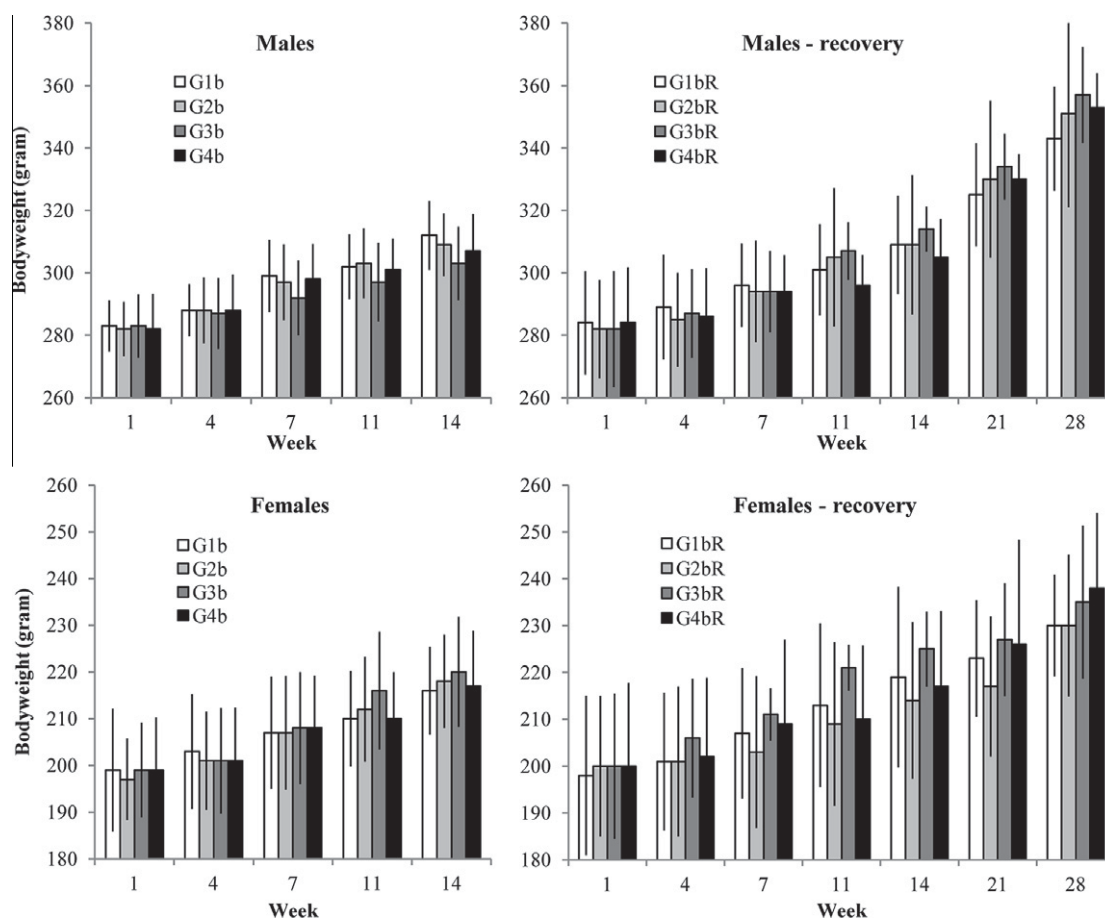


Fig. 2. Mean body weights of rats in main groups (G1b–G4b), and recovery groups (G1bR–G4bR) receiving 0, 1000, 2000, or 2500 mg/kg/day of Enzogenol® pine bark extract.

≥5 units due to the preferential ionisation of the lower molecular weight compounds (Fig. 1c).

3.2. Bacterial reverse mutation test (Ames test)

No increase in the number of revertant colonies occurred in the five test strains at any concentrations of Enzogenol® in either assay series in the presence or absence of S-9 mix indicating that Enzogenol® is non-mutagenic in the Ames test (see Ames test results in Supplementary data).

3.3. Oral toxicity and maximum tolerated dose study in rats

There were no deaths prior to sacrifice and no adverse clinical signs observed in any of the animals in either phase I or phase II of the study.

There were no treatment related eye abnormalities in either sex in any dose groups at the end of the treatment period or after the recovery period in phase II of the study. The treatment did not affect body weight gains at any dose level in either phase I or phase II (Fig. 2). There were no differences in food consumption between dosage groups in phase I. In phase II, there was no differences in food consumption in the main groups (G1b–G4b) for males or females, and in the recovery group females. In the male rats of the recovery group there were statistically significant marginal increases in food intake over some periods: G2bR, days 21–28 +11.8%; G3bR, days 11–14 +14.8%, and days 21–28 +13.7%; G4bR, days 14–21 +6.1% and 21–28 +15.1%. This marginal increased food intake is not considered a treatment related effect, because it was only seen in the recovery group males but not the

females. However, if this effect was treatment related it could reflect a rebound occurring after mild appetite suppression during the treatment period. This interpretation appears unlikely as group G3bR showed increased food consumption already during days 11–14 while still receiving Enzogenol, and group G2bR showed increased food consumption only from day 21, but not in days 14–21 when treatment had already stopped at day 14. Furthermore, there were no significant reductions or discernable trends of reduced food intake in males and females of the main treatment groups compared to controls.

There were no treatment related alterations in any of the haematology, coagulation, clinical chemistry or urine parameters evaluated in phase II in either males or females (detailed data tables in online Supplementary data).

No treatment related pathological changes were observed in this study. There were no significant intergroup differences in the terminal fasting body weights in males or females. For organ weights, a significant increase in mean gross liver weight was observed in recovery group males: G3bR +16% and G4bR +14%. This increase was considered incidental as there was no significant difference in relative liver weight (liver to body weight ratio), and no related microscopic changes in liver were found on histopathology. Organ weight changes were observed for uterus in different groups. These were found to be associated with the gross and histopathology finding of dilation of uterus observed in the control and treated groups, and, hence, were considered physiological, associated with the stage of the oestrous cycle.

The study indicates that on single dose administration the MTD for Enzogenol® in rats is greater than 2500 mg/kg body weight, and the NOAEL in rats is 2500 mg/kg/day under this 14-day treatment.

3.4. Oral toxicity and maximum tolerated dose study in dogs

There were no pre-terminal deaths in either phase of the study. There were no treatment related changes in terminal fasting body weight, organ weights and organ weight ratios. Pathology examination found one small white focus in the stomach of the male dog in Phase I, and single incidences of small testes were observed in mid and high dose males of Phase II microscopically confirmed as immature testes. These findings were considered not treatment related. There were no significant differences in the parameters of haematology, coagulation, urine analysis, and ECG (Phase II) in any of the male and female dogs in Phase I and II groups. On clinical chemistry, blood urea nitrogen (BUN) showed a significant decrease in mid (32.7%) and high dose (32%) males and low (28.8%) and high dose (29.7%) females (Table 1.). However, since low dose males (G2) and mid dose females (G3) did not show this decrease, BUN values for all groups were within the expected normal range of 2.14–7.50 mmol/L (Evans, 2009), and there was no liver pathology, the decrease in BUN was considered toxicologically insignificant. There were no differences in any of the other clinical chemistry parameters (detailed data tables in online Supplementary data).

One prominent, treatment related clinical sign observed in both phases in males and females was brownish emesis and brownish soft faeces or diarrhoea. In Phase I, emesis and soft faeces occurred at various time points post treatment (between 30 min to 4 h) at all three dose levels in both sexes in the fasted state. Under fed condition, there were no clinical observations at 300, 750, and 1250 mg/kg in either sex in phase I. During the 14-day phase II, with repeat-dose treatment under fed conditions, no toxic signs were observed in the low dose group; a single incidence of emesis and diarrhoea occurred in the mid dose group at 750 mg/kg. In the high dose group at 1250 mg/kg/day (G4/G4R), emesis with undigested food and brownish diarrhoea was observed in both sexes during some of the treatment days. The total incidence of emesis and diarrhoea over all treatments in Phases I and II was 18.6% (26 occurrences out of 140 treatments). No abnormalities were observed during the recovery period.

Toxicokinetics found plasma concentrations for unconjugated catechin, unconjugated taxifolin, and total catechin below the limit of quantification in low dose treatments. Total taxifolin was quantifiable at the low dose, indicating that the dogs had been exposed to the test article at this dose level. At dose levels of 750 and 1250 mg/kg, concentrations for all four analytes were observed for up to 2 h after dosing. Total taxifolin concentrations were observed in samples collected from animals at all dose levels. There was no quantifiable concentration of any of the analytes in plasma samples from control group animals. There was no evidence of any accumulation of the analytes in the dogs on repeat administration for 14 days.

In conclusion, the results indicated that the oral administration of Enzogenol® at 300, 750, and 1250 mg/kg/day had no effect on general health of the male and female dogs. The only observed adverse effect was emesis and diarrhoea, primarily in dogs in the fasted state in phase I, and to a lesser degree in the high dose, fed animals in phase II. Therefore, the MTD for Enzogenol® in fed Beagle dogs is considered to be 1250 mg/kg/day, and the NOAEL on repeated oral administration (14-days) to fed Beagle dogs is 750 mg/kg/day.

3.5. Human clinical studies

3.5.1. Five-week brain function trial

Results for brain function measurements have been published previously by Pipingas et al. (2008). Here we present the safety relevant blood analyses. Descriptive exploration of data followed by

analysis of variance showed that the Enzogenol® and the control group were not significantly different from each other at baseline and at the end of the study for all blood results including urea and electrolytes, liver function tests, full blood estimates and differential counts, and lipid profiles. Analysis of variance also showed that there were no significant changes in any of these parameters from measurements at baseline to the measurements after five weeks of taking 960 mg Enzogenol® per day (Table 2.). These findings indicated that Enzogenol® did not influence any of these safety parameters. There were no adverse events reported by any participant from either arm of the study. All participants completed the 5-week treatment period.

3.5.2. Six-month general health trial in elderly retirement village residents

There were no significant differences in age, gender, and assessment parameters at baseline indicating that treatment and control groups were well matched. Results of blood analyses in Table 3 show no significant differences between treatment and control groups on clinical chemistry, haematology, and liver and kidney function tests. In the treatment group, there were 38 adverse events, 4 of these serious. In the control group, there were 43 adverse events, 7 of these serious, one resulting in death. The number of adverse events in this study were due to the particular study population being elderly rest-home residents, average age 82. None of the adverse events were treatment related; details are shown in Table 4.

The study, did not find any differences between groups on the general health outcome measures of SF-36 and WHOQOL questionnaires, Berg Balance Test and Timing Up and Go Test. Eye exams at baseline and after 6 months of treatment showed no difference in cataract degeneration and macular degeneration. However, there were differences in distant vision tests for the left eye, and for the right and left eye combined (Table 5), and vision acuity decimal score decreased by 0.17 in the Enzogenol group versus a decrease of 0.92 in the controls group ($p = 0.075$) indicating a possible trend to better eye function in the treatment group over the six month period.

In conclusion, Enzogenol® appeared to have no effect in this elderly population on quality of life and sense of wellbeing, but may have had some benefit for eye sight in reducing deterioration of vision. Consumption of 480 mg Enzogenol® per day for six months appeared to be safe and well tolerated without any influence on blood parameters, and without causing any adverse effects.

4. Discussion

The presented studies provide evidence for lack of toxicity of the PBE, Enzogenol®. Ames tests have demonstrated lack of mutagenic activity. Single dose and 14-day repeat dose studies have shown no obvious toxicity at doses of up to 2500 mg/kg body weight in Sprague Dawley rats and up to 750 mg/kg body weight in Beagle dogs. The human studies presented here have used doses of 960 mg/day for 5 weeks, and 480 mg/day for 6 months without any ill-effects in people. These findings are consistent with other human clinical studies on Enzogenol® that reported no change in biochemical and haematological indices, and absence of any treatment related adverse events. In one study, 24 participants received a combination of 480 mg Enzogenol® and 240 mg vitamin C per day for 12 weeks (Shand et al., 2003); a second study used 480 mg of Enzogenol® plus 60 mg of vitamin C in 22 patients compared to a control group receiving vitamin C only for 12 weeks (Young et al., 2006); and a third study used 1200 mg Enzogenol® plus 600 mg vitamin C plus 300 IU vitamin E for 3 months in 12 individuals (Chayasirisobhon, 2006). A further human clinical

Table 5
Change in vision scores from baseline to 6 months.

		Deteriorated/Stable/Improved		P value
		Enzogenol® group	Control group	
Distant vision	Right eye	2/23/2	4/18/1	0.275
	Left eye	2/22/3	5/18/0	0.049
	Combined	4/45/5	9/36/1	0.028
Acuity change (decimal score)	Combined	−0.172	−0.920	0.075

Table 6
Example of PA-rich foods that may be consumed in a day as part of a healthy diet.

Food	PA content (mg/100 g) ^a	Daily consumption	PA intake (mg)
Tea (black)	13.42	2 cups (400 g)	53.68
Granny Smith apple	136.01	1 (180 g)	244.82
Nectarine	29.18	1 (110 g)	32.10
Plum, black diamond	247.27	1 (40 g)	98.91
Grapes, green	81.54	50 g	40.77
Almonds	184.02	30 g	55.21
Pecan nuts	494.05	25 g	123.51
Chocolate (72% cocoa)	1373.32 × 0.72	4 pieces (20 g)	381.48
Hot chocolate (Cocoa, dry powder)	1373.32	1 cup (5 g of cocoa)	68.67
Chilli with kidney beans	510.35	1 bowl (50 g beans)	255.18
Red wine	61.63	1 glass (200 g)	123.26
		Total:	1478 mg

^a US Department of Agriculture database for proanthocyanidin content of selected foods. August, 2004. Available at: <<http://www.nal.usda.gov/fnic/foodcomp/Data/PA/PA.html>>. Accessed 21.09.2011.

study with 60 participants that had sustained a mild traumatic brain injury using treatment with 1000 mg Enzogenol® in a single daily dose for 6 or 12 weeks has also found no treatment related adverse events (Feigin, 2012). The sum of these studies shows that this water-only extract of *P. radiata* appears to be safe for human consumption and well tolerated at dosages of up to 1200 mg per day.

Toxicological and human studies for other pine bark and grape seed extracts (GSE) lend further support to the safety of PA-rich flavonoid extracts for human consumption. Considerable overlap in composition between pine barks of different species and grape seeds exists and warrant the comparison to the present data.

A review on the pharmacology of a PBE from *Pinus pinaster* (Pycnogenol®) includes a summary on safety concluding that the extract had no mutagenic or teratogenic activity, a low acute oral toxicity with a no observed adverse effect level (NOAEL) in dogs of 150 mg/kg body weight, and no effects in chronic toxicity tests on blood chemistry, behaviour, body weight or food consumption in rats or guinea pigs after intakes of 1.8–7.1 mg/kg (Rohdewald, 2002). Adding to the evidence for the safety of this *P. pinaster* extract are several human clinical studies that have consistently found few adverse events after treatments with 30–450 mg per day for 6–12 weeks, recently reviewed by the American Botanical Council (2010). The composition of *P. pinaster* and *P. radiata* bark extracts are greatly similar with PA, taxifolin, monomeric catechins, and phenolic acids being the main constituents. They both contain 65–87% PA with mean degree of polymerisation of 7.9–10.6, respectively, and taxifolin is the second most abundant flavonoid in Enzogenol® and Pycnogenol® (Jerez et al., 2007; Grimm et al., 2006).

Several toxicity studies on different grape seed extracts further contribute to the evidence for the safety of PA extracts. IH636 (ActiVin®), a grape seed extract with 76% PA, was found to have an LD50 greater than 5000 mg/kg, a no-observed effect level of 2000 mg/kg in rats, and no adverse effects in rats and mice during 6 months chronic administration of up to 500 mg/kg/day (Ray et al., 2001; Wren et al., 2002). A GSE called Gravinol Super™ con-

taining 89% PA was found to lack mutagenic activity, acute and sub-chronic toxicity with a NOAEL of 1500 mg/kg/day in rats (Yamakoshi et al., 2002). A third GSE (Meganatural™) has been shown to lack mutagenic and *in vivo* clastogenic activity, did not show any sub-chronic toxicity in rats with an NOAEL of 2150 mg/kg (Bentivegna and Whitney, 2002; Erexson, 2003).

As introduced above, it is difficult to determine precisely how much PA and flavonoids we consume with our diet, since this varies greatly with individual food choices. Table 6 shows PA contents for a selection of PA-rich foods that one could easily consume in a day. In this example the total amount of PA consumed is 1478 mg. Even greater amounts of PA could be consumed if one would eat, for example, an entire 100 g block of dark chocolate (989 mg PA), or five Granny Smith apples (1224 mg PAs) in a day. Another food not listed in Table 6, since uncommon in a western diet, is Sorghum grain. In Africa, Central America, and Asia, Sorghum grain is widely consumed and is a food staple for poor and rural populations. Sorghum grain contains 1902 mg PA per 100 g and hence would be a highly consumed component of the diet for these populations. These considerations show that PA-intake will generally be higher in healthy diets rich in fruits, nuts, seeds, and vegetables. Hence, it does not seem surprising that PA-rich extracts have so far not shown any toxic effects in animal or human safety studies.

In conclusion, we have shown details of production and composition for the Enzogenol® *P. radiata* bark extract, and demonstrated lack of mutagenicity, lack of toxicity in single and 14-day repeat dose trials in rats and dogs, and a positive safety profile on human clinical studies supporting the safe use of Enzogenol® in food and other nutritional applications.

Conflict of Interest

M.A.E. Frevel is employed by ENZO Nutraceuticals as Chief Science Officer. The company produces and sells the food ingredient, Enzogenol pine bark extract, that is the subject of this paper. As such Dr. Frevel has a commercial interest in the product and publication of the presented studies.

N. Gilchrist is the principle investigator, and C. Frampton is the statistician on the clinical trial in elderly rest-home residence that is part of this paper. A. Pipingas is the principle investigator on the cognitive function trial that is part of this paper. W. Grigsby is the principle scientist who carried out NMR and Mass spec analyses. Gilchrist, Frampton, Pipingas and Grigsby have no commercial interest in the Enzogenol product and do not gain any commercial advantage from this publication; their interest is only in publication of their research findings.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fct.2012.08.051>.

References

- American Botanical Council, 2010. Scientific and clinical monograph for Pycnogenol®. <http://abc.herbalgram.org>.
- Ames, B.N., McCann, J., Yamasaki, E., 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutat. Res.* 31, 347–364.
- Aron, P.M., Kennedy, J.A., 2008. Flavan-3-ols: nature, occurrence and biological activity. *Mol. Nutr. Food Res.* 52, 79–104.
- Bentivegna, S.S., Whitney, K.M., 2002. Subchronic 3-month oral toxicity study of grape seed and grape skin extracts. *Food Chem. Toxicol.* 40, 1731–1743.
- Chayasisobhon, S., 2006. Use of a pine bark extract and antioxidant vitamin combination product as therapy for migraine in patients refractory to pharmacologic medication. *Headache* 46, 788–793.
- Cho, K.J., Yun, C.H., Packer, L., Chung, A.S., 2001. Inhibition mechanisms of bioflavonoids extracted from the bark of *Pinus maritima* on the expression of proinflammatory cytokines. *Ann. N.Y. Acad. Sci.* 928, 141–156.
- Erexson, G.L., 2003. Lack of in vivo clastogenic activity of grape seed and grape skin extracts in a mouse micronucleus assay. *Food Chem. Toxicol.* 41, 347–350.
- Evans, G.O., 2009. *Animal Clinical Chemistry*, second ed. CRC Press, Taylor & Francis Group, Boca Raton, Florida. Appendix C, pp. 322.
- Feigin, V., 2012. Personal communication, submitted for publication.
- Grimm, T., Skrabala, R., Chovanova, Z., Muchova, J., Sumegova, K., Liptakova, A., Durackova, Z., Hogger, P., 2006. Single and multiple dose pharmacokinetics of maritime pine bark extract (pycogenol) after oral administration to healthy volunteers. *BMC Clin. Pharmacol.* 6, 4.
- Gu, L., Kelm, M.A., Hammerstone, J.F., Beecher, G., Holden, J., Haytowitz, D., Gebhardt, S., Prior, R.L., 2004. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr.* 134, 613–617.
- Guri, A., Kefelas, P., Roussis, V., 2006. Antioxidant potential of six pine species. *Phytother. Res.* 20, 263–266.
- Jerez, M., Tourino, S., Sineiro, J., Torres, J.L., Nunez, M.J., 2007. Procyanidins from pine bark: relationships between structure, composition and antiradical activity. *Food Chem.* 104, 518–527.
- Ku, C.S., Mun, S.P., 2007. Characterization of proanthocyanidin in hot water extract isolated from *Pinus radiata* bark. *Wood Sci. Technol.* 41, 235–247.
- Markham, K.R., Porter, L.J., 1973. Extractives of *Pinus radiata* bark. *N.Z. J. Sci.* 16, 751–761.
- Maron, D.M., Ames, B.N., 1983. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* 113, 173–215.
- Packer, L., Rimbach, G., Virgili, F., 1999. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free Radical Biol. Med.* 27, 704–724.
- Peng, Z., Hayasaka, Y., Iland, P.G., Sefton, M., Hoj, P., Waters, E.J., 2001. Quantitative analysis of polymeric procyanidins (tannins) from grape (*Vitis vinifera*) seeds by reverse phase high-performance liquid chromatography. *J. Agric. Food Chem.* 49, 26–31.
- Pipingas, A., Silberstein, R.B., Vitetta, L., Rooy, C.V., Harris, E.V., Young, J.M., Frampton, C.M., Sali, A., Nastasi, J., 2008. Improved cognitive performance after dietary supplementation with a *Pinus radiata* bark extract formulation. *Phytother. Res.* 22, 1168–1174.
- Porter, L.J., 1974. Extractives of *Pinus radiata* bark. *N.Z. J. Sci.* 17, 213–218.
- Prior, R.L., Gu, L., 2005. Occurrence and biological significance of proanthocyanidins in the American diet. *Phytochemistry* 66, 2264–2280.
- Ray, S., Bagchi, D., Lim, P.M., Bagchi, M., Gross, S.M., Kothari, S.C., Preuss, H.G., Stohs, S.J., 2001. Acute and long-term safety evaluation of a novel IH636 grape seed proanthocyanidin extract. *Res. Commun. Mol. Pathol. Pharmacol.* 109, 165–197.
- Rohdewald, P., 2002. A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. *Int. J. Clin. Pharmacol. Ther.* 40, 158–168.
- Santos-Buelga, C., Scalbert, A., 2000. Proanthocyanidins and tannin-like compounds – nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* 80, 1094–1117.
- Senthilmohan, S.T., Zhang, J., Stanley, R.A., 2003. Effects of flavonoid extract Enzogenol® with vitamin C on protein oxidation and DNA damage in older human subjects. *Nutr. Res.* 23, 1199–1210.
- Shand, B., Strey, C., Scott, R., Morrison, Z., Gieseg, S., 2003. Pilot study on the clinical effects of dietary supplementation with Enzogenol®, a flavonoid extract of pine bark and vitamin C. *Phytother. Res.* 17, 490–494.
- Wang, Y., Chung, S.J., Song, W.O., Chun, O.K., 2011. Estimation of daily proanthocyanidin intake and major food sources in the U.S. diet. *J. Nutr.* 141, 447–452.
- Wood, J.E., Senthilmohan, S.T., Peskin, A.V., 2002. Antioxidant activity of procyanidin-containing plant extracts at different pHs. *Food Chem.* 77, 155–161.
- Wren, A.F., Cleary, M., Frantz, C., Melton, S., Norris, L., 2002. 90-day oral toxicity study of a grape seed extract (ih636) in rats. *J. Agric. Food Chem.* 50, 2180–2192.
- Yamakoshi, J., Saito, M., Kataoka, S., Kikuchi, M., 2002. Safety evaluation of proanthocyanidin-rich extract from grape seeds. *Food Chem. Toxicol.* 40, 599–607.
- Young, J.M., Shand, B.I., McGregor, P.M., Scott, R.S., Frampton, C.M., 2006. Comparative effects of Enzogenol® and vitamin C supplementation versus vitamin C alone on endothelial function and biochemical markers of oxidative stress and inflammation in chronic smokers. *Free Radical Res.* 40, 85–94.