Food and Chemical Toxicology 50 (2012) 2845–2853

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

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

Safety evaluation of Evesse™ EPC, an apple polyphenol extract rich in flavan-3-ols

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ARTICLE INFO

Article history:
Received 28 February 2012
Accepted 9 May 2012
Available online 18 May 2012

Keywords:
EVESSE™ EPC
Apple polyphenols
Epicatechin
Ninety-day toxicity
Genotoxicity

ABSTRACT

The safety of the apple polyphenol extract Evesse™EPC, which is rich in flavan-3-ols, particularly epicatechin, was evaluated.

Both in a bacterial reverse mutation test and a mouse lymphoma assay, Evesse™EPC showed a positive response *in vitro*. *In vivo* studies (UDS test in hepatocytes, bone marrow micronucleus test and comet assay in intestinal cells) were all negative and hence Evesse™ EPC is considered not to have genotoxic properties *in vivo*.

In a 90-day study in rats, Evesse™EPC was administered at dietary levels of 0%, 1.25%, 2% and 3.25%. Body weights were decreased in the high-dose group in both sexes without effects on feed or water intake. In the high-dose group, thrombocytes (males) and creatinine (both sexes) were decreased, prothrombin time (males) was increased, and liver, kidneys and spleen weights were increased (males), without histological correlates. Diffuse acinar cell hypertrophy, observed in the parotid salivary glands in all treatment groups, was not considered as adverse and presumably reflected a local, reversible and adaptive response to direct contact with Evesse™EPC. The NOAEL for Evesse™EPC in rats was 2% in the diet, equivalent to an overall average intake of 1.3 and 1.5 g/kg body weight/day for males and females, respectively.

1. Introduction

Polyphenols are secondary plant metabolites characterised by the presence of more than one phenol unit or building block per molecule, and generally involved in defence against UV radiation or aggression by pathogens. Several thousand molecules have been identified, and polyphenols can be classified according to their structure as phenolic acids, flavonoids, stilbenes and lignans (Manach et al., 2004).

Flavonoids share a common structure consisting of 2 aromatic rings (A and B) bound together by 3 carbon atoms forming an oxygenated heterocycle (C ring), and have been further subclassified as flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavan-3-ols (catechins and their oligomers proanthocyanidins) (Manach et al., 2004; Hooper et al., 2008; Hollman et al., 2011). Flavonoids are present in significant amounts in many commonly consumed fruits, vegetables, grains, herbs, and beverages.

Epidemiological studies have examined cardiovascular effects of diets rich in flavonoids with the majority of studies showing positive associations, which may be attributable to beneficial activities related to oxidation, inflammation, platelet aggregation, endothelial function and blood flow (Erdman et al., 2007).

Specific interest in the flavonoid subclass of flavan-3-ols arose from observations that Kuna Indians of the San Blas Islands who consume large amounts of flavan-3-ols from cocoa have a very low blood pressure and lower cardiovascular mortality (Hollenberg et al., 2009). In addition, cardiovascular benefits of flavan-3-ols from cocoa or pure epicatechin have also been demonstrated in human intervention studies in relation to reducing blood pressure or improving endothelium function (increase in flow-mediated dilatation) (Engler et al., 2004; Heiss et al., 2003, 2005, 2007, 2010; Grassi et al. 2005, 2008; Balzer et al., 2008; Muniyappa et al. 2008; Davison et al., 2010; Schroeter et al., 2006; Hooper et al., 2008). Pure epicatechin increased flow-mediated dilatation acutely after ingestion of 1 mg epicatechin/kg body weight in humans (Schroeter et al., 2006). Flavan-3-ols may act by increasing the activity of endothelial nitric oxide synthase (eNOS) and as a consequence nitric oxide (NO) production. NO is an important vasoactive signalling molecule that diffuses from the endothelial to vascular smooth muscle cells where relaxation is induced (Corti

Abbreviations: dp, degree of polymerisation; E, erythrocytes; MPE, micronucleated polychromatic erythrocytes; MNE, micronucleated normochromatic erythrocytes; NE, normochromatic erythrocytes; PE, polychromatic erythrocytes.

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http://dx.doi.org/10.1016/j.fct.2012.05.013

et al., 2009). Increased levels of plasma or urinary NO metabolites were found following consumption of cocoa flavan-3-ols and pure epicatechin (Heiss et al., 2003, 2005, 2010; Loke et al., 2008).

Apples (*Rosaceae malus* sp.) have been part of the human diet since ancient times and are one of the world most commonly consumed fruits either in raw form or processed. Apples contain a variety of polyphenols including flavan-3-ols, quercetin, phloridzin and chlorogenic acid, but the polyphenol composition varies greatly between different varieties (Boyer and Liu, 2004). For example, Evesse™ Apples with the botanical classification *Malus pumila* MILLER are varieties selected specifically for their high flavan-3-ol, and specifically (−)-epicatechin content. Evesse™ Apples are the source material for the apple polyphenol extract Evesse™ EPC, which is rich in flavan-3-ols and contains a minimum of 30% epicatechin.

Safety of an apple polyphenol extract rich in proanthocyanidins has been demonstrated in genotoxicity and subchronic toxicity tests (Shoji et al., 2004). In addition, safety of polyphenols has also been shown in subchronic toxicological studies with Oligonol™, an optimised phenolic product containing catechin-type monomers and lower proanthocyanidin oligomers (Fujii et al., 2007) and various grape seed extracts containing oligomeric and polymeric proanthocyanidins (Yamakoshi et al., 2002; Bentivegna and Whitney, 2002; Wren et al., 2002).

However, the compositions of these extracts differ from that of Evesse™ EPC, and they have a considerably lower content of epicatechin monomer compared to Evesse™ EPC. which is rich in flavan-3-ols and contains a minimum of 30% epicatechin monomer, Therefore, we investigated the safety of the apple polyphenol extract Evesse™ EPC in a range of toxicological studies.

2. Materials and methods

The studies were conducted in accordance with the OECD Principles of Good Laboratory Practice (OECD, 1998a). The welfare of the animals was maintained in accordance with the general principles governing the use of animals in experiments of the European Communities (European Council Directive, 1986 86/609/EEC) and the Netherlands legislation (Experiments on Animals Act, 1977).

2.1. Manufacturing process and composition of the apple polyphenol extract Evesse™ EPC

Evesse™ Apples have the botanical classification *Malus pumila* MILLER, more generally known as *Malus domestica* or the domestic modern apple. Evesse™ Apples are selected from the Herefordshire Pomona in the UK, a 19th Century catalogue of variety type/names of apples and pears known to have been grown in Herefordshire (Hogg et al. 1884), and are the source material for the apple polyphenol extract hereafter mentioned as Evesse™ EPC. Evesse™ Apples are harvested at maturity in a period between August and November each year. Post harvesting, Evesse™ Apples are either freeze dried and powdered or stored in controlled atmosphere and/or coldstores at chilled conditions (2–5 °C) until processed. Whole fresh apples are de-waxed by an ethanol wash then combined with a proportion of freeze dried apple granules and hot water. While continually stirring, the extraction mixture is rapidly cooled and enzymes are added to assist in the liberation of flavan-3-ols from the apple granule matrix. Denaturing of the enzymes by heating and filtration yields a sugar-rich syrup. Residual carbohydrate polymers are further degraded with enzymes before the sugar-rich intermediate extract is passed over an ion-exchange column to remove sugars. Evesse™ EPC is precipitated using food-grade ethanol and dried. Evesse™ EPC has a high content of polyphenols (around 90% total polyphenols as catechin equivalents), specifically a minimum of 30% (−)-epicatechin, at least 20% flavan-3-ol oligomers (dp2-dp7),and minor amounts of oligomers with dp > 7 (max. 5%) and other catechins. In addition, the extract contains caffeoylquinic acid, phloridzin and quercetin glycosides, however, at concentrations below 5%.

2.2. Genotoxicity studies

The potential genotoxicity of Evesse™ EPC was evaluated using two *in vitro* genotoxicity tests; (1) a bacterial reverse mutation test (Ames test) and (2) a mammalian cell gene mutation test with mouse lymphoma L5178Y cells at the thymidine kinase (TK) locus, and three *in vivo* genotoxicity tests; (1) a bone marrow micronucleus test in rats, (2) an *in vivo* comet assay in rat intestinal cells and (3) an unscheduled DNA synthesis (UDS) test using rat hepatocytes. Positive and negative control substances were run simultaneously for all tests. The *in vitro* genotoxicity tests were performed in the presence and absence of S9 metabolic activation, which was prepared in house from homogenates of male Wistar rat livers following single intraperitoneal dosing (420 mg/kg body weight) of Aroclor 1254 (20% w/v in soy bean oil). The studies all met the acceptance criteria for validity; positive control substances induced the expected genotoxic effects and the results for the negative control substances were within the historical ranges.

2.2.1. Bacterial reverse mutation test

The bacterial reverse mutation test (Ames test) was performed in accordance with OECD testing guideline No. 471 (OECD, 1997a). The standard plate incorporation method with the histidine-requiring *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100 (courtesy of Dr. B.N. Ames, University of California Berkeley, USA) and the tryptophan-requiring *E. coli* strain WP2 uvrA (courtesy Dr. C. Voogd, National Institute of Public Health, Bilthoven, The Netherlands) was used. Evesse™ EPC was dissolved in dimethyl sulfoxide (DMSO) at a maximum concentration of 50 mg/ml (expressed as epicatechin; 33.4% of Evesse™ EPC). Final test concentrations ranged from 62 to 5000 μg/plate in triplicate cultures, with 5000 μg/plate being the recommended limit dose for this test system Approximately 10–16 h after preparation of fresh bacterial cultures, bacterial cultures were mixed with top agar, dose solutions of Evesse™ EPC, positive or negative controls, and S9 or 100 mM sodium phosphate, poured on minimal glucose agar plates and incubated for 48–72 h at 37 °C. His + and trp + revertants were counted using Sorcerer Ames software (Perceptive Instruments, Suffolk, UK). A more than 2-fold and/or dose-related increase in the mean number of revertant colonies compared to the concurrent negative controls was considered a positive mutagenic response.

2.2.2. Gene mutation in mammalian cells

The mouse lymphoma assay was performed in accordance with OECD testing guideline No. 476 (OECD, 1997b). Approximately one week before treatment, L5178Y cells (courtesy of Dr. J. Cole, University of Sussex, UK) were generated from frozen stock cultures. Evesse™ EPC was dissolved in DMSO at a maximum concentration of 370 and 100 mg/ml, with and without S9, respectively. L5178Y cells were treated in duplicate at 37 °C and 5% CO_2 for 4 h with S9 at final test concentrations ranging from 7 to 3700 μg/ml, and for 24 h without S9 at final test concentrations ranging from 8 to 1000 μg/ml. The maximum concentrations were limited by solubility and cytotoxicity, respectively. Cytotoxicity of Evesse™ EPC was determined by (1) the relative initial cell yield, (2) the relative suspension growth (RSG) and (3) the relative total growth (RTG). Approximately 48 h after treatment, subsamples were collected and incubated for 10–14 days at 37 °C and 5% CO_2 before determination of cloning efficiency and frequency of trifluorothymidine (TFT) resistant mutants. A positive mutagenic response was considered when the induced mutant frequency (*i.e.* the mutant frequency induced by Evesse™ EPC minus the mutant frequency induced by the negative control) was >126 mutants/1000,000 clonable cells; an equivocal response was >88 mutants/1000,000 clonable cells (Aaron et al., 1994; Clive et al., 1995).

2.2.3. Bone marrow micronucleus test and in vivo comet assay in rats

The study was conducted with male Wistar WU rats (Crl:WI(WU), outbred) aged 7–8 weeks. Further details with respect to supplier, allocation and maintenance are given in §2.3.1. Five rats per treatment group were used. The bone marrow micronucleus test was performed in accordance with OECD testing guideline No. 474 (OECD, 1997c). An *in vivo* comet assay in intestinal cells was conducted using the same animals. Incorporation of *in vivo* genotoxicity studies in other studies is highly encouraged from an ethical and economical point of view and is in line with the 3R principle on the replacement, reduction and refinement of animal testing. No OECD testing guideline of the *in vivo* comet assay currently exists, but the method is extensively described in literature (Hartmann et al., 2003; Smith et al., 2008; Tice et al., 2000) and is currently undergoing international validation. Moreover, the European Scientific Committee on Food (SCF) considers the comet assay useful for providing additional information on the genotoxic potential of food additives (SCF, 2001) and recently the European Food Safety Authority (EFSA) included the *in vivo* comet assay in their guidance as replacement for the UDS test (EFSA, 2011).

The rats were dosed orally with Evesse™ EPC at a dose level of 2000 mg/kg body weight (limit dose) or vehicle (corn oil) on three consecutive days. The third dose was not mandatory for the bone marrow micronucleus test, but was included for the comet assay. The interval between the first and second dosing was approximately 24 h and the interval between the second and third dose was approximately 21 h. Animals were sacrificed by decapitation under CO_2/O_2 anaesthesia approximately 3–6 h after the third dose.

Comet assay: Immediately after sacrifice, the first part of the jejunum (approximately 20 cm from the stomach) was isolated and rinsed with ice-cold Krebs–Ringer buffer. The intestinal segment was cut into small pieces in a collagenase B solution (1 mg/ml in Krebs–Ringer buffer at 37 °C). After approximately 5 min incubation at room temperature the cell suspension was filtered, centrifuged, and the cells were suspended in a sufficient volume for preparation of comet assay slides. Viability of the cell suspension was determined using tryphan blue dye exclusion. Comet assay slides were processed as described by Singh et al. (1988) with minor modifications. Slides were stained with ethidium bromide and analyzed using Co-