Safety evaluation and nutritional composition of a *Fraxinus excelsior* seed extract, FraxiPure™

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ABSTRACT

A natural extract obtained from the seeds of *Fraxinus excelsior* L. (FraxiPure™) has been previously reported to reduce glycemia in animal models and in humans. The objective of this work was to evaluate the safety of FraxiPure™ at *in vitro, in vivo* and human levels. In addition, nutritional analyses revealed an extract high in carbohydrates, with minor levels of protein, dietary fiber, glucose and sucrose. IC_{50} and IC_{90} values of 1.447 and 2.530 mg/mL, respectively, after 72 h incubation were calculated using the MTT assay. FraxiPure™ conferred a magnitude of protection of 69.2% against the formation of micronuclei in irradiated human lymphocytes as determined by the micronucleus assay. An LD_{50} of greater than 2500 mg/kg was concluded following an acute oral toxicity study in Sprague–Dawley rats. A human safety evaluation in a double-blind, placebo-controlled parallel study of 100 healthy volunteers revealed no significant differences between daily consumption of 1000 mg of FraxiPure™ for 90 days and placebo (maltodextrin) for any of the biochemical or hematological parameters studied. Numbers of adverse events were similar in both groups, and were deemed mild to moderate. These results demonstrate, for the first time, the safety and tolerability of FraxiPure™ for consumption in healthy subjects.

1. Introduction

The common ash (*Fraxinus excelsior* L.) is a species of Fraxinus that grows in temperate regions, and is native to most of Europe, western Asia and exists throughout southeastern Morocco (Maghrani et al., 2004). The Fraxinus species have been used in traditional medicine in different parts of the world for the treatment

of various conditions (Kostova and Iossifova, 2007), and the traditional use of the seeds of *F. excelsior* (as shown in Fig. 1) as a carminative and to break down gallstones in the bladder has been reported in Iran (Parsa, 1959).

Scientific evaluation of *F. excelsior* has revealed several biological activities including anti-oxidative (Meyer et al., 1995; Middleton et al., 2005; Schempp et al., 2000), anti-inflammatory (El-Ghazaly et al., 1992; von Kruedener et al., 1996), analgesic (Okpanyi et al., 1989), antipyretic, both individually (Okpanyi et al., 1989) and as part of a combined plant drug (Strehl et al., 1995), hypoglycemic (Eddouks and Maghrani, 2004; Maghrani et al., 2004), and hypotensive effects (Eddouks et al., 2005).

More recent studies on a patented seed extract of *F. excelsior*, marketed as FraxiPure™, have confirmed the ability of this seed extract to reduce glycemia both *in vivo* and in an acute clinical study (Visen et al., 2009; Ibarra et al., 2011). Mice fed a high-fat diet supplemented with FraxiPure™ displayed significant

Fig. 1. *Fraxinus excelsior* seeds from which FraxiPure™ was obtained.

reductions in fasting blood glucose and fasting plasma insulin levels at study end compared to the high-fat diet control (Ibarra et al., 2011). Body weights were significantly decreased in mice fed FraxiPure™ compared to mice fed the high-fat diet from week 9 of the 20-week study. Administration of FraxiPure™ in the diet ameliorated incidences of fatty liver and reduced liver weight and plasma ALT induced by the high fat diet. The results suggested that the extract itself was without adverse effects on the liver and that FraxiPure™ may possess hepatoprotective properties.

The effects of FraxiPure™ on postprandial glycemia and insulin secretion were evaluated in healthy human volunteers in an acute randomized, double-blind, placebo-controlled, crossover study (Visen et al., 2009). Participants (with a mean blood fasting glucose level of 4.4 ± 0.09 mmol/L and a mean BMI of 26 ± 2.2 kg/m^2) consumed 1000 mg of FraxiPure™ after overnight fasting and were immediately challenged using the oral glucose tolerance test. Ingestion of FraxiPure™ resulted in a significant decrease in the mean area under the plasma time-concentration curve (AUC) for glucose levels compared to the placebo group ($p = 0.02$). A significant increase (55.5 ± 4.6 mU/L; $p = 0.02$) in insulin secretion was induced following consumption of FraxiPure™ at 90 min compared to placebo (43.5 ± 5.0 mU/L).

The mechanism of action behind these effects may be related to the ability of individual secoiridoid glucosides isolated from *F. excelsior* seeds to activate PPARα *in vitro*; this effect was also observed with the unfractionated extract (Bai et al., 2010). PPARα is a nuclear receptor which has been shown to improve symptoms of the metabolic syndrome (Lefebvre et al., 2006).

Detailed information regarding the safety and nutritional composition of the seeds of *F. excelsior* appears to be lacking from the scientific literature. The aim of this study was to evaluate the safety of a *F. excelsior* seed extract, FraxiPure™, by combining information from cytotoxicity and genotoxicity studies *in vitro*, an oral acute toxicity study *in vivo*, and biochemical and hematological results from a human clinical trial. A further objective was to provide a detailed nutritional analysis of FraxiPure™.

2. Materials and methods

2.1. Raw material

The seeds of *F. excelsior* L. were collected from rural communities in Morocco and deposited into the herbarium (voucher specimen J02/02/A7; Reference #RB3524) at Naturex Maroc, Technopole Nouasser BP 42, Casablanca 20240, Morocco. Harvesting, collection and analysis of the seeds were performed as described by Visen et al. (2009).

2.2. Extract preparation

Extracts from *F. excelsior* L. seeds were prepared as described (Visen et al., 2009) using an established industrial process at Naturex France (FraxiPure™, Reference: EA149251, Naturex SA, Site d'Agroparc BP 1218, 84911 Avignon Cedex 9, France). Samples were standardized by their active compounds, nuzhenide and GI3. All samples contained greater than 10% of nuzhenide and GI3, the markers of these extracts.

2.3. Reagents

All reagents were HPLC or chemical grade and obtained from local suppliers. Nuzhenide (85%) and GI3 (70%) standards were prepared in-house as previously described (Bai et al., 2010).

2.4. Nuzhenide and GI3 analysis

HPLC-DAD analysis of nuzhenide and GI3 in the FraxiPure™ samples was conducted as described by Ibarra et al. (2011) with the following modification; a Zorbax SB$_{18}$ column (25 cm × 4.6 mm, 5 µm) was used under identical conditions of flow rate, temperature and solvent gradient.

2.5. Nutritional composition and evaluation of contaminants

A total of five different samples of *F. excelsior* L. seed extract (with batches codes A140/058/A11, A119/053/A11, A138/052/A11, A150/029/A11 and A147/045/A10) were used for characterization of nutritional composition and evaluation of contaminants.

2.5.1. Macronutrients

The samples were analyzed for chemical composition (moisture, protein, fat and ash) based on official methods (MAPA, 1994). Moisture was determined at 105 °C in a laboratory oven for 4 h (until constant weight). Protein content of the samples was determined by the Dumas procedure using an elemental analyzer LECO TruSpec N (St. Joseph, MI, USA). The sample was submitted to a pyrolysis process and nitrogen was determined in the combustion gas. A factor of 6.25 was applied to convert nitrogen to protein. For fat content determination, a known weight of powdered sample was submitted to an acid hydrolysis step, the obtained powder was extracted with petroleum ether in an automatic Soxhlet system and the fat content was determined by gravimetric difference. The ash content was determined by incineration at 550 °C for 4 h. Total carbohydrates were calculated by difference using the following formula: Carbohydrate content = 100% − (%moisture + %protein + %fat + %ash). Total energy was expressed as kilocalories per 100 g, using the factors described under Spanish Legislation (Real Decreto, 2009): Energy kcal/100 g = 4(g protein + g carbohydrate) + 9(g lipid) + 2(g dietetic fiber).

2.5.2. Sugars

Simple sugars, including fructose, glucose, sucrose, maltose and lactose were determined by HPLC coupled to a refractive index detector (HPLC-RI) based on a modification of standardized methods (AOAC, 2006a; Scott, 1992). Duplicate samples of 1.0 g were extracted with 25 mL of 50% aqueous acetonitrile at 60 °C for 20 min. The samples were left to cool to room temperature and were diluted between 10 and 25 times with acetonitrile. The solution was filtered using a 0.45 µm polypropylene filter and 20 µL was injected. The elution of the sugars was performed with an apHera NH$_2$ Polymer column (25 cm × 4.6 mm, 5 µm) using a pre-column NH$_2$ or equivalent, operating at 35 °C in isocratic mode, with acetonitrile/deionized water, 77:23 (v/v) as mobile phase, at a flow rate of 1 mL/min. The refractive index detector was set at 30 °C. Detection and quantification were based on relative retention times and signal response of the commercial standards of each compound (fructose, glucose, sucrose, maltose and lactose). The results were expressed in grams per 100 g of dry weight.

2.5.3. Dietary fiber

Total, soluble and insoluble dietary fiber contents were determined by the AOAC official method (AOAC, 1995).

2.5.4. Microbiological analysis

Microbiological analysis was carried out to international standards: aerobic mesophiles at 30 °C (ISO, 2003a), molds and yeast (European Pharmacopoeia, 2009) and Enterobacteriaceae, Escherichia coli and Staphylococcus aureus (ISO, 2000). Salmonella was analyzed by PCR in real time (ISO, 2003b).

2.5.5. Heavy metals

The heavy metals cadmium (Cd), lead (Pb), and arsenic (As) were determined following minor modifications of official AOAC methodologies. For the quantification of cadmium and lead, duplicated samples were mineralized (AOAC, 2006c) and then dry ashed at 550° C. Subsequently, a solution of HCl (12 M) was added and the solution was evaporated until dry. The residue was dissolved in 6 M HCl and cadmium and lead were determined by Inductively Coupled Plasma Atomic Emission Spectrometry (AOAC, 2002). For the quantification of arsenic, duplicate samples were digested with 5 mL of HNO_3 65% and 2 mL of H_2O_2 30% in a reflux system (AOAC, 2006b). Arsenic was measured by Inductively Coupled Plasma Atomic Emission Spectrometry (AOAC, 2002) with previous generation of the hydride compound (AsH_3) by the reaction between $NaBH_4$, HCl and the metal. Arsenic was determined measuring the light emitted by the hydride compound. The mercury content of the samples was determined by an Atomic Absorption Spectrometer AMA254 (LECO Corporation, St. Joseph, MI, USA). Duplicate samples of known weight were placed on a sampling tray and introduced into a catalytic tube. By controlled heating of a decomposition furnace, the samples were first dried and then thermally decomposed or burned. The decomposition products of the samples were transferred by oxygen stream to the second part of the catalytic tube. Here the oxidation was finalized and halogens, nitrogen and sulfur oxides were trapped. The decomposition products were then passed to an amalgamator for selective trapping of mercury. After this step, the amount of mercury was measured by Atomic Absorption (Szakova et al., 2004).

2.5.6. Pesticides

Pesticides were determined according to standardized guidelines (European Pharmacopoeia, 2008). All pesticides included in the European Pharmacopeia proposed list were analyzed. Sample analysis of pesticides was performed by GC–MS/MS after a previous extraction step. Samples were weighed (0.3–3 g) and 4 g of $MgSO_4$, 1 g of NaCl, 9 mL distilled water and 9 mL of an ethyl acetate/cyclohexane/acetone 1:1:4 mixture were added. The mixture was stirred vigorously for 1 min and the solution was separated by centrifugation at 8000 rpm for 5 min. Supernatant was recovered (0.975 mL) and 25 µL of a 3 mg/mL internal standard solution of triphenylphosphate, 150 mg of primary and secondary amine, 150 mg of anhydrous $MgSO_4$, 30 mg of C18 and 30 mg of graphite carbon (Envi-Carb; Supelco) were added. The mixture was shaken vigorously and centrifuged at 5000 rpm for 3 min. The supernatant was recovered and filtered through a 0.45 µm filter prior to injection.

Separation and identification of pesticides were performed using an HP-5MS column (30 m × 0.25 mm i.d., 0.25 µm) and triple quadrupole detector. The oven temperature program was as follow: the initial temperature of the column was 70 °C, held for 2 min: 25 °C/min ramp to 150 °C; a 3 °C/min ramp to 200 °C; a 8 °C/min ramp to a 280 °C and held for 6 min. The carrier gas (helium) flow-rate was 3.2 mL/min (30.89 psi), measured at 70 °C. For each analysis, 1 µL of sample was injected in the gas chromatograph. Pesticides confirmation is based on the ratio between the intensity of the quantifier and qualifier ion. The results were expressed in mg/kg.

2.6. Cytotoxicity

In vitro cytotoxicity studies in Vero cell lines at varying concentrations were completed by performing the MTT cell viability assay as described by Carmichael et al. (1987) and Alley et al. (1988). The Vero cell line (European Collection of Cell Cultures, Wiltshire, UK) corresponds to non-tumor adherent cells established from renal cell tubules of the African green monkey (Cercopithecus aethiops). The culture medium used was DMEM (Dulbecco's Modified Eagle's Medium) with pH indicator (red phenol) and supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, penicillin (100 IU/mL) and streptomycin (100 µg/mL).

Cells were seeded in 96 well plates at an appropriate density (Vero: 1600 cells/well) to ensure exponential growth throughout the test. After 24 h, different concentrations of freshly prepared stock solution were applied. Addition of Fraxipure™ (batch No. A147/045/A10) to the cell medium did not cause any changes in osmolarity (vapor pressure osmometer Wescor Vapro 5520). In Vero cells, concentrations of 0.006, 0.028, 0.055, 0.139 and 0.277 mg/mL of FraxiPure™ were applied. Each plate held six replicates for each concentration and 12 untreated control replicates. The plates were incubated under the conditions described for 24, 48 and 72 h after addition of the stock solution. After incubation, the culture medium was removed and freshly dissolved MTT (1 mg/mL) in medium without phenol red was added. The plates were further incubated for 4 h in the dark at 37 °C.

Subsequently, the medium was extracted, leaving only the cells adhered to the bottom. Formazan was solubilized by adding 100 µl of dimethyl sulfoxide to each well. After shaking the plate for 10 min at 80 rpm, absorbance was recorded on a spectrophotometer (Multiskan MCC/340P, Labsystems, Finland) at wavelengths of 570 nm (reading) and 690 nm (reference). All tests were performed in duplicate. The results obtained were expressed as a percentage of the change in growth of the control. Growth curves were plotted as a function of concentration, and a linear relationship was constructed to determine the correlation of fit. If the correlation of fit was greater than 50% ($r^2 > 0.500$), the slope and intercept of the fit was used to determine IC_{50} and IC_{90} values.

2.7. Radio-protective properties

2.7.1. Blood samples and irradiation procedure

Heparinized samples of human peripheral blood were obtained from two consenting, healthy, young, non-smoking female donors. Twenty and 40 µL of FraxiPure™ (batch No. A147/045/A10) were added to 2 mL of human blood to obtain concentrations of 0.144 and 0.288 mg/mL, respectively, 5 min before irradiation. A control blood sample without FraxiPure™ was also prepared. The blood samples were exposed to X-rays using an Andrex SMART 200E (YXLON International, Hamburg, Germany) operating at 120 kV, 4.5 mA, DFO 74.5 cm at room temperature for 19 min, 29 s with a dose rate of 103 mGy/min at a total dose of 2 Gy ± 3%. The radiation doses were monitored by a UNIDOS® Universal Dosimeter with PTW Farme® ionization chambers TW 30010 (PTW-Friburg, Freyburg, Germany) in the radiation cabin and the X-radiation dose was confirmed by means of thermoluminescent dosimeters (TLDs) (GR-200®, Conqueror Electronics Technology Co. Ltd, China). The TLDs were supplied and monitored by CIEMAT (Ministry of Industry and Energy, Spain). The absence of Mycoplasma spp. was verified by direct fluorescence with a specific colorant for DNA (Hoechst 33258).

2.7.2. Cell culture technique

The micronucleus assay was carried out on irradiated human lymphocytes after X-irradiation (with and without FraxiPure™, and with rosmarinic acid as a positive control), with the cytokinesis-blocking method described by Fenech and Morley (1985) and adapted by IEAE (2001). Whole blood (1 mL) was cultured at 37 °C for 72 h in 9 mL of F-10 medium containing 15% fetal bovine serum, 1.6% phytohemaglutinin and 1% penicillin/streptomycin. Forty-four hours after initiation of the culture of the lymphocytes, cytochalasin B was added at a concentration of 3 µg/mL. At 72 h, the lymphocytes were treated with hypotonic solution (KCl, 0.075 M) for 3 min and fixed using methanol:acetic acid (3:1). Air-dried preparations were made and slides were stained with May Grünwald–Giemsa stain.

2.7.3. Scoring of micronuclei (MN)

Triplicate cultures were analyzed. In each, the micronuclei of at least 3000 cytokinesis-blocked cells (CB cells) (MN/500 CB) were examined by two experts using a Zeiss light microscope (Oberkochem, Germany). A magnification of 400× was used for slide examination and 1000× to confirm the presence or absence of MN in the cells (3000 CB), according to the criteria of Fenech (2007). The results were used to obtain the Magnitude of Protection (Sarma and Kesavan, 1993):

$$\text{Magnitude of protection } (\%) = ((F_{control} - F_{treated}) \times 100)/F_{control})$$

where $F_{control}$ = frequency of MN in irradiated blood lymphocyte controls and $F_{treated}$ = frequency of MN in irradiated blood lymphocytes treated with FraxiPure™.

2.8. Oral acute toxicity study

An oral acute toxicity study was conducted in male and female (nulliparous and non-pregnant) Sprague–Dawley (Crl: SD) rats (International Genetic Standard) obtained from Charles River Laboratories (France) to OECD standards (OECD, 1996). The rats were approximately 9–10 weeks old on the day of dosing. All animals were housed in groups of three in Tecniplast Makrolon cages (48 × 27 × 20 cm) with a bed of wood shavings in animal rooms maintained at a temperature of 21 °C (±2 °C) and a relative humidity of 30–80%. Animal rooms were artificially illuminated (fluorescent light) on a 12 h light/dark cycle. Tap water and standard chow were available ad libitum except during the fasting period. The air was changed 15 times per hour, and was pre-filtered to 5 µm. Animals were inspected daily during a 5-day acclimatization–quarantine period, which passed without incident. Rats were fasted overnight prior to dosing. The test substance (FraxiPure™; batch number A021/010/A11) was administered to three male and three female rats by gavage of a single oral dose of 2000 mg/kg body weight at a volume of 2 mL/100 g body weight. Food was returned to the animals 3 h after dosing. Individual weights were determined shortly before the test substance was administered (Day 0), and on test days 7 and 14. The animals were observed for clinical signs of toxicity while handled before and after fasting, frequently within 4 h after dosing and daily thereafter. Observations for mortality and signs of illness, injury, or abnormal behavior were conducted daily. Surviving animals were sacrificed by sodium pentobarbital overdose and given a complete gross pathology examination.

2.9. Human safety evaluation

2.9.1. Study subjects

The clinical safety and tolerability of FraxiPure™ was assessed in a sub-chronic, 90-day study conducted by Optimed Clinical Research in the Rhône-Alpes region, France. The study protocol and informed consent documents were approved by local ethics committee in Biomedical Research (Comité de Protection des Personnes – CPP Sud-Est III, Lyon, France). All participants gave written informed consent. The study was conducted in accordance with the International Conference on Harmonization (ICH)-Good Clinical Practice (GCP) guideline, the International Ethical Guidelines for Biomedical research Involving Human Subjects (CIOMS, 2002) and the World Medical Association declaration of Helsinki and its amendments concerning research in humans (Helsinki, 1964, amended Seoul 2008).

One hundred healthy men and women aged between 18 and 65 years inclusive were randomized into two treatment groups (FraxiPure™ or placebo), stratified by gender (25 men and 25 women per group). Each volunteer's duration of participation was a total of 100 days.

The subjects were initially screened to determine whether they met the inclusion criteria 10 days prior to commencement of the 90-day treatment period. Subjects were excluded if it was considered on the basis of their medical history that they may potentially have some underlying compromised health condition. Women who began taking oral contraceptives or hormone replacement therapy (within the previous 6 months) and women with polycystic ovarian syndrome were not included. Frequent users of alcohol or drugs in the 2 years prior to study initiation, or at any time during the study, and volunteers who had quit smoking in the 6 months preceding the study commencement or were planning on quitting/altering smoking were also excluded. The demographic characteristics of the subjects in each group, collected during the screening visit, are presented in Table 3.

2.9.2. Study design

The study was a mono-centric, double-blinded, placebo-controlled and parallel evaluation in which 25 men and 25 women per group were given capsules containing either FraxiPure™ or maltodextrin (placebo) three times per day during a period of 90 days. During the entire study period, subjects were advised to follow a calorie-restricted diet (minus 500 kcal from the balanced caloric intake according to the Harris Benedict equation). Subjects were required to return to the study center every month after the baseline visit for a routine check, giving a total of 4 visits (i.e., on days 0, 30, 60 and 90).

2.9.3. Study treatments

FraxiPure™ (batch A129/062/A11) was obtained following GMP. The extract was standardized to 14.01% nuzhenide and GI3 as its active ingredients. The placebo consisted of a color-matched potato-derived maltodextrin (batch A104/036/A11). Each capsule contained 334 mg of maltodextrin (placebo group) or 334 mg of FraxiPure™ (FraxiPure™ group). For encapsulation purposes, excipients were added to the products. The composition of the FraxiPure™ capsules content was as follow: 334 mg FraxiPure™ (82.67%), 62 mg corn starch (15.35%), 2 mg colloidal silica (0.49%) and 6 mg magnesium stearate (1.48%). The total weight of each FraxiPure™ capsule was 404 mg. The composition of the placebo capsules content was as follow: 334 mg maltodextrin (81.86%), 70 mg corn starch (17.16%), 2 mg colloidal silica (0.49%) and 2 mg magnesium stearate (0.49%). The total weight of each placebo capsule was 408 mg. The two treatments were indistinguishable in terms of color, smell and visual appearance.

2.9.4. Subject randomization and dosing

Allocation concealment was performed by an off-site person who generated active and placebo random numerical sequences using the online software for random sequence generator provided by www.random.org.

The participants were instructed to take one capsule (334 mg active or placebo) with a glass of water three time daily (equivalent to a daily dose of 1000 mg active or placebo), immediately before each of the three main meals.

On days 0, 30 and 60, subjects were dispensed with new supply of investigational product (100 capsules, a 33-day supply) and were asked to return the bottle with unused product on their return to study center, on days 30, 60 and 90 (end of study). Intake compliance was monitored by counting the remaining pills at the return of each bottle at each visit (compliance was considered acceptable if 90% of the capsules had been taken between two visits).

2.9.5. Evaluation procedure

Evaluation of subjects was performed at screening (day −10), at baseline (day 0) and at days 30, 60 and 90 of study. During the screening visit (day −10), the following information were recorded for each subjects wishing to participate in the study: demographic characteristics (date of birth, age, sex, height and weight), nature and duration of medical and surgical histories, nature and duration of previous medication, habits (alcohol, caffeine and tobacco consumption, uncommon dietary habits). A pregnancy test was also conducted for women of child-bearing potential.

Hematological and blood biochemical parameters were analyzed using standard methodologies at baseline (day 0) and at the end of the 90-day treatment period (day 90).

During study center visits (days 30, 60 and 90), inclusion/exclusion criteria were reviewed and subjects were asked "How have you been feeling". Tolerability was evaluated by recording unsolicited adverse events (AEs) occurring at any time during the study.

2.9.6. Adverse events evaluation

AEs were recorded throughout the study period. An AE has been defined by ICH E6 as "any untoward medical occurrence in a patient or clinical investigation subject administered an investigational product regardless of its causal relationship to the study treatment". All AEs occurring during the course of the study were recorded on the volunteer's CRF. An AE could therefore have been any unfavorable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with the use of a test material, whether or not related to the material. A serious AE (SAE) was defined as any untoward medical occurrence or effect that resulted in death, was life-threatening, required inpatient hospitalization or prolongation of existing hospitalization, resulted in congenital anomaly or birth defect, resulted in a persistent or significant disability/incapacity, or was an otherwise important medical event based upon appropriate medical judgment.

The occurrence of an AE may have come to the attention of study personnel during study visits and interviews of a study subject, or upon review of subject data by a study monitor. Any medical condition that was present at the time that the subject was screened was considered as baseline and not reported as an AE. However, if it deteriorated at any time during the study, it was recorded as an AE.

Information collected for assessment and documentation included event description, date and time of onset, time between AE onset and last study product administration, date and time of resolution/stabilization of the event, actions taken (none, study product interrupted or discontinued, concomitant medications administered, hospitalization, etc.) and the outcome. All AEs were graded by the clinician for seriousness, frequency (isolated, intermittent, permanent), intensity (mild, moderate or severe) and causal relationship to study treatment (none, remote, possible, probable, highly probable). Any AE that occurred was followed to resolution or until determined to be clinically insignificant by the physician.

2.9.7. Analysis of samples

2.9.7.1. Blood. Biochemistry analysis was conducted on 9 mL of venous blood samples collected into serum preparation tubes (SST) during the study visit (day 0, before treatment started) and the last study day (day 90). Variables assessed included: alkaline phosphatase, total bilirubin, creatinine, aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyltransferase (GGT), total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, glucose, glycosylated hemoglobin (HbA1C) and insulin.

Hematological measurements were performed using 4 mL of blood in ethylenediamine tetraacetic acid (collected at the same time as blood for the biochemistry evaluation). Measurements or calculations included: hemoglobin, red cells count (RBC), white cells count (WBC), hematocrit (as vol.%), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), differential counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils) and platelet count.

2.9.7.2. Urine. Urine pregnancy testing for women of childbearing potential was undertaken (beta-hCG) at baseline.

2.9.8. Data collection and statistical analysis

The data were collected in electronic case report forms (eCRF) and verified with source documents by the data manager at the study center. Data on each case report form was entered into a Microsoft Access database and then transferred into Genstat version 14.0 for statistical analysis. Differences between groups means was calculated as a 95% confidence interval and significance calculated using an unpaired two-tailed t-test of unequal variance (Genstat, version 14.0). Intragroup differences were calculated using a paired two-tailed t-test. Significance was set at $p < 0.05$.

3. Results and discussion

3.1. Chromatographic profile

The principal bioactive compounds present in FraxiPure™ are proposed to be the secoiridoids (Bai et al., 2010). Nuzhenide and GI3 are the main secoiridoids in FraxiPure™, and combined are present at levels greater than 10% in the extract. A chromatographic profile showing the elution of nuzhenide and GI3 of batch number A147/045/A10 is presented in Fig. 2.
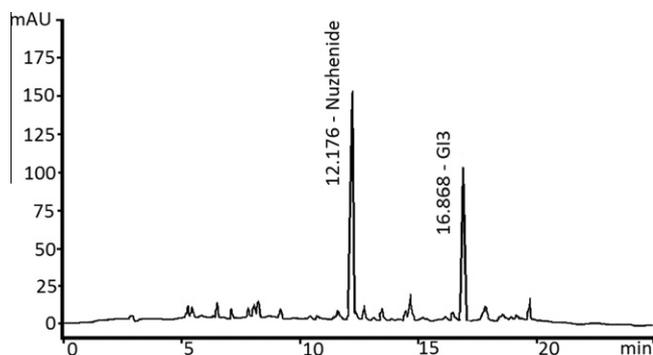
Fig. 2. Chromatographic profile of FraxiPure™, with elution of the major secoiridoids, nuzhenide and GI3 at 12.176 and 16.868 min, respectively.

3.2. Nutritional composition

The results of the nutritional composition, moisture and energetic value obtained following analysis of FraxiPure™ are provided in Table 1. FraxiPure™ was found to be primarily composed of carbohydrates (72.4 g/100 g), along with low levels of protein and total dietary fiber (approximately 6 g/100 g each). Insoluble dietary fiber constituted approximately 1 g/100 g of the nutritional composition. Further analysis of the simple sugars contained in the extract revealed the presence of glucose and sucrose to relatively high concentrations (3.34 and 3.84 g/100 g, respectively); maltose and lactose were not detected (Table 2).

This appears to be the first time that a nutritional analysis of *F. excelsior* seed extract has been reported. Carbohydrates, more specifically complex carbohydrates, are the predominant nutrients present in *F. excelsior* seed extract, and are likely the source of the health benefits of FraxiPure™. The phenolic and secoiridoid compounds in FraxiPure™ have been profiled previously, with a total phenolic composition of 20.35% being reported. The secoiridoids nuzhenide and GI-3 were found to account for 11.42 and 6.15%, respectively, of the overall phenolic content (Ibarra et al., 2011), and these compounds, along with other secoiridoids present in *F. excelsior*, have previously been shown to induce activation of

Table 1
Nutritional composition (% dry weight), moisture (% wet weight) and energetic value (kcal/100 g dry weight) of five FraxiPure™ samples (mean ± standard deviation; $n = 5$).

Carbohydrates	72.40 ± 6.45
Protein	6.54 ± 1.27
Fat	0.98 ± 0.97
Ash	10.88 ± 2.46
Total dietary fiber	6.94 ± 1.99
Soluble dietary fiber	5.94 ± 1.28
Insoluble dietary fiber	1.02 ± 0.76
Moisture	2.56 ± 0.68
Energy	335.80 ± 8.26

Table 2
Simple sugar composition of FraxiPure™ samples (mean ± standard deviation; $n = 5$; results adjusted for moisture and ash).

	Mean ± s.d. (%)
Fructose	0.98 ± 0.48
Glucose	3.34 ± 2.26
Sucrose	3.84 ± 1.34
Maltose	<0.5
Lactose	<0.5

PPAR-α (Bai et al., 2010). PPAR-α pathways are known to be involved in lipid homeostasis and inflammation, and also play a role in obesity-related diabetes (Finck et al., 2005).

3.3. Evaluation of contaminants

Microbiological, heavy metal and pesticide analyses were conducted on five batches of FraxiPure™. Results were found to be within normally accepted limits for each parameter tested (results not shown). Full details of experimental analyses can be found in Section 2.

3.4. Cytotoxicity

The cytotoxicity of FraxiPure™ was determined by comparing the growth of a Vero cell line in the presence of varying concentrations of FraxiPure™ using the MTT assay.

Growth curves constructed at 24, 48 and 72 h post-incubation with FraxiPure™ are presented in Fig. 3. Typical growth curves were observed, with slightly decreasing cell growth (i.e. increasing inhibition) as concentration of FraxiPure™ increased at all time points. After incubation of FraxiPure™ for 72 h, IC_{50} and IC_{90} values were calculated at 1.447 and 2.530 mg/mL, respectively.

3.5. Radio-protective properties

A micronucleus assessment of human lymphocytes exposed to X-rays was used to determine whether FraxiPure™ could affect the biological damage caused by the irradiation. The *in vitro* micronucleus assay is a mutagenicity test system used for the detection of chemicals that induce the formation of small membrane-bound DNA fragments such as micronuclei (MN) in the cytoplasm of interphase cells and has the potential to detect the activity of both clastogenic and aneugenic chemicals (OECD, 2004). Identification of nuclei that have undergone one division as binucleates is possible through the use of actin polymerization inhibitor cytochalasin B.

Examination of the non-irradiated cells revealed the presence of 7 ± 2 MN cells per 500 binucleated cells (Fig. 4). Exposure of the blood cells to irradiation increased the number of cells containing MN to 26 ± 2 per 500 binucleated cells. The presence of 0.139 mg/mL of FraxiPure™ with the blood cells during irradiation resulted in a dramatic reduction in the number of cells containing MN to 8 ± 2 per 500 binucleated cells (Fig. 4). This corresponds to a magnitude of protection of 69.2%, which was a higher level of protection compared to that afforded by rosmarinic acid (50.0%).
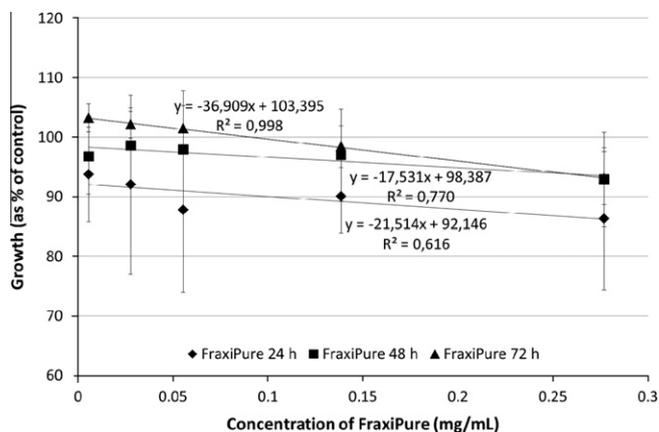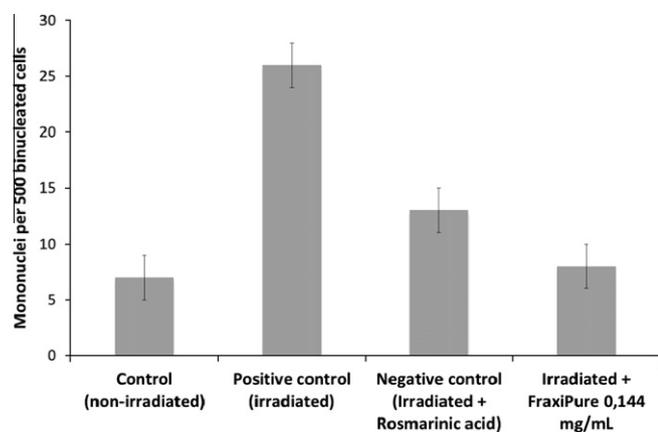

Fig. 3. Growth curves of Vero cell lines following incubation with varying concentrations of FraxiPure™ for 24, 48 and 72 h. Error bars represent standard deviations; $n = 6$.

Fig. 4. Effect of FraxiPure™ on the appearance of mononucleated cells per 500 binucleated cells following irradiation of human lymphocytes, compared to negative (rosmarinic acid), positive (irradiated) and non-irradiated controls. Error bars represent standard deviations. FraxiPure™ gave 69.2% protection compared to the positive control.

Table 3
Baseline characteristics of volunteers. Values reported are means ± standard deviations; $n = 50$.

Baseline parameter (units of measure)	Placebo	FraxiPure™	p Value
Age (years)	43.7 ± 11.9	41.3 ± 12.9	0.337
Weight (kg)	84.3 ± 11.0	83.2 ± 12.5	0.650
Height (cm)	169 ± 10	169 ± 10	0.859

Rosmarinic acid was previously reported to offer a magnitude of protection against X-ray induced biological damage of 57.7% (Alcaraz et al., 2011).

3.6. Oral acute toxicity study

An oral acute toxicity study was conducted in Sprague–Dawley rats by a single oral dose of 2000 mg/kg body weight. No abnormal behavior or signs of toxicity attributable to administration of the test sample were observed in any of the experimental animals. All the animals that received the test product survived in normal conditions up to day 14 of observation, at which point the animals were sacrificed. Postmortem studies did not detect significant gross changes. Following the diagrams of interpretation of results (OECD, 1996), FraxiPure™ was classified as having an LD$_{50}$ of greater than 2500 mg/kg body weight. This appears to be the first time that an LD$_{50}$ value has been reported for *F. excelsior* seed extracts.

3.7. Human safety and tolerability evaluation

The safety and tolerability of FraxiPure™ was assessed in a sub-chronic 90 day double-blinded, placebo-controlled randomized

clinical trial of 100 healthy volunteers. Analysis of the properties of the populations at baseline did not reveal any significant differences between groups, in terms of age, weight or height (Table 3).

Overall, treatments were well tolerated and drop-out rates were comparable between FraxiPure™ and placebo groups: four subjects were withdrawn from FraxiPure™ group (three for protocol deviation and one AE not related to study product but necessitating study interruption) and four subjects from placebo group (three for protocol deviation and one AE not related to study product but necessitating study interruption). The AE in the FraxiPure™ group was due to breast cancer rated of moderate intensity and with no causal relation to the study product. The AE in the placebo group was a gastric ulcer rated of moderate intensity and with no causal relation to the study product. Nevertheless, the subject chose to withdraw from the study. A follow-up abdominal echography was normal.

AEs recorded in both groups were deemed mild to moderate and not related to the test substance by the attending medical practitioner. Due to lack of AEs, both in terms of severity and causal relationship to treatments, the data were not subject to statistical analysis but are summarized in Supplementary Table 1. The NCI Common Terminology Criteria for Adverse Events (CTCAE) v4.0 (National Cancer Institute, 2009) descriptive terminology was used for Adverse Event (AE) reporting.

Administration of 1000 mg of FraxiPure™ for 90 days did not result in any significant differences in the changes in clinical chemistry parameters measured as compared to the changes caused by the maltodextrin control (Table 4). The effect of the calorie-restricted diet may have resulted in the significant ($p < 0.05$) intra-group changes in ALAT and creatine observed in both FraxiPure™ and placebo groups, although certain parameters were only significantly modulated in the FraxiPure™ group (ASAT, ALP, GGTP).

A hematological analysis of the samples collected at day 0 and day 90 did not reveal any significant differences between the FraxiPure™ and the placebo groups (Table 5). Nevertheless, significant changes were observed between values observed on day 0 compared to day 90 in both the FraxiPure™ and placebo groups for RBC, MCV, MCH, MCHC, lymphocytes and platelet count. These changes may be explained by the change in the number of calories ingested by all volunteers in the study. Changes in hematological parameters have previously been observed on restricting caloric intake (Kang et al., 2004).

Previous studies which reported on the safety of *F. excelsior* focused on extracts produced from the leaves or from the bark of the plant. Middleton et al. (2005) used the brine shrimp mortality test to determine the toxicity of the leaves of *F. excelsior*. High levels of toxicity (<1 mg/mL) were observed for the *n*-hexane and dichloromethane extracts of the leaves of *F. excelsior*. In a review of the available published literature on Phytodolor®, an extract comprised of a mixture of the fresh stem bark of *F. excelsior*, *Populus tremula* (fresh bark and leaves) and *Solidago virgaurea*, Gundermann and Muller (2007) reported a lack of toxicity (doses not

Table 4
Results of clinical chemistry parameters observed after administration of 1000 mg of FraxiPure™ for 90 days to healthy individuals compared to placebo. Values reported are means ± standard deviations; $n = 50$; differences between groups are expressed at the 95% confidence interval.

Parameter (units of measure)	Placebo ($n = 50$)		FraxiPure™ ($n = 50$)		Difference (95% CI)
	Day 0	Day 90	Day 0	Day 90	
Alanine transaminase (IU/L)	27.9 ± 19.1	23.3 ± 12.7[*]	28.2 ± 17.1	24.5 ± 12.1[*]	(−4.1, 4.9)
Aspartate transaminase (IU/L)	22.1 ± 7.8	20.4 ± 5.9	22.1 ± 6.4	20.6 ± 5.6[*]	(−2.3, 2.4)
Total bilirubin (μmol/L)	10.2 ± 7.7	9.8 ± 5.3	8.5 ± 3.4	9.2 ± 3.9	(−0.5, 2.6)
Alkaline phosphatase (IU/L)	68.3 ± 14.7	66.0 ± 15.0	68.5 ± 16.4	64.8 ± 14.3[*]	(−5.5, 2.7)
Creatine (μmol/L)	74.4 ± 16.6	78.0 ± 15.7[*]	73.8 ± 14.7	77.6 ± 13.4[*]	(−2.3, 2.7)
GGTP (IU/L)	31.5 ± 38.2	27.6 ± 43.6	30.5 ± 18.6	24.5 ± 14.4[*]	(−7.4, 3.2)

GGTP, gamma-glutamyl transpeptidase.
[*] Significant (p < 0.05) intragroup difference between day 0 and day 90.

Table 5
Results of hematological parameters observed after administration of 1000 mg of FraxiPure™ for 90 days to healthy individuals compared to placebo. Values reported are means ± standard deviations; n = 50; differences between groups are expressed at the 95% confidence interval.[a]

Parameter (units of measure)	Placebo (n = 50)		FraxiPure™ (n = 50)		Difference (95% CI)
	Day 0	Day 90	Day 0	Day 90	
WBC ($\times 10^9$/L)	6.76 ± 1.56	6.83 ± 1.78	7.30 ± 1.84	7.04 ± 1.75	(−0.94, 0.27)
RBC ($\times 10^{12}$/L)	4.81 ± 0.36	4.95 ± 0.43[*]	5.00 ± 0.54	5.09 ± 0.53[*]	(−0.13, 0.03)
Hematocrit (%)	42.7 ± 3.3	43.3 ± 3.4	43.8 ± 4.0	43.9 ± 3.8	(−1.2, 0.3)
Hemoglobin (g/L)	145.2 ± 13.5	145.7 ± 13.1	148.5 ± 15.1	147.6 ± 14.1	(−3.9, 1.2)
MCV (fL)	88.8 ± 4.6	87.6 ± 4.5[*]	87.8 ± 5.9	86.7 ± 5.8[*]	(−0.4, 0.6)
MCH (pg/RBC)	30.3 ± 1.9	29.6 ± 1.8[*]	29.9 ± 2.4	29.3 ± 2.2[*]	(−0.2, 0.3)
MCHC (g/L)	339.9 ± 9.7	336.8 ± 8.0[*]	339.5 ± 9.8	336.7 ± 9.4[*]	(−3.1, 3.7)
Neutrophils ($\times 10^9$/L)	3.90 ± 1.23	4.20 ± 1.62	4.35 ± 1.51	4.31 ± 1.44	(−0.87, 0.18)
Eosinophils ($\times 10^9$/L)	0.16 ± 0.08	0.16 ± 0.13	0.20 ± 0.12	0.18 ± 0.11	(−0.07, 0.02)
Basophils ($\times 10^9$/L)	0.03 ± 0.02	0.04 ± 0.02[*]	0.03 ± 0.02	0.04 ± 0.02[*]	(−0.00, 0.01)
Lymphocytes ($\times 10^9$/L)	2.26 ± 0.59	2.02 ± 0.49[*]	2.31 ± 0.53	2.12 ± 0.48[*]	(−0.12, 0.23)
Monocytes ($\times 10^9$/L)	0.41 ± 0.12	0.42 ± 0.14	0.41 ± 0.11	0.40 ± 0.11	(−0.06, 0.01)
Platelet count ($\times 10^9$/L)	260.0 ± 55.6	279.8 ± 61.4[*]	268.4 ± 49.5	286.9 ± 58.8[*]	(−14.2, 11.4)

[a] RBC, red blood cells; WBC, white blood cells; MCV, mean corpuscular value; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.
[*] Significant ($p < 0.05$) intragroup difference between day 0 and day 90.

specified) in acute and short-term repeated dose oral toxicity studies conducted in rodents and dogs. No adverse effects were observed in two reproductive toxicity studies (species not specified) at doses of up to 10 mL/kg body weight/day of Phytodolor® (equivalent to a *F. excelsior* fresh stem bark extract of 950 mg/kg body weight/day). Furthermore, the authors reported that Phytodolor® was non-mutagenic in *in vitro* and *in vivo* genotoxicity assays, consisting of an unscheduled DNA test in rat hepatocytes, a mammalian spot test, and a mouse bone marrow cell micronucleus test. While this information on Phytodolor® may have been used to support the safe use of extracts of *F. excelsior*, it was indefinitive as Phytodolor® contains an extract of the fresh stem bark of *F. excelsior* and the existence of interactions between the three different extracts present in Phytodolor® is unknown.

4. Conclusion

The safety of FraxiPure™ has clearly been demonstrated by means of the absence of changes in biochemical or hematological parameters observed between FraxiPure™ and control groups in a double-blinded, placebo-controlled, randomized clinical study of 100 volunteers. FraxiPure™ was deemed to be well tolerated and any adverse events encountered were not deemed to be related to the product. An acute oral toxicity test *in vivo* and an *in vitro* toxicity study corroborated the safety of this *F. excelsior* extract.

Conflict of Interest

Acknowledgements

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.2012.11.030.

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