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Assessment of the genotoxic risk of *Punica granatum* L. (Punicaceae) whole fruit extracts

Angel Sánchez-Lamar[a,*], Gladys Fonseca[a], Jorge Luis Fuentes[b], Renata Cozzi[c], Enrico Cundari[d], Mario Fiore[d], Ruggero Ricordy[d], Paolo Perticone[d], Francesca Degrassi[d], Rosella De Salvia[d,**]

[a] *Departamento de Biología Vegetal, Laboratorio de Toxicología Genética, Facultad de Biología, Universidad de la Habana, Calle 25 No. 455, e/ I y J, Vedado, C. Habana, Cuba*
[b] *Centro de Aplicaciones Tecnológicas y Desarrollo Nuclear (CEADEN), Apartado Postal 6122, Calle 30 #502, e/ 5ta y 7ma, Miramar, Playa, C. Habana, Cuba*
[c] *Dipartimento di Biologia, Universitá degli Studi "Roma Tre", via G. Marconi 446, 00146 Roma, Italy*
[d] *Istituto di Biologia e Patologia Molecolare, CNR, via degli Apuli. 4, 00185 Roma, Italy*

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Abstract

Punica granatum L. (Punicaceae) whole fruit extracts, have been used in Cuban traditional medicine as an effective drug for the treatment of respiratory diseases. This species showed interesting anti-viral activity, e.g. aqueous or hydroalcoholic extracts of whole fruits have proved highly active against the influenza virus. However, some toxic properties of this extract have also been reported and, to date, very little is known about its genotoxic properties. In the present study, the genotoxicity of a *Punica granatum* (pomegranate) whole fruit extract was assessed using different *in vitro* and *in vivo* assays that detect DNA damage at different expression levels. Results from reversion and gene-conversion test in microorganisms, sister chromatid exchanges, micronuclei and sperm-shape abnormality assays in mice, clearly showed that the hydroalcoholic extract of *P. granatum* whole fruits is genotoxic when tested both *in vitro* and *in vivo*.

Keywords: *Punica granatum*; Genotoxicity; Reversion test; Conversion test; Sister chromatid exchanges; Micronuclei; Sperm-shape abnormality

1. Introduction

The species *Punica granatum* L. (Punicaceae) has been widely used by traditional medicine in America, Asia, Africa and Europe for the treatment of different types of diseases (Gracious Ross et al., 2001; Kim et al., 2002; Murthy et al., 2004). Many pharmacological properties have been experimentally demonstrated (House and Lagos-Witte, 1995; Alekperov, 2002; Cerda et al., 2003; Vasconcelos et al., 2003; Afaq et al., 2005a,b; Seeram et al., 2005). In Cuban traditional medicine the fruits of *P. granatum* (pomegranate) have been used to treat acidosis, dysentery, microbial infections, diarrhoea, helminthiasis, haemorrhage, and respiratory pathologies (Fuentes and Expósito, 1995). Furthermore, this species appears to have interesting antiviral activity. Extracts have been shown to be effective against the herpes virus (Zhang et al., 1995) and hydroalcoholic extracts of whole fruits have exhibited high activity against the influenza virus (Caballero et al., 2001; Peña and Martínez, 2001).

Studies carried out with aqueous extracts of pomegranate in conditions similar to those used by traditional medicine, showed no toxic effects (Desta, 1995). Nevertheless, it has been demonstrated that some parts of the plant, as the root and the bark, are toxic (Fuentes et al., 1985). The toxic activity of some galenic preparations of pomegranate extract was related to its alkaloid content (Ferrara et al., 1989; Tripathi and Singh, 2000). Vidal et al. (2003), reported that the toxic effects of *P. granatum* whole

* Corresponding author. Tel.: +53 7 8328542; fax: +53 7 8321321.
** Corresponding author. Tel.: +39 06445727, fax: +39 06445729.
E-mail addresses: alamar@fbio.uh.cu (A. Sánchez-Lamar), desalvia@ascaspur.it (R. De Salvia).

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fruit extract took place at higher doses than those effective as anti-viral or those used in Cuban traditional medicine. These authors have also indicated that the *P. granatum* fruit extract was innocuous when directly administered via the nasal cavity.

As these complex mixtures from medicinal plants could contain genotoxic compounds, there is an increasing interest in determining their genotoxicity. The only available study on genotoxicity of pomegranate (Amorin, 1995), showed no mutagenic effect in mouse treated with an aqueous extract similar to those used in folk-medicine. Since hydroalcoholic extracts of *P. granatum* whole fruits have been currently evaluated in preclinical trials for their application in the treatment of viral diseases, the present work aims at assessing the potential genotoxic risk of such extracts. To this purpose, different genetic end-points were assayed, in order to evaluate DNA damage at different genetic expression levels.

2. Methodology

2.1. Plant extract

Fresh *P. granatum* (pomegranate) fruits were collected from wild plants in the Havana province in July 2001 and their identities confirmed by Dr. Alvarez et al. The voucher specimen is deposited at the Havana Botanical Garden (HAJB) with the number 40619 HAJB, Havana, Cuba. The mature fruits (including the peel) were macerated in a 50% (v/v) ethanol solution in a ratio of 1:3 (w/v), during 15 days. The extract was filtered and the ethanol removed by vacuum evaporation at 55 °C, then the extract was lyophilized and stored at −20 °C.

2.2. Negative control

In all cases (*in vivo* and *in vitro* assays) the negative control was the appropriate solvent used to dissolve plant extract and all the employed substances.

2.3. Positive control

The following positive controls were used: 2AF (Amino Fluorene) in Ames test; CP (Cyclophosphamide) in *Saccharomyces cerevisiae*; mouse bone marrow micronucleus and sperm-shape abnormality assays; MMC (Mitomycin-C) in Sister Chromatid Exchanges and in Chromosome Aberrations assays in CHO (Chinese hamster ovary) cells; EMS (ethyl methane sulphonate) in *Saccharomyces cerevisiae* assay.

2.4. In vitro assays

2.4.1. Ames assay

The plate-incorporation mutagenicity assay was performed as described (Maron and Ames, 1983) using *Salmonella typhimurium* tester strains TA100. Experiments with metabolic activation were conducted using rat liver S9 at a concentration in the activation mix of 4%. Plates were incubated for 72 h and counted.

2.4.2. Saccharomyces cerevisiae assay

The testing procedure described by Zimmermann et al. (1975) was used with strain D7 (MATa/MATα, ade2-40/ade2-119, trp5-12/trp5-27, ilv1-92/ilv1-92). Cells from the stationary phase of growth were treated with the fruit extract. Afterwards, cell suspensions were processed following the methodology described by Bronzetti et al. (1981). For assays with metabolic activation, rat-liver S9 fraction was used.

2.4.3. Cytogenetic assays on Chinese hamster ovary (CHO) cells

The CHO cells were grown in Ham F10 medium supplemented with foetal calf serum (10%), penicillin (100 IU/ml), streptomycin (100 IU/ml) and L-glutamine (2 mM) in a CO_2 humidified atmosphere (5%) at 37 °C. All experiments were performed seeding an initial cell density of 1.5×10^5 cells per 25 cm^2 flask. After 12 h, exponentially growing cells were treated with plant fruit extract in the presence or absence of rat S9 mix and the following genotoxicity tests were performed:

2.4.3.1. Sister chromatid exchanges (SCE) assay. After a 3 h fruit extract treatment, the cultures were incubated in fresh complete medium containing BrdUrd at the final concentration of 1.5 μg/ml. The cells were fixed after 26 h for SCE analysis. Colchicine (5×10^{-7} M) was always added 2 h before the cells were fixed. As previously described FPG (Hoechst-Giemsa technique modified by De Salvia et al., 1999), was used for differential staining of sister chromatids. In order to determine the SCE, 40 s mitoses (M_2) were blind scored from coded slides for each experimental point. The mitotic index (MI) was determined on 1000 scored cells and 100 metaphases were analysed for first (M_1); second (M_2) and third (M_3) mitoses.

2.4.3.2. Chromosome aberration (CA) assay. The chromosome aberration test was conducted according to the international guidelines for this assay (ICH, 1995). Exponentially growing cells were treated with plant extract for 3 h without S9 fraction. After 18 h of post-treatment, cells were harvested. Colchicine (5×10^{-7} M) was added 2 h before harvesting. After trypsinisation, the cells were treated with hypotonic solution, fixed with acetic acid and methanol (1+3) solution and stained as reported by Galloway et al. (1994). At least 200 metaphases were scored for each dose of fruit extract.

2.5. In vivo assays

Balb/C mice (*Mus musculus*) obtained from the National Centre for Production of Laboratory Animals (CENPALAB, Cuba) were bred in our laboratory animal-care facility, and employed in the present work. Adult healthy animals, 8–10 weeks old, weighing 25–30 g of either sex were used. Each experimental group consisted of five animals.

All the animal studies reported in this work were carried out in accordance with the Cuban regulations on the protection of animals (Código Práctico para el Uso de los Animales de