Iraqi Journal of Cancer and Medical Genetics

Assessment of mutagenic and antimutagenic effects of *Punica granatum* against ifosfamide induced chromosomal aberrations in male albino mice

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Abstract:

The present study has been done to assess the mutagenic and antimutagenic effects of Punica granatum (Pomegranate) seed extract against the genotoxic effects ifosfamide (IFO) in male albino mice Mus musculus of BALB/c strain. The pomegranate seed extract (tablets) was provided by Source naturals, INC. Santa Cruse Company, Canada (from Egypt). Two independent experiments were performed. In the first, mice was treated daily and orally with phosphate buffer saline (PBS) as negative control group, and the other three groups were treated daily and orally with three different doses of the P. granatum seed extract (12.5, 25, and 50 mg/kg b.w.) for five weeks in order to test the clastogenetic effects of the plant seed extract. In the second experiment, the first group was positive control group which weekly and intraperitonially was treated with ifosfamide (100 mg/kg b.w.), the other three groups were treated daily and orally with three different doses of the pomegranate seed extract (12.5, 25, and 50 mg/kg b.w.) and also intraperitonially treated at the end of each week with IFO for five weeks to test the protective effects P. granatum seed extract. The results of the first experiment indicated that the P. granatum seed extract has no significant clastogenetic effects on chromosomal aberrations of the bone marrow cells of treated mice. The results of the second experiment was that the P. granatum seed extract, especially at the third dose (50 mg/kg b.w.) exhibited well protective and anti-clastogenetic effect against the genotoxic actions of the ifosfamide on bone marrow cells.

Keywords: *Punica granatum, Ifosfamide (IFO), Clastogenetic effects, Anti- clastogenetic effect, Genotoxic actions.*

Introduction:

Punica granatum L., belonging to family (Punicaceae), commonly known as pomegranate, is a small tree with potential human health benefits. Edible parts of pomegranate fruit comprise 78% juice and 22% seed (Kullkarni and Aradhya, 2005).

In fact, studies on *P. granatum* phytochemistry and pharmacological actions suggest a wide range of potential clinical applications: Antitumour (Lanskey and Newman, 2007), antibacterial (Aqil and Ahmad, 2007), antifungal (Vasconcelos *et al*., 2006), antiulcer (Gharzouli *et al*., 1999).

Recently, the antioxidant activity of *P. granatum* associated with its phytochemicals, such as, polyphenols, flavonoids, and anthocyanidins has gained importance (Guo *et al*., 2007; Zaid *et al*., 2007). Guo *et al*. (2007) demonstrated *in vitro,* a powerful DNA damage prevention ability of *P. granatum*. The strong antioxidant activities of the hydro-ethanolic extract can be found in seed oils (Adsule and Patil, 1995) and in fruit juices (Zaid *et al*., 2007). Such activity is mainly due to the hydrolyzed tannins which are converted during metabolism into ellagic acids, also known as ellagitannins (punicalagin is a major ellagitannin) (Chen *et al.*, 2007). Ellagic acid, a known antioxidant and free-radical scavenger, was found to significantly inhibit DNA breaks (Moktar *et al.*, 2009). The antigenotoxic potential of ellagic acid was demonstrated, and found that ellagic acid significantly reduces the percentage of aberrant cells and frequencies of aberration (Sheeba and Yadav, 2012).

Ifosfamide (IFO) is a highly effective chemotherapeutic agent for treatment of a variety of pediatric and adult solid tumors (Straka *et al*., 2003). Common metabolites of ifosfamide include, acrolein (Alarcon *et al.*, 1972), isophosphoramide mustard (Bryant *et al.*, 1980), induce DNA adducts, single strand breaks, crosslinks, sister chromatid exchanges, and chromosomal aberrations (Bishop *et al*., 1997),

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and N- Dechloroethylifosfamide (Struck, 1976), induce DNA crosslinking (Hilton *et al.*, 1981).

The present work was designed to investigate the protective effects of *P. granatum* seed extract and aimed to study their ability to induce mutagenicity or modulate the genotoxic effects induced by the alkylating agent ifosfamide (IFO) in mice, using chromosomal aberrations in bone marrow cell test.

Materials and Methods:

Pomegranate and chemicals :

The pomegranate seed extract (tablets) was provided by Source naturals, INC. Santa Cruse Company, Canada (purchased from Egypt). The mutagenic material been used in this study was the ifosfamide (Baxter Oncology GmbH Company). The other chemicals were obtained from local markets.

Mice treatment:

The experiments were carried out on adult male albino mice (16-20 weeks, weighed 30-35 g), and were kept under constant environmental conditions with a 12:12 light-dark cycle. The animals were fed standard granulated chow. In the present work, two independent experiments were performed. In the first experiment, mice were divided into four groups each with six mice; the first group was treated daily and orally with phosphate buffer saline (PBS) as negative control group. The other three groups were treated daily and orally with three different doses of the P. granatum seed extract (12.5, 25, and 50 mg/kg b.w.) for five weeks in order to test the clastogenetic effects of the plant seed extract.

In the second experiment, mice were was also divided into four groups each with six mice, the first group was positive control group which weekly and intraperitonially treated with ifosfamide (100 mg/kg b.w.). The other three groups were treated daily and orally with three different doses of the pomegranate seed extract (12.5, 25, and 50 mg/kg b.w.) and also intraperitonially treated at the end of each week with IFO for five weeks to test the protective effects P. granatum seed extract.

Chromosome preparation:

Chromosomal preparations from bone marrow cells were done by standard method of Evans et al., (1964).

Microscopic examination:

Microscopic examinations for prepared chromosome slides were done by using novel digital microscope (XSZ-N107T, made in China) with ocular lens 10X and objective lens 100X, and also the photos were taken by the same microscope.

Statistical analysis:

All data are expressed as means ± standard error (M±SE) and statistical analysis was carried out using statistically available software (Statistical Package for the Social Sciences (SPSS) version 16). Comparisons between groups were made using one-way analysis of variance (ANOVA) in combination with Duncan t-test post hoc analysis. Duncan t-test treats one group as a control, and compares all other groups against it. P values ≤ 0.05 were considered significant.

Results:

Clastogenetic effects of P. granatum seed extract:

Table (1) shows the results of chromosomal aberrations which indicate non significant difference ($P<0.05$) between treated groups and negative control in parameters (centromeric gap, centromeric break, chromatid gap, chromatid break, ring chromosome, dicentric chromosome, pulverization, acentric fragment and polyploidy) (Figure 1), but the third concentration had significantly decreased ($P<0.05$) total abnormal chromosome in comparison with the negative control. The highest mean values of most abnormalities were observed in the first concentration (12.5 mg/kg b.w.) and the lowest one was observed in the third concentration (50 mg/kg b.w.).

Protective effect of P. granatum seed extract against ifosfamide:

Table (2) shows the protective treatment effect of the three doses of P. granatum seed extract against ifosfamide induced chromosomal aberrations: a significant increase ($P<0.05$) was found in ifosfamide treated group (positive control) on most chromosomal aberrations parameters that were studied like (total abnormal chromosome, centromeric gap, centromeric break, chromatid gap, chromatid break, ring chromosome, pulverization, and polyploidy) when compared with negative control, but there were non significant effects ($P<0.05$) observed on (dicentric chromosome, and acentric fragment).

Treatment with P. granatum seed extract showed significant decrease ($P<0.05$) of all three doses of the plant extract on total abnormal metaphase and most other aberrations were studied compared with positive control and there was non significant difference between these groups and negative control in most of the analyzed parameters. It was clear from the Table (2) that all three doses of plant extract were minimized the effect of ifosfamide in respect with chromosome structure, but the highest protective dose was the third dose (50 mg/kg b.w.) and the lowest effective dose was the first dose (12,5 mg/kg b.w.).

Discussion:

Clastogenetic effects of P. granatum seed extract:

Although the fruits of P. granatum are commonly eaten, its roots and bark are toxic (Fuentes et al., 1985). Thus, an assessment of their cytotoxic and mutagenic potential is necessary to ensure a relatively safe to use medicinal plants (De Souza et al., 2006).

The results of Table (1) show non significant difference ($P<0.05$) between treated groups and negative control in almost all parameters, except the third concentration which had significantly increased ($P<0.05$) total normal chromosome and significantly decreased ($P<0.05$) total abnormal chromosome when compared with the negative control. Meerts et al., (2009) evaluated the possible mutagenicity and toxicity of pomegranate seed oil (PSO) using the in vitro Ames and in vitro chromosomal aberration test (in cultured peripheral human lymphocytes). No mutagenicity of PSO was observed in the absence and presence of metabolic activation up to pre-

cipitating concentrations of 5000 µg/plate (Ames test) or 333 µg/ml (chromosome aberration test), the results of the mutagenicity studies reveal that PSO is neither mutagenic nor clastogenic, both in the absence or presence of metabolic activation. Valadares et al., (2010) evaluated the mutagenic and anti-mutagenic potential of P. granatum ethanolic leaf extract (PGL) and P. granatum ethanolic fruit extract (PGF) in mouse bone marrow cells. Normal mice orally treated for 10 days with both (PGL) and (PGF) showed micronuclei frequency similar to that found in the control group. Amorin (1995) did not observe genotoxic effect using mouse bone marrow micronucleus (MN) assay in mouse treated orally with fruit aqueous extracts of this plant at dose of l000 and 2000 mg/kg b.w.

Protective effect of P. granatum seed extract against ifosfamide:

From the results of (2), ifosfamide significantly increased most chromosomal aberrations parameters that were studied like (total abnormal chromosome, centromeric gap, centromeric break, chromatid gap, chromatid break, ring chromosome, pulverization, and polyploidy) when compared with negative control. This outcome is similar to the results obtained by others (Rohrborn and Basler, 1977; Ursini et al. 2006; Donya et al, 2010).

It was clear from Table (2) that treatment with P. granatum seed extract showed significant decrease of all three doses of the plant extract on total abnormal metaphase and most other aberrations which were studied compared with positive control and there was non significant difference between these groups and negative control in most of the analyzed parameters. This is mostly because pomegranate (PG) here acts as desmutagens, either because of the antagonistic effect of the P. granatum seed extract against ifosfamide (acting as a mutagen blocker or inhibitor) or inhibition of the clastogenic effect of the ifosfamide by enhancing the detoxification enzymes or due to the high antioxidant properties of the P. granatum. Negi et al. (2003) reported that 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. West et al. (2007) showed that polyphenols present in this plant protect neonatal mouse brain against hypoxic-ischemic injury. Zaid et al., (2007) showed the beneficial antioxidant activity effects of P. granatum by inhibition of ultraviolet B (UVB)-mediated oxidative stress in immortalized HaCaT keratinocyte cells.

Valadares et al., (2010) noted that the oral administration of pomegranate fruit (PGF) or leaf extract for 10 days prior to exposure had reduced, in a dose dependent manner, the frequency of micronucleated polychromatic erythrocytes (MNPCE) induced by cyclophosphamide (CPH) 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 dose-dependent protective effects against CPH-induced oxidative DNA damage.

Sheeba and Yadav, (2012) demonstrated the antigenotoxic potential of ellagic acid against the Aflatoxin B1 induced genotoxicity. Five optimum doses of ellagic acid (100, 200, 300, 400, and 450 mg /kg body weight) in vivo were studied, and found that ellagic acid significantly reduces the percentage of aberrant cells (chromatid and chromosomes breaks exchanges, fragments, gaps, pulverized, chromosome) and frequencies of aberration. They had noticed that the antigenotoxic potential of ellagic acid is depend on doses and duration of treatment. Thresiamma et al, (1998), studied induction of micronuclei and chromosomal aberration sproduced by whole body exposure of γ-radiation (1.5-3.0 Gray(Gy)) in mice was found to be significantly inhibited by oral administration of ellagic acid (200 micro moles) per kilogram body weight.

Table (1): Effects of Punica granatum seed extact on chromosomal aberrations in male albino mice (Mean ± SE) (P<0.05).

Parameters / Groups	Total normal Chromosome	Total abnormal Chromosome	Centrom-eric gap	Centrome-ric break	Chromatid gap	Chromatid break	Ring Chromosome	Dicentric Chromosome	Pulverization	Acentric fragment	Polyploidy
Control (PBS.)	96 ± 0.516^{a}	4 ± 0.516^{b}	1.333 ± 0.333^{a}	1.333 ± 0.210^{a}	0 ± 0.000	0.333 ± 0.210^{a}	0 ± 0.000	0.166 ± 0.166^{a}	0.166 ± 0.166^{a}	0.666 ± 0.333^{a}	0 ± 0.000
Dl (12.5 mg/kg b.w.)	96 ± 0.447^{a}	4 ± 0.447^{b}	1.5 ± 0.223^{a}	1.166 ± 0.166^{a}	0 ± 0.000	0.333 ± 0.210^{a}	0 ± 0.000	0.166 ± 0.166^{a}	0.333 ± 0.210^{a}	0.5 ± 0.233^{a}	0 ± 0.000
D2 (25 mg/kg b.w.)	96.833 ± 0.307^{ab}	3.1 ± 0.307^{ab}	1 ± 0.000^{a}	1.166 ± 0.166^{a}	0 ± 0.000	0.333 ± 0.210^{a}	0 ± 0.000	0 ± 0.000	0.333 ± 0.210^{a}	0.333 ± 0.210^{a}	0 ± 0.000
D3 (50 mg/kg b.w.)	97.5 ± 0.428^{b}	2.5 ± 0.428^{a}	0.833 ± 0.166^{a}	0.833 ± 0.166^{a}	0 ± 0.000	0.166 ± 0.166^{a}	0 ± 0.000	0 ± 0.000	0.166 ± 0.166^{a}	0.5 ± 0.233^{a}	0 ± 0.000

Note: Similar letters in each column refer to non significant difference while different letters refer to significant difference between them. (P.B.S. = Phosphate buffer saline, D= Dose).

Table (2): Protective effects of Punica granatum seed extract against ifosfamide induced chromosomal aberrations in male albino mice. (Mean ± SE) (P<0.05).

Parameters / Groups	Total normal Chromosome	Total abnormal Chromosome	Centromeric gap	Centromeric break	Chromatid gap	Chromatid break	Ring Chromosome	Dicentric Chromosome	Pulverization	Acentric fragment	Polyploidy
Control (PBS.)	96 ± 0.516^{d}	4 ± 0.516^{a}	1.333 ± 0.333^{a}	1.333 ± 0.210^{a}	0 ± 0.000^{a}	0.333 ± 0.210^{a}	0 ± 0.000^{a}	0.166 ± 0.166^{a}	0.166 ± 0.166^{a}	0.666 ± 0.333^{a}	0 ± 0.000^{a}
IF (lOOmg/kg b.w.)	76.166 ± 0.872^{a}	23.833 ± 0.872^{d}	6.166 ± 0.401^{d}	7 ± 0.258^{e}	0.5 ± 0.223^{b}	2.666 ± 0.333^{c}	2.666 ± 0.494^{c}	0.5 ± 0.223^{a}	1 ± 0.258^{b}	1 ± 0.258^{a}	2.333 ± 0.494^{b}
D1 (12.5 mg/kg b.w.) (lOOmg/kg b.w.)	83.333 ± 0.714^{b}	16.666 ± 0.714^{c}	5 ± 0.000^{c}	5.666 ± 0.210^{d}	0.166 ± 0.166^{ab}	1.5 ± 0.223^{b}	1.333 ± 0.210^{b}	0.166 ± 0.166^{a}	1 ± 0.000^{b}	1 ± 0.258^{a}	0.833 ± 0.307^{a}
D2 (25 mg/kg b.w.) (lOOmg/kg b.w.)	90 ± 0.966^{c}	10 ± 0.966^{b}	3.333 ± 0.333^{b}	4.166 ± 0.307^{c}	0 ± 0.000^{a}	0.833 ± 0.166^{ab}	0.166 ± 0.166^{a}	0 ± 0.000^{a}	0.5 ± 0.233^{ab}	0.833 ± 0.166^{a}	0.166 ± 0.166^{a}
D3 (50 mg/kg b.w.) (lOOmg/kg b.w.)	94.166 ± 0.477^{d}	5.833 ± 0.477^{a}	1.8 ± 0.307^{a}	2.166 ± 0.307^{b}	0 ± 0.000^{a}	0.5 ± 0.223^{a}	0.166 ± 0.166^{a}	0 ± 0.000^{a}	0.667 ± 0.210^{ab}	0.5 ± 0.223^{a}	0 ± 0.000^{a}

Note: Similar letters in each column refer to non significant difference while different letters refer to significant difference between them. (P.B.S. = Phosphate buffer saline, D= Dose).

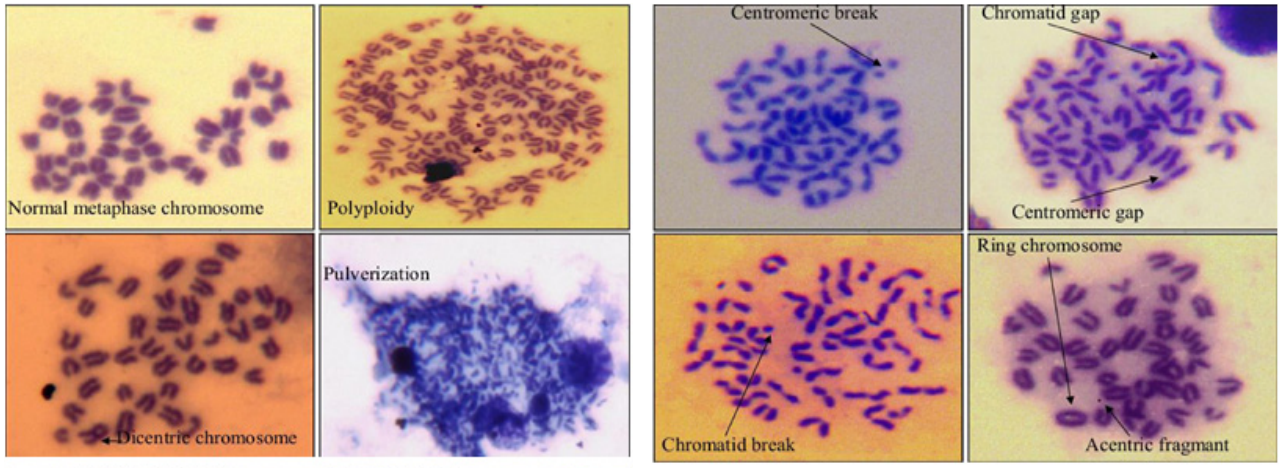

Figure (1): Types of structural and numerical chromosomal aberrations induced by ifosfamide (1000 X).

Conclusions:

We concluded the followings from the present work: first, seed extract of P. granatum extract has no mutagenic effects on chromosomal aberrations assay under experimental conditions in vivo. Second, ifosfamide has ability to produce chromosomal aberrations in bone marrow cells in male albino mice. Third, P. granatum seed extract (especially at dose 50 mg/kg b.w.) showed a significant protective activity against mutagenic effects of ifosfamide.

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تقييم التأثير التطفيرى و المضاد للتتطفر لمستخلص بذور الرمان *Punica granatum. L* ضدّ عقار الأيفوسفامايد المحفز للتغيرات الكروموسومية في ذكور الفئران البيضاء

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الخلاصه:

أُجريت هذه الدراسة لبحث وتقييم التأثيرات الوراثية الخلوية السمية والتأثيرات الوقائية لمستخلص بذور نبات الرمان (Punica granatum) ضد التأثيرات الخلوية الناتجة عن استخدام عقارالأيفوسفامايد ifosfamide)) في ذكور الفئران البيضاء (Mus musculus) من سلالة BALB/c.

شملت الدراسة الحالية تجربتين مختلفة. شملت التجربة الاولى (24) فأرًا حيث قسمت الى اربع مجاميع (كل مجموعة إحتوت على 6 فئران). أُعطيت للمجموعة الاولى يوميًا وعن طريق الفم محلول دارى الفوسفات الفسيولوجي (PBS) كمجموعة المقارنة السالبة. وأعطيت للمجاميع الاخرى يوميًا وعن طريق الفم تراكيز مختلفة من مستخلص بذور الرمان (12,5 و 25 و 50 ملغم/كغم من وزن الجسم) على التوالي ولمدة خمسة أسابيع لإختبار التأثيرات الوراثية الخلوية لهذا المستخلص.

بينما شملت التجربة الثانية (24) فأرًا, وتم تقسيمها أيضا الى اربع مجاميع (إحتوت كل مجموعة على 6 فئران). أعطيت المجموعة الأولى إسبوعيا وعن طريق الغشاء البريتوني (100 ملغم/كغم من وزن الجسم) من عقار الأيفوسفامايد (IFO) كمجموعة المقارنة الموجبة. بينما أعطيت المجاميع الأخرى يوميًا وعن طريق الفم تراكيز مختلفة من مستخلص بذور الرمان (12,5 و 25 و 50 ملغم/كغم من وزن الجسم) على التوالي وأعطيت أيضا عند نهاية كل أسبوع عقار الأيفوسفامايد (100 ملغم/كغم من وزن الجسم) عن طريق الغشاء البريتوني ولمدة خمسة أسابيع متتالية لإختبار التأثيرات الوقائية لمستخلص بذورالرمان.

وقد اظهرت نتائج التجربة الأولى بأن مستخلص بذور نبات الرمان ليس له أي تأثير معنوي سلبي على تغيرات الكروموسومية في خلايا نخاع العظم في الفئران البيضاء المعاملة. بينما أظهرت نتائج التجربة الثانية أن لمستخلص بذور الرمان (بالأخص عند تركيز 50 ملغم/كغم من وزن الجسم) تأثيرًا إيجابيًا وكفاءة تثبيطية عالية تجاه التأثيرات الوراثية الخلوية السلبية لعقار الأيفوسفامايد في خلايا نخاع العظم, حيث أدت الى تقليلها.