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Article

Assessment of mutagenic and antimutagenic effects of *Punica granatum* in mice

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In the present study, the ability of *Punica granatum* ethanolic leaf extract (PGL) and Punica granatum ethanolic fruit extract (PGF) to induce mutagenicity or to modulate the genotoxic effects induced by the alkylating agent cyclophosphamide (CP) was evaluated. Swiss male mice were treated by gavage for 10 days with PGL or PGF (12.5, 25, 50, and 75 mg/kg/day) prior to exposure to CP (i.p. 200 mg/kg), 24 h after the end of the treatment. Initial observations revealed that normal mice treated with both extracts (12.5, 25, 50, and 75 mg/kg/day) showed a similar micronucleated polychromatic erythrocyte (MNPCE) frequency to that of the control group. Investigation of the protective effect of PGL and PGF based on data analysis revealed that, irrespective of dose or extract, oral administration of PGL or PGF for 10 days prior to exposure had reduced, in a dose-dependent manner, the frequency of MNPCE induced by CP in all groups studied. Higher reductions were observed at PGF doses of 50 and 75 mg/kg. Taken together, these results demonstrate that mice treated with *P. granatum* showed an absence of mutagenic effects and dose-dependent protective effects against CP-induced oxidative DNA damage.

Uniterms: *Punica granatum*/mutagenic effects. *Punica granatum*/antimutagenic effects. Micronucleic. Genotoxicity. Cyclophosphamide.

No presente estudo investigamos o potencial do extrato etanólico das folhas da *Punica granatum* (PGFO) e do extrato etanólico dos frutos da *Punica granatum* (PGFR) de induzir mutagenicidade ou de proteger contra efeitos genotóxicos induzidos pela ciclofosfamida (CF). Camundongos machos Swiss foram tratados por 10 dias, via oral, com PGFO ou PGFR (12,5, 25, 50 e 75 mg/kg/dia), previamente a exposição à CF (i.p. 200 mg/kg) 24 horas após término do tratamento. Observamos que os animais tratados por 10 dias com ambos os extratos (12,5, 25, 50 e 75 mg/kg/dia) demonstraram a frequência de micronúcleo policromático eritrocitário (MNPCE) similar ao grupo controle. Quando aos efeitos protetores dos extratos foram investigados, a análise dos dados revelou que, independentemente da dose ou do extrato usado, a administração oral por 10 dias, previamente à exposição, reduziu, de forma dose-dependente, a frequência de MNPCE induzidos pela CF, em todos os grupos estudados. As maiores reduções foram observadas com PGFR nas doses de 50 e 75 mg/kg. Em conjunto, sob as condições testadas, camundongos tratados com *P. granatum* demonstraram ausência de efeitos mutagênicos e, de forma dose-dependente, efeitos protetores contra os danos oxidativos do DNA induzidos pela CF.

Unitermos: *Punica granatum*/efeitos mutagênicos. *Punica granatum*/efeitos antimutagênicos. Micronúcleo. Genotoxicidade. Ciclofosfamida.

INTRODUCTION

Punica granatum Linn., a Punicaceae, commonly known as pomegranate, has a vast ethnomedical history and represents a phytochemical reservoir of heuristic medicinal value (Lansky and Newman, 2007). The plant as a whole and especially its flowers, have been extensively used in the ancient Ayurvedic health care system (Sivarajan and Balachandran, 1994).

In fact, studies on *P. granatum* phytochemistry and

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pharmacological actions suggest a wide range of potential clinical applications. Antitumour (Afaq *et al*., 2005; Lansky and Newman, 2007), antibacterial (Prashanth *et al*., 2001; Braga *et al*., 2005; Menezes *et al*., 2006; Aqil, Ahmad, 2007), antidiarrhoeal (Das *et al*., 1999; Mathabe *et al*., 2006), antifungal (Dutta *et al*., 1998; Vasconcelos *et al*., 2003; Vasconcelos *et al*., 2006), antiulcer (Gharzouli *et al*., 1999), and antioxidant pharmacological properties have been reported for various extracts/constituents of different parts of this plant species.

Recently, the antioxidant activity of *P. granatum* associated with its phytochemicals, such as, polyphenols, flavonoids, and anthocyanidins has gained importance (Faria *et al*., 2007; Guo *et al*., 2007; Kulkarni *et al*., 2007; Mirdehghan *et al*., 2007; Reddy *et al*., 2007; Rout, Banerjee, 2007; Sestili *et al*., 2007; Zaid *et al*., 2007). In this regard, Negi *et al*. (2003) demonstrated that the *in vitro* antioxidant ability of the pomegranate fruit, rich in polyphenols and anthocyanidins, was higher than that found in green tea, also considered a powerful antioxidant. Several extracts/constituents of *P. granatum* have been found to prevent low-density lipoprotein oxidation and hence are antiarthrogenic (Aviram *et al*., 2002; Wang *et al*., 2004), modulating the expression of oxidation-sensitive genes *in vitro* and in hypercholesterolemic mice (De Nigris *et al*., 2005), as well as inhibiting the nuclear factor κβ, which is activated by reactive oxygen species (ROS) (Afaq *et al*., 2005). Moreover, Guo *et al*. (2007) demonstrated *in vitro,* a powerful DNA damage prevention ability of *P. granatum*. Although extracts from many parts of this plant, including its fruit, seeds, and peel, have been reported to exhibit strong antioxidant activity *in vitro*, important confirmation and characterization of these effects using biological systems *in vivo* were lacking. Cerda *et al*. (2004, 2006) suggested that the clinical antioxidative efficacy of pomegranate could be impaired by the poor bioavailability found in its active compounds.

P. granatum is generally considered safe due to the fact that it is widely consumed by numerous populations in many different countries. However, toxic effects have been ascribed to the consumption of this plant, including acute gastric inflammation, congestion of internal organs with elevated creatinine *in vivo* (Vidal *et al*., 2003), and even death, as well as, allergic reactions (Gaig *et al*., 1999; Hegde *et al*., 2002; Igea *et al*., 1991; Lansky, Newman, 2007).

Genetic toxicology tests are assays designed to detect direct or indirect genetic damage induced by chemical compounds. Fixation of DNA damage can result in gene mutations, loss of heterozygosity, chromosome loss or gain, and chromosome aberrations. These events may play an important role in many malignancies. Thus, identifying genotoxic/mutagenic effects is important for the risk/benefit assessment of substances, in particular those which are part of the dietary habits of any populations (Doppalapudi *et al*., 2007). The *in vivo* mouse bone marrow micronucleus (MN) assay is currently recommended by the International Conference on Harmonization (ICH, 1997), the Food and Drug Administration, and other regulatory agencies (Cimino, 2006) to investigate the genotoxic/chemopreventive potential of new agents. In addition, a direct connection between increase in MN frequency and development of carcinogenesis has been documented in the literature (Olaharski *et al*., 2006).

The present work was designed to further investigate the effects of *P. granatum* on biological systems and aimed to study the ability of both its ethanolic leaf extract (PGL) and ethanolic fruit extract (PGF) to induce mutagenicity or modulate the genotoxic effects induced by the alkylating agent cyclophosphamide (CP) in mice, using the micronucleus test.

MATERIAL AND METHODS

Mice treatment

The experiments were carried out on adult male Swiss mice (8-12 week) obtained from Indústria Química do Estado de Goiás (IQUEGO). All mice weighed between 28 g and 30 g and were kept under constant environmental conditions with a 12:12 light-dark cycle. The animals were fed standard granulated chow and had access to drinking water *ad libitum*. Animal experiments were done in accordance with Institutional Protocols of Animal Care. The mice groups (n=5) received PGL or PGF daily doses of 12.5; 25; 50 or 75 mg/kg (0.2 mL/mouse), for 10 consecutive days by gavage, prior to exposition. For the antimutagenic assay, mice were exposed to cyclophosphamide, (CP) (Genuxal®, Baxter Oncology, Lot# 7K585B, Expiration date 11/2010) i.p., (200 mg/kg) 24 h after the end of the treatment, administrated as a single dose diluted in physiological saline. Each experiment included parallel control groups of normal and exposed mice treated with an equivalent volume (0.2 mL/animal) of the vehicle (n=5). The micronuclei frequency quantification was performed 24 h after exposure to cyclophosphamide.

Extract preparation

Plant material from *P. granatum*, leaves and fruits, was collected in September 2005 in Goiânia, Goiás State, Brazil. The fruits or leaves were dried and ground to a po-

wder and both, PGL and PGF, were prepared according to guidelines of the Brazilian Pharmacopoeia. After filtration, both PGL and PGE were rotaevaporated to dryness under low pressure and temperature. The extracts obtained were kept at 4 °C.

Micronucleus test

The mutagenicity and antimutagenicity of PGL and PGF were evaluated using the micronucleus test by scoring 1000 bone marrow cells for each of the 5 animals per treatment group, as described by MacGregor *et al*. (1987). To detect possible cytotoxic effects, the proportion of polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) in 1000 erythrocytes/animal was calculated. We stained the cells with Leishman, coded the slides, and scored in a blind test using a light microscope (1000 x magnification). The frequency of micronucleated polychromatic erythrocytes (MNPCE) in individual mice was used as the experimental unit, with variability (standard deviation) based on differences among animals within the same group.

Statistical analysis

The data from the micronucleus assay were statistically analyzed using Student's t-test, comparing the treated groups with controls (Pereira, 1991). The significance level considered was $P<0.05$. The percentage of reduction in the frequency of CP-induced DNA damage was calculated according to Manoharan and Banerjee (1985), by the following formula: % reduction = ((mean frequency of damage in A - mean frequency of damage in B)/(mean frequency of damage in A - mean frequency of damage in C)) x 100.

Where, A = positive control group treated with CP; B = group treated with the PGL or PGF + CP; C = negative control group.

RESULTS

The frequency of MNPCE in bone marrow cells of mice orally treated with different doses of PGL and PGF (12.5, 25, 50, 75 mg/kg) are shown in Figure 1. Oral treatment with PGL and PGF for 10 days did not induce any mutagenic effects in mouse bone marrow cells, i.e., no statistically significant difference in the frequency of MNPCE or the ratio of PCE to NCE between the negative control and the groups treated with PGL and PGF was detected. In fact, a tendency toward a decrease in normal MNPCE frequency in control mice at the dose of 75 mg/kg of PGF was observed.

The effects of the prophylactic treatment with PGL and PGF on the frequencies of MNPCE in mice bone marrow 24 h after exposure to CP, an indirect alkylant agent, are shown in Figure 2. Examination of the antimutagenicity profile of the extracts revealed significant decrease ($P < 0.05$), in a dose-dependent manner, in the frequency of CP-induced MNPCE in all groups. The highest doses of PGF were found to be more effective against CP-induced DNA damage. Figure 3 shows the percentage reduction in the frequency of MNPCE in mice pre-treated with PGL

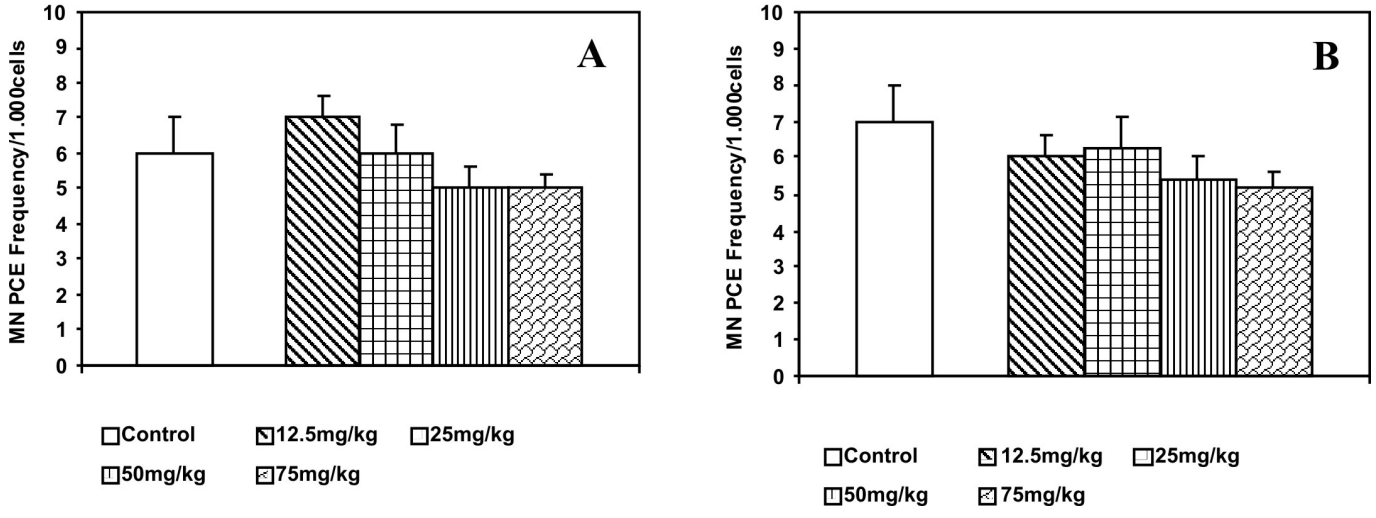

FIGURE 1 - Effects of prophylactic oral treatment with *Punica granatum* ethanolic leaf extract - PGL (A) and ethanolic fruit extract - PGF (B) (12.5, 25, 50, 75 mg/kg/day) for 10 days, on the frequency of micronucleated polychromatic erythrocytes (MNPCE) in mice (n = 5).

and PGF. Daily PGL doses of 12.5, 25, 50, or 75 mg/kg reduced the frequency of MN by 35, 56, 61 and 60%, whereas treatment with 12.5, 25, 50, or 75 mg/kg of PGF led to 45, 69, 80 and 80% reduction, respectively.

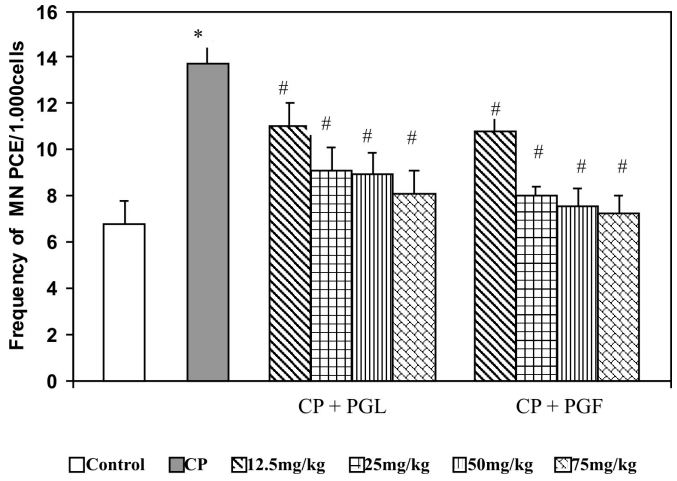

FIGURE 2 - Effects of prophylactic oral treatment with *Punica granatum* ethanolic leaf extract - PGL and the ethanolic fruit extract - PGF (12.5; 25; 50 or 75mg/kg/day) for 10 days on the frequency of micronucleated polychromatic erythrocytes (MNPCE) in mice (n = 5) exposed to cyclophosphamide (CP). $^{*}P < 0.05$ when compared to negative control; $^{\#}P < 0.05$ when compared to positive control.

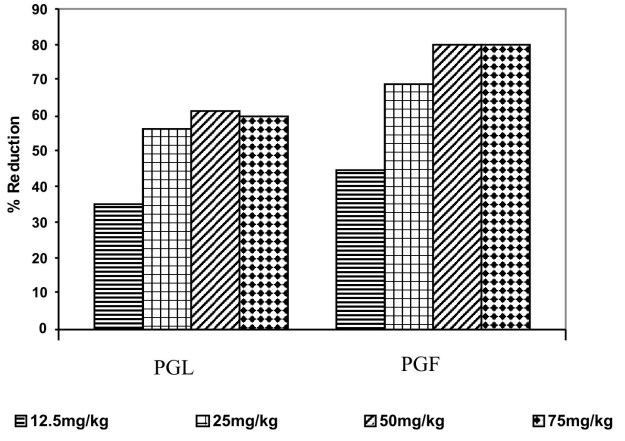

FIGURE 3 - Percentage micronucleated polychromatic erythrocyte (MNPCE) reduction in mice (n = 5) orally pretreated with *Punica granatum* ethanolic leaf extract - PGL or ethanolic fruit extract - PGF (12.5, 25, 50, 75 mg/kg/day) for 10 days and exposed to cyclophosphamide (CP).

DISCUSSION

The concept that environmental chemical exposure could induce DNA damage has led to the introduction of requirements for testing mutagenic properties of new and/or frequently consumed substances, especially food. However, it has been documented in the literature that antioxidant intake can reduce cancer risk and may also mitigate the effects of oxidative DNA damage (Watters *et al*., 2007). In the present study, we evaluated the mutagenic and anti-mutagenic potential of PGL and PGF in mouse bone marrow cells. Our first observation was that normal mice orally treated for 10 days with both extracts showed micronuclei frequency similar to that found in the control group. When the protective effect of PGL and PGF were investigated in mouse bone marrow cells, the data analysis revealed that, irrespective of dose or extract, the treatments reduced the frequency of MNPCE induced by CP exposure. The oral administration of 12.5, 25, 50, and 75 mg/kg/day of PGL or PGF for 10 days prior to CP exposure reduced, in a dose-dependent manner, the frequency of MNPCE induced by CP in all groups studied. Higher reductions were observed at higher PGF doses (50 and 75 mg/kg). These results are in accordance with the findings of Guo *et al.* (2007), which demonstrated *in vitro* that besides scavenging free radicals and ROS, *P. granatum* also prevents DNA damage. Therefore, our results confirm and extend our knowledge on the ability of *P. granatum* to protect DNA, showing that both PGL and PGF prevent chromosome damage after CP exposure in mice.

In recent years, attention has been focused on the antioxidant properties elicited by plants or food against ROS, lipid peroxidation, protein damage, and DNA strand breaking. Several plants can positively modulate biological systems against damaging effects produced by active oxygen species by several means, including free radical scavengers and enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Srinivasan *et al.*, 2007).

The antioxidant activities of *P. granatum* are associated with different bioactive components, mainly polyphenols, ellagitannins, condensed tannins, and anthocyanins (Kulkarni *et al*., 2004; Li *et al*., 2006; Negi *et al*., 2003; Noda *et al*., 2002). In this regard, West *et al*. (2007) showed that polyphenols present in this plant protect neonatal mouse brain against hypoxic-ischemic injury. Other examples of beneficial antioxidant activity effects of *P. granatum* include inhibition of UVB-mediated oxidative stress in immortalized HaCaT keratinocyte cells (Zaid *et al.*, 2007), augmentation of the human immune system's antioxidant and thus antimalarial and antimicrobial capacities *in vitro* (Reddy *et al.*, 2007), decrease in the expression of the vascular inflammation markers thrombospondin and cytokine TGFbeta1, along with an increase in plasma nitrate and nitrite levels and endothelial NO synthase expression in obese Zucker rats (De Nigris *et al.*, 2007). Moreover, Kaur *et al.* (2006) demonstrated

that *P. granatum* extract afforded up to 60% protection against hepatic lipid peroxidation due to the maintenance of the glutathione levels and activities of CAT, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase.

Although the biochemical mechanisms underlying PGL and PGF activities are not yet clear, our results demonstrated *in vivo* that *P. granatum* has a preventive effect against chromosome fragmentation and/or damage to the mitotic apparatus, probably due to its free radical scavenging capability. Taken together, under the conditions tested herein, mice treated with *P. granatum* showed an absence of mutagenic effects and dose-dependent protective effects against CP induced oxidative DNA damage.

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