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Toxicological evaluation of pomegranate seed oil

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ABSTRACT

In this manuscript, the toxicology and safety of pomegranate seed oil (PSO) was evaluated by *in vitro* (Ames, chromosomal aberration), and *in vivo* toxicity tests (acute toxicity and 28-day toxicity in Wistar rats).

No mutagenicity of PSO was observed in the absence and presence of metabolic activation up to precipitating concentrations of 5000 μg/plate (Ames test) or 333 μg/ml (chromosome aberration test). The acute oral toxicity study revealed no significant findings at 2000 mg PSO/kg body weight. In the 28-day dietary toxicity study PSO was dosed at concentrations of 0, 10,000, 50,000 and 150,000 ppm, which resulted in a mean intake of 0–0, 825–847, 4269–4330 and 13,710–14,214 mg PSO/kg body weight per day in males–females, respectively. At 150,000 ppm dietary exposure to PSO, a much higher dose than the level of PSO that elicits antidiabetic and anti-inflammatory efficacy, increased hepatic enzyme activities determined in plasma (aspartate, alanine aminotransferase and alkaline phosphatase) and increased liver-to-body weight ratios were observed. However, these effects might be the result of a physiological response to exposure to a very high level of a fatty acid which is not part of the normal diet, and are most likely not toxicologically relevant. The no observable adverse effect level (NOAEL) was 50,000 ppm PSO (=4.3 g PSO/kg body weight/day).

1. Introduction

The pomegranate (*Punica granatum*) is an ancient fruit which has been widely consumed in many different cultures for thousands of years, largely without incidents, and thus is considered generally safe by the general public. It belongs to the family Punicaceae which grows only in Socotra, an island off the Yemeni coast (Lansky and Newman, 2007). The pomegranate is native from Iran to the Himalayas in northern India, and has been cultivated and naturalized over the whole Mediterranean region since ancient times. Over 1000 cultivars of *P. granatum* exist (Levin, 1994). It is widely cultivated throughout Iran, India, the drier parts of Southeast Asia, Malaysia, the East Indies, and tropical Africa. Pomegranate is now cultivated mainly in the drier parts of California and Arizona for its fruits exploited commercially as juice products gaining in popularity since 2001, especially because of the appearance of several reports presenting the antioxidant activities of pomegranate fractions *in vitro* (Schubert et al., 1999; Gil et al., 2000; Singh et al., 2002).

Pomegranate seed oil (PSO) comprises 12–20% of total seed weight. The oil is characterized by a high content of conjugated linolenic acids (CLNA, about 31.8–86.6%), followed by linoleic acid (0.7–24.4%), oleic acid (0.4–17.7%), stearic acid (2.8–16.7%) and palmitic acid (0.3–9.9%) (El-Shaarawy and Nahapetian, 1983; Ozgul-Yucel, 2005; Fadavi et al., 2006). Variability in concentration is due to differences between cultivars. The CLNA found in PSO are all 9,11,13 isomers, with punicic acid (9 cis, 11 trans, 13 cis) as the predominant conjugated triene (Ozgul-Yucel, 2005). Cold-pressed PSO has been commercially available during the last 5–6 years. The pomegranate seeds are cleaned and pressed giving virgin oil which is further purified by centrifugation, sedimentation or filtration. The cold-pressed oil obtained can then be used for skin care recipes or food use as a dietary supplement.

Although pomegranate has been widely consumed for thousands of years, little is known about the possible toxicity and safety

Abbreviations: 2-AA, 2-aminoanthracene; CLNA, conjugated linolenic acids; DMSO, dimethyl sulfoxide; EEC, European Economic Committee; GLP, Good Laboratory Practice; MMS, methylmethanesulfonate; 4-NQO, 4-nitroquinoline N-oxide; OECD, Organisation for Economic Co-operation and Development; PSO, pomegranate seed oil.

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of the pomegranate or the PSO. Several studies indicate that cold-pressed PSO is able to reduce tumor occurrence, tumor incidence and multiplicity in both *ex vivo* models and *in vivo* in mice and rats. In an *ex vivo* model using mouse mammary organ culture (Mehta and Lansky, 2004), cold-pressed PSO reduced the tumor occurrence up to 87%. In an *in vivo* study with female CD-1 mice with skin tumors induced by topical application of 7,12-dimetylbenz[*a*]anthracene and promoted by 12-*O*-tetradecanoylphorbol 13-acetate, PSO applied topically (5%) resulted in significant decreases in tumor incidence and multiplicity (Hora et al., 2003). In male F344 rats treated with the carcinogen azoxymethane, dietary PSO at concentrations of 0.1% already reduced the incidence and multiplicity of colon tumors (Kohno et al., 2004).

However, besides several very intriguing and promising studies on the possible beneficial effects of PSO, little is known about the possible toxicity and safety of pomegranate fruits or PSO, and standard *in vitro* and *in vivo* toxicology studies have not been performed with PSO. Therefore, in the current investigations we studied the possible mutagenicity of PSO using two *in vitro* mutagenicity studies (the Ames test and chromosomal aberration test), and the possible *in vivo* toxicity of PSO using Wistar rats (acute oral toxicity study and 28-day toxicity test).

2. Materials and methods

2.1. Pomegranate seed oil (PSO)

Cold-pressed PSO was obtained from Lipid Nutrition, Wormerveer, The Netherlands. The composition of the PSO was determined using GC analysis and is presented in Table 1.

2.2. Chemicals and bacterial strains

The *Salmonella typhimurium* strains TA1535, TA1537 and TA98 were obtained from Dr. B.N. Ames (University of California, Berkeley, USA) and the strain TA100 was obtained from Xenometric (Boulder, Co, USA). The *Escherichia coli* strain was obtained from Prof. Dr. B.A. Bridges (University of Sussex, Brighton, UK). Positive controls used in the Ames test without metabolic activation were sodium azide (TA1535), 9-aminoacridine (TA1537), 2-nitrofluorene (TA98), methylmethanesulfonate (TA100) and 4-nitroquinoline N-oxide (WP_2uvrA). In the presence of 5 and 10% S9-mix as metabolic activation, 2-aminoanthracene (2AA) was used as positive control. Sodium azide, methylmethanesulfonate, 4-nitroquinoline N-oxide, 2-aminoanthracene, β-naphthoflavone were obtained from Sigma, Zwijndrecht, The Netherlands. 9-Aminoacridine and colchicine were from Acros Organics, Geel, Belgium. Dimethyl sulfoxide, 2-nitrofluorene and Giemsa were obtained from Merck, Darmstadt, Germany.

2.3. Animal husbandry

All procedures concerning the use of animals were approved by the Animal Experimental Committee of NOTOX. The animals were housed in a controlled environment, with approximately 15 air changes per hour, a temperature of 21 ± 3 °C and a relative humidity of 30–70%. The rooms were illuminated with 12 h artificial fluorescent light and 12 h darkness per day. Unless otherwise stated, the animals were provided a standard pelleted laboratory animal diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany) that met or exceeded all nutritional needs and tap water *ad libitum*. The animals were allowed to acclimatize for at least 5 days before the start of the treatment. Results of analysis for ingredients and/or contaminants of diet, bedding, paper and water were assessed and did not reveal any findings that were considered to have affected the study integrity.

Table 1
Chemical composition of the two PSO batches used.

	Batch A[a] (%)	Batch B[b] (%)
Triglycerides	94	91
Diglycerides	2.3	3.2
Monoglycerides	0.4	1.0
Free fatty acids	0.9	2.2
Polymers	2.1	2.8
Total composition (%)	99.7	100.2
Fatty acid analysis (%)		
C16:0 stearic acid	2.7	2.9
C18:0 palmitic acid	2.6	2.2
C18:1 oleic acid	5.9	7
C18:2 linoleic acid	5.8	6.5
CLNA	82.8	79.6
Total fatty acid analysis (%)	99.8	98.2

[a] Batch A is used for the two *in vitro* mutagenicity assays and the *in vivo* acute oral toxicity study.
[b] Batch B is used for preparation of the diets for the 28-day dietary toxicity study.

2.4. Preparation of rat liver S9-fraction for in vitro mutagenicity studies

Rat liver S9 was prepared from young adult male Wistar rats (8 weeks of age), which were obtained from Charles River (Sulzfeld, Germany). Five male rats per S9-batch were group-housed in labeled polycarbonate cages (type MIV height: 18 cm) containing Woody Clean bedding (Woody-Clean type 3/4; Technilab-BMI BV, Someren, The Netherlands) and paper as cage-enrichment (Technilab-BMI BV).

The rats were orally dosed for three consecutive days with a suspension of phenobarbital (80 mg/kg body weight) and β-naphthoflavone (100 mg/kg body weight) in corn oil. The rats were denied access to food for 3–4 h preceding each dosing. One day after the final exposure, the rats were sedated using oxygen/carbon dioxide and sacrificed by decapitation. The rats received a limited quantity of food during the night before sacrifice. The livers of the rats were removed aseptically, and washed in cold (0 °C) sterile 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 mM Na_2-EDTA (Merck, Germany). Subsequently the livers were minced in a blender and homogenized in 3 volumes of 0.1 M sodium phosphate buffer (pH 7.4) with a Potter-Elvehjem tube and Teflon pestle. The homogenate was centrifuged for 15 min at 9000*g* (4 °C). The resulting supernatant (S9) was transferred into sterile ampules, which were stored in liquid nitrogen (−196 °C) for a maximum of 1 year. All batches were checked for sterility and the concentration of cytochrome P450 was calculated from the CO-reduced difference spectrum according to Omura and Sato (1964).

2.5. In vitro mutagenicity tests

2.5.1. Ames test

PSO was tested in the *S. typhimurium* reverse mutation assay with four histidine-requiring strains of *S. typhimurium* (TA1535, TA1537, TA98 and TA100) and in the *E. coli* reverse mutation assay with a tryptophan-requiring strain of *E. coli* (WP_2uvrA). The test was performed in two independent experiments in the presence and absence of metabolic activation (phenobarbital- and β-naphthoflavone-induced rat liver S9-mix), and was performed under GLP conditions according to OECD and EEC guidelines (OECD 471 and 2000/32/EC).

PSO was dissolved in dimethyl sulfoxide and tested in TA100 and WP_2uvrA at concentrations of 0 (solvent control), 3, 10, 33, 100, 333, 1000, 3330 and 5000 μg/plate in the absence and presence of 5% (v:v) S9-mix (first experiment). Since PSO precipitated on the plates at concentrations of 3330 μg/plate and 5000 μg/plate, in the strains TA1535, TA1537 and TA98, PSO was tested at concentrations of 33, 100, 333, 1000 and 3330 μg/plate. An independent repeat assay was performed, in which PSO was tested at concentrations of 33, 100, 333, 1000 and 3330 μg/plate in all bacterial strains in the absence and presence of 10% (v:v) S9-mix (second experiment). Appropriate negative and strain-specific positive controls were added.

2.5.2. In vitro chromosomal aberration test using cultured peripheral human lymphocytes

A chromosomal aberration test using cultured peripheral human lymphocytes was performed under GLP conditions according to OECD and EEC guidelines (OECD 473 and 2000/32/EC). Two independent experiments were performed. In the first experiment, PSO was tested at concentrations of 33, 100 and 333 μg/ml for a 3 h exposure time with a 24 h fixation time in the absence and presence of 1.8% (v:v) S9-fraction. PSO precipitated in the culture medium at a concentration of 333 μg/ml. In the second experiment, PSO was again tested at concentrations of 33, 100 and 333 μg/ml, with a change in incubation time and fixation time. In the absence of S9-mix, 24 h and 48 h continuous exposure times were used with a 24 and 48 h fixation time. In the presence of S9-mix, a 3 h exposure time and 48 h fixation time was used. As positive controls, mitomycin (Sigma, Zwijndrecht, The Netherlands) was used for incubations without metabolic activation, and cyclophosphamide (Endoxan-Asta, Asta-Werke, Germany) for incubations with metabolic activation. As negative control, dimethyl sulfoxide was used since this was the solvent used to dissolve PSO.

During the last 2.5–3 h of the culture period, cell division was arrested by the addition of the spindle inhibitor colchicine (0.5 μg/ml medium). Thereafter, the cell cultures were centrifuged and the remaining pellet was treated for 5 min with hypotonic 0.56% (w:v) potassium chloride solution at 37 °C. Subsequently, the cells were fixed with 3 changes of methanol:acetic acid 3:1 (v:v). Fixed cells were dropped onto clean slides. Slides were allowed to dry and stained for 10–30 min with 5% (v:v) Giemsa solution in tap water. Thereafter, slides were rinsed in tap water, allowed to dry, embedded in MicroMount and mounted with a coverslip. All slides were randomly coded before examination of chromosome aberrations and scoring was performed in a blind fashion. Per culture, 100 metaphase chromo-