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Cytogenetic Activity of Methanolic Extract of Aerial Parts of *Plumbago europaea* on Balb/C Mouse Bone Marrow Cells

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ABSTRACT

Background & Aim: The World Health Organization (WHO) estimated that majority of the inhabitants of the world rely chiefly on folk medicine. It, therefore, approved the use of herbal products for national policies and drug regulatory measures in order to strengthen research and evaluation of the safety and efficacy of herbal products. We have evaluated the cytotoxic, mutagenic and antimutagenic potential of the methanolic extract of the aerial parts of *P. europaea*, a common species in the Mediterranean and Central Asia.

Experimental: Male Balb/C mice were intraperitoneally (i. v.) injected with varying doses of the extract dissolved in dimethyl sulfoxide (DMSO). The i.v. LD50 of the extract was determined to be 58.33mg/kg body weight. Bone marrow cells were processed and screened for chromosomal aberration and micronucleus formation. Mitomycin C (MMC, 2mg/kg b. w.) and DMSO (0.5%) served as positive and negative control, respectively. Antimutagenecity was followed by administration of 2mg MMC/kg in the beginning of the first or the last 24h of applying 14.6mg/kg of the extract daily for 7 consecutive days.

Results: The selected doses of the extract elevated mitotic index and increased percentage of aberrant cells compared to the negative control. However, at a dose of 14.6mg/kg, the extract was enough to reduce significantly the toxic effects induced by MMC. This indicated that the *P.europaea* extract by itself is mutagenic, but antimutatgenic probably at small doses and can modulate the mutagenicity of MMC.

Recommended applications/ industries: These data may help in understanding of therapeutic properties of *P. europaea* claimed by folk medicine. However, caution regarding indiscriminate use of this plant by the public is necessary. Multiple experiences are needed to rule out any possible side effects and to prove health safety of this natural product before introducing it into the market for therapeutic purposes.

1. Introduction

The World Health Organization estimated that perhaps 80% of the world population use herbal medicines for some aspect of routine health care (Pravin *et al.*, 2013).Scientists are visualizing a great future for the pharmaceutical industry in the development of plant based drugs since they appear to be an excellent source of new bioactive compounds (Matthys *et al.*, 2007). Recent epidemiological studies have shown that plant based medicines may be involved in preventing or delaying the development of cancer, chronic diseases and viral infections (Gangabhagirathi and Joshi, 2015;

Shawarb *et al.*, 2017). Medicinal plants may act on different targets in signal transduction pathways that may modulate gene expression, cell cycle progression, cellular proliferation, and/or apoptosis (Subramaniya *et al.*, 2011).

The medicinal plant *Plumbago europaea* (Linn) is distributed in the Mediterranean area, Italy and in south Europe (Serrilli *et al.*, 2010, Shawarb *et al.*, 2017). It has been used extensively in China and other Asian countries for treatment of cancer, rheumatoid arthritis, dysmenorrhea, rheumatoid arthritis, and skin diseases (Chaplot *et al.*, 2006). The chemotherapeutic potentials of this plant may be due to the presence of some active compounds like plumbagin (5-hydroxy-2-methylnaphthalene-1, 4-dione), naphthoquinones, terpenoids and flavonoids in its extract (Muhammad et al., 2009). Plumbagin has been tested for cytotoxicity, genotoxicity, mutagenicity, and antimutagenicity using several standard assays (Anuf *et al.*, 2014; Santhakumari *et al.*, 1980; Wang *et al.*, 2008).

In Jordan, the local Bedouins and villagers know many plant species; 363 species of medicinal vascular plants were recorded (Oran and Al-Eisawi, 2015). Despite of this, the rich diversity of Jordanian medicinal plants have not yet systematically screened for bioactivity (Afifi *et al.*, 2011; Alkofahi *et al.*, 1996; Tawaha, 2006). Based on the chemical diversity of known active phytoantimutagens (Afifi *et al.*, 2011) many of them are traditionally used. Jordanian medicinal plants may exhibit desired therapeutic properties due to similarity in the major class of phytocompounds. Hence the aim of this research works was directed towards evaluation of the cytogenetic effects of the extract of the aerial parts of *P. europaea* using a mammalian test system.

2. Materials and Methods

2.1. Chemicals

Mitomycin C (MMC) and Giemsa stain were purchased from Sigma (USA). Dimethyl sulfoxide (DMSO) was obtained from Gainland Chemical Company (UK). Colcemid was acquired from Life Technologies Corporation (USA). All other chemicals were of analytical grade.

2.2. Plant Materials and Extraction

Fresh above ground biomass of the leaves, stems and flowers of *P. europaea* were collected from the farms and fields of the village of Zoubia in the southwestern area of Irbid Directorate in the northern part of Jordan during summer of 2015. The plant was identified by a plant taxonomist and checked with www.theplantlist.org Voucher specimens were deposited at the herbarium allocated at the Department of Biological Sciences at Yarmouk University in Irbid (Jordan). All the national and institutional rules concerning the biodiversity and conservation rights were followed. The plants were thoroughly cleaned under running tap water and left in door at room temperature (RT) to air dry and to protect it from direct light. The dried coarsely powdered aerial parts (500g) were soaked in 1000 ml of absolute methanol for 7d at RT with occasional stirring using a sterilized glass rod. The extract was filtered through Whatman filter paper No.1 (Whatman Ltd., England) and concentrated to dryness under vacuum on rotary evaporator at 40°C. The concentrated extract was weighed according to the body weight (b. w.) and then accurately reconstituted in minimum amount of DMSO and stored at 4°C for further use.

2.3. Experimental Animals

Male Balb/C albino mice (8-10-w-old) weighing 25-30g were used for all the experiments. Animals were obtained from the Animal House Facilities at Yarmouk University. Mice were randomly distributed into groups of six animals and housed in sanitized polyethylene cages containing paddy husk as bedding. Animals were kept under closely controlled environmental conditions (12 h light/dark cycle, lights on between 7 and 19h, 25°C, 50-70% humidity) and allowed free access to food and water supply. The study was commenced in accordance with the internationally accepted principles for laboratory animal use and care. The protocol was approved by Yarmouk University Animal Ethical Committee for Purpose of Control and Supervision of Experiments on Animals. All efforts were made to minimize unwanted stress or discomfort to the animals during experimental procedures.

2.4. Determination of LD50

The stock fraction of the extract was further diluted homogenously in 0.5% DMSO to get methanol extract of known concentration. Acute intraperitoneal (i. p.) LD50 of the extract was determined using the method described before (Karber, 1931) and reported recently by Ahmed (2015). Graded single doses (50, 100, 150, 200, 250, 500, and 750 mg/kg) of the extract

(0.2ml/mouse) were administrated to 8 different groups of mice, six animals in each. Animals were observed continuously for the first 2 h and then for 6 h for any toxic symptoms. Finally, the number of survivors was recorded after 24 h. Toxicological effects were assessed on the basis of mortality, expressed as LD50. The LD50 value of the extract was calculated according to the following formula:

$$LD50 = LD\ 100 - \sum(a * b)/n$$

LD100: Lethal dose causing the 100% death of mice; a: the difference between two successive doses of administered extract/substance; b: the average number of dead animals in two successive doses. N: total number of animal in a group.

2.5. Cytogenetic Toxicity

Extract solution (0.2ml) at doses, chosen on the basis of LD50 value obtained in this study (58mg/kg b.w), 14.6, 29.2, 58.33, or 87.5 mg/kg b.w. was given i. p. to the experimental mice (4 animal/dose) daily for 7 consecutive days. Every mouse in the negative control group i. p. received same volume of 0.5% DMSO (extract solvent), daily for 7 d. The positive control group received i.p. injection of 0.2 ml of MMC solution (2mg/kg).

Based on the results of experiments on cytotoxicity (mitotic index; MI) and mutagenicity (% aberrant cells; %Abc), a single dose (14.6mg/kg) of the extract was selected to study ability of the extract to modify mutagenic action of MMC. In the first treatment (pre-treatment), each animal was injected with 0.2ml of MMC (2mg/kg) alone for 24h and then treated with a single dose of the extract on each of the following 7 d. In the second treatment (post-treatment), each animal was injected with 14.6 mg extract for 7 consecutive days and on day 8, it received MMC (2mg/kg).

Mice were kept in their cages and food and water were changed daily at the same time. To accumulate mitotic cells, each mouse was injected with 0.2ml of 0.1 µg/ml colcemid solution 2-3h prior to termination of the experiment. A triplicate for each dose level was done for each time interval.

2.6. Slide Preparation

Animals were sacrificed by cervical dislocation at 24h after the seventh injection and bone marrow cells from femur and tibia bones were obtained and prepared according to a standard protocol (Khalil and Da'dara, 1994) with little modification. Two slides from each animal were made and stained with Giemsa. The slides were coded before scoring.

2.7. Cytogenetic Analysis

Mitotic index was calculated as percentage of dividing cells among 2000 cells per animal, totaling 8000 cells/group. For chromosomal aberrations (CA) analysis, a total of 800 cells for each dose were examined for the appearance of micronuclei, polyploidy, breaks, fragments, bridges, rings or dicentric chromosomes and the percentage of cells with aberrations (%Abc) was recorded. Antimutagenicity of the extract was expressed as percentage reduction of MMC-induced %AbC which was calculated by the following formula (Akinboro *et al*., 2016):

$$RCA = a\text{-}b/a\text{-}c \times 100\%$$

RCA: percentage reduction of MMC-induced %Abc; a: percentage of MMC-induced %Abc; b: percentage of plant extract plus MMC-induced %Abc; c: percentage of DMSO-induced %Abc.

2.8. Statistical analysis

Collected data were analyzed using Minitab Statistical Software Version 14 (Minitab Inc., State College, USA). Experimental groups were compared with the negative and positive control groups using the Student's t-test. Means, with 95% confidence limit and the standard errors of each of the data obtained were calculated. Significant difference between control groups and samples was set at $P \leq 0.05$.

3. Results and discussion

In this study, the calculated 24h LD50 value of the methanolic extract of the aerial parts of *P. europaea* was 58.33 mg/kg. This value is higher than (5mg/kg) that was demonstrated in mice by Berdy (1982) for roots of the same species given i. p. In Swiss albino mice, the reported (Siva Kumar and Devaraj, 2006) LD50 dose for pure plumbagin was 16 mg/kg b. w. Differences in LD50 could be attributed to either differences in tolerance of mice strains, or the used part of the plant, extracting solvent and route of administration. The concentrations of plumbagin in dry stems and leaves of *P. europaea* were 0.560%, and 1.5002%, respectively (Al-Nuri *et al*., 1994). Another study estimated that *P. europaea* leaves extract contains about 1.5% plumbagin (Muhammad *et al*.,

2009). Thus, we can predict an average percent of plumbagin in our preparation as 1.03%. Based on this, it could be expected that the active component i. p. injected in the present study is about 0.6 mg/kg at the LD50 of the extract (58.33 mg/kg). Our finding appears to be more in agreement with the CSIR report (CSIR, 1969) which stated that 0.1 mg/kg as minimum lethal dose of plumbagin for mice, even though route of administration is not specified. It is also consistent with a similar finding (Solomon *et al.*, 1993) that active component of P. rosea root extract i. p. injected in mice at LD50 was about 2 mg/kg.

The observed signs of toxicity were similar to those reported earlier (Ganesan and Gani, 2013) with *P. zeylanica*. Animals expressed a high tolerance to the extract given by i.p. route. No death was observed at doses ≤ 200 mg/kg. Compared to the control group, mice treated with doses ≥ 250mg/kg showed some behavioral changes including increased pulse rate, loss of appetite, and sluggish activity with decreasing response to outside stimuli. No animals survived doses ≥500 mg/kg; death was observed after 6 h of extract application. These observations may need further extensive studies to investigate the effects on organ systems, for example, the hematological system and the nervous system in either a subacute or chronic fashion. Such experiments are related to risk assessment because these pathological changes are highly predictive for human toxicity, when data are extrapolated from animal studies (Shatoor, 2011).

The extract exerted dose-dependent stimulatory effect on MI, but not significant at the lower doses. However, statistically significant increases (P< 0.05) in MI were recorded at the highest two doses of the extract (58.33 and 87.5 mg/kg, 15.55% and 20.17%, respectively (Table 1). In comparison, the MI value reached 10.45% in bone marrow cells from the negative control mice.

Thus, the extract could not be considered as cytotoxic. In this regard, plumbagin at 5 mM was proven to be noncytotoxic to resting mouse lymphocytes, but at a concentration of 2 mM was found to induce apoptosis in human resting lymphocytes, indicating species-specific differences in the activity of plumbagin (Checker *et al.*, 2010). Plumbagin from *P. zeylanica* extract was reported be toxic to proliferating Swiss albino mice bone marrow cells, as evidenced from decrease in polychromatic/normochromatic erythrocytes ratio and induction of MN (Siva Kumar and Devaraj, 2006). For other Plumbago species, ethanolic extract of *P. indica* root was cytotoxic and genotoxic in human lymphocytes in vitro (Thitiorul *et al.*, 2013).

Table 1. Mitotic index (MI) and the percent aberrant cells (%Abc) induced in the bone marrow cells of mice following i. p. exposure to methanolic *P. europaea* extract for 7 consecutive days.

Treatment	Concentration (mg/kg b. w.)	Mitotic Index ± SE*	% Aberrant Cells ± SE**
Negative Control (0.5% DMSO)	-	10.45 ± 0.03	8.67 ± 0.17
Positive Control (MMC)	2.0	4.11 ± 0.33	31.30 ± 0.33
¼ LD50	14.6	9.42 ± 0.02	10.00 ± 0.29
½ LD50	29.2	10.20 ± 0.06	14.00 ± 0.29
LD50	58.3	15.55 ± 0.03	19.56 ± 0.30
1.5 LD50	87.5	20.17 ± 0.02	21.67 ± 0.15

Number of screened cells * 6000. ** 600

Micronuclei are biomarkers of chromosome damage and/or whole chromosome loss, nucleoplasmic bridges are indicators of DNA misrepair and/or telomere end fusion, while nuclear duds are considered as biomarkers of elimination of amplified DNA and DNA repair complexes (Makhafola, 2014).

Furthermore, dose-dependent increases of CA with highly significant (P < 0.05) increases in % of aberrant cells (%Abc) (Table 1) were observed in all treated groups when compared to the negative control. All doses of the extract induced both structural and numerical CA (Figure 1). In addition, different nuclear abnormalities (NA) (Figure 2) were demonstrated with all dose range of the extract studied. Percent NA induced by all doses of extract (Table 2) was statistically higher (P<0.05) relative to negative control. The highest incidence (9.2%) was scored in bone marrow cells treated with 87.5 mg/kg of the extract. The cell death seen in the present study in the form of shrinkage in nuclear volume, nuclear fragmentation, bridge between two interphase nuclei, nuclear budding, and sticky nuclei was noted in previous investigation (Makhafola, 2014). Consistent with this are the nucleotoxic effects of plumbagin at higher concentrations (Santhakumari *et al.*, 1980). Therefore, our data prove and extend previous findings that plumbagin is a genotoxic agent.

Table 2. Type and incidence of nuclear abnormalities (NA) induced in the bone marrow cells of mice following exposure to methanolic of *P. europaea* extract.

Treatment	Concentration (mg/kg b. w.)	BN*	BN+bridge	BN+bud	BN+MN	R	L	NL	%NA± SE
-Ve Control (0.5% DMSO)	-	56	11	1	2	40	3	60	1.90±0.011
+V Control (MMC)	2.0	91	55	0	0	35	5	71	2.84±0.029
¼ LD50	14.6	132	4	0	0	48	3	252	4.88±0.033
½ LD50	29.2	65	3	0	0	35	8	138	2.79±0.022
LD50	58.3	93	7	0	0	38	13	293	5.00±0.038
1.5 LD50	87.5	380	75	1	0	124	16	233	9.20±0.019

* BN: binucleated; L: lobed nucleus; MN: micronucleus; NA; nuclear abnormality; NL: nuclear lesion; R: reniform nucleus. Total number of screened cells is 600.

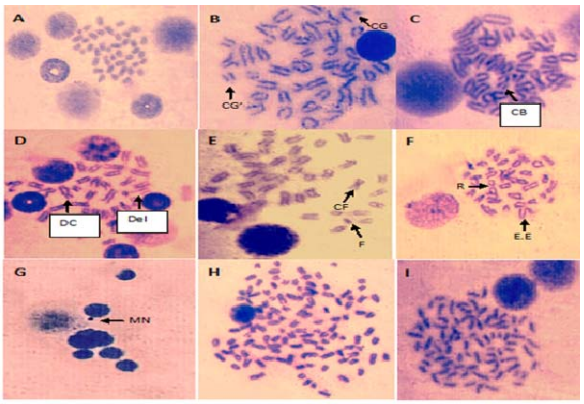

Figure 1. Chromosomal aberrations in bone marrow cells of mice treated with methanolic extract of *P. europaea*. A: normal metaphase, B: CG: Chromatid gap and CG': Chromosome gap, C: CB: Chromatid break, D: Del: Deletion and DC: Dicentric chromosome, E: CF: Centric fusion and F: fragment, F: E.E: End-to-end association, G: MN: micronucleus, H: Polyploid cell, I: Diploid cell. Giemsa Staining.1000X.

Two major mechanisms have been proposed for plumbagin cytogenetic action in various biological systems. The first is associated with excessive generation of reactive oxygen species (ROS) such as superoxide radicals, singlet oxygen, and hydrogen peroxide (Castro *et al.*, 2008).The second involves redox and oxidation cycle of quinones namely ''redox cycles'' which lead to their ability to act as potent electrophiles to inhibit the electron transportation, as uncouplers of oxidative phosphorylation, as intercalating agent in the DNA double helix and as bio-reductive alkylating agents in the biomolecules (Babula *et al.*, 2006). Pretreatment of mice with 14.6mg/kg of the extract significantly (P<0.05) modulated the effect of MMC on MI (Figure 3; 7.58% and 4.11% for the extract and MMC, respectively). The %Abc was significantly reduced (P <0.05) when the *P. europaea* extract was given before MMC treatment (Figure 4) and reached 13 % in comparison with the positive control 31.3%.

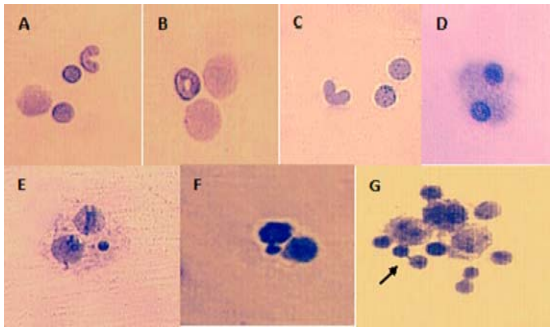

Figure 2. Nuclear abnormalities in bone marrow cells of mice induced by methanolic *P. europaea* extract. **A**: lobed, **B**: nuclear lesion, **C**: reniform, **D**: normal binucleated cell, **E**: binucleated cell with micronucleus, **F**: binucleated cell with bud, **G**: binucleated cell with nucleoplasmic bridge. Giemsa Staining.400X.

Thus, pre-treatment with *P. europaea* plant extract provided 80.87% protection against the mutagenic action of MMC. However, the % NA in bone marrow cells were significantly elevated (P<0.05) as a

consequence of pre-treatment (4.45%) compared to the corresponding positive control; 2.84% (Figure 5).

Similarly, as Figure 3 shows, posttreatment caused significant increase (P<0.05) in MI (10.84%) relative to MI in positive control (4.11%) and % Abc after treatment with extract was 19% compared to that of positive control (31.3%); providing 54.35% protection against MMC-induced CA (Figure 4). The % NA induced in bone marrow cells was significantly higher (P<0.05) following post-treatment (4.23%) as compared to the corresponding positive control; 2.84% (Figure 5).

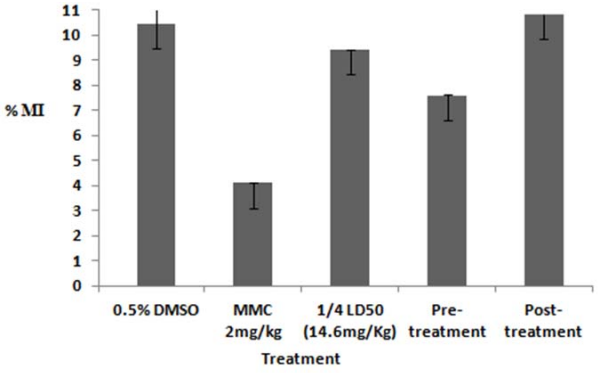

Figure 3. Dose-dependent increase in the incidence of mitotic indices in mice bone marrow cells following interaction between *P. europaea* (14.6mg/kg) and MMC (2mg/kg) (pre- and post-treatment).

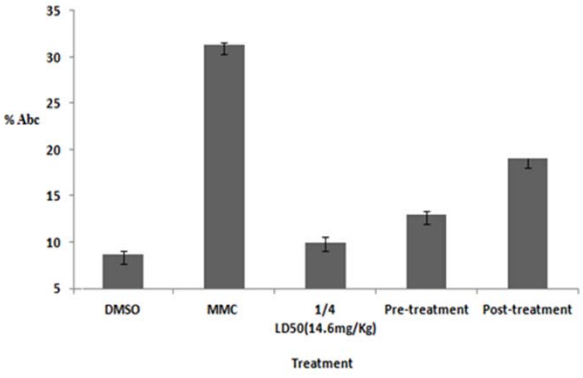

Figure 4. Dose-dependent increases in the % of aberrant cells (% Abc) in mice bone marrow cells resulting from i. p. injection of *P. europaea* (14.6mg/kg) before or after and MMC. (2mg/kg).

The significant antimutagenic activity in *P. europaea* extract against MMC suggests that this extract may directly protect DNA damage from mutagen. However, the inhibition of mutagenesis is often complex, acting through multiple mechanisms (Edenharder and Grunhage, 2003; Musarrat *et al.*, 2006).The post-treatment mechanism of action occurs when antimutagenic substance acts upon process that induces formation of mutation or process that repair DNA damage (Kuroda *et al.*, 1992).

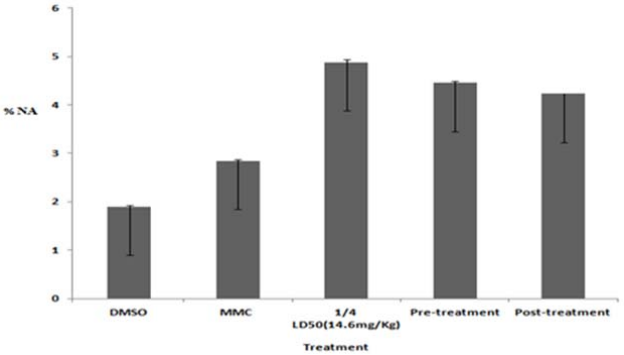

Figure 5. Incidence of nuclear abnormalities in mice bone marrow cells following exposure to *P. europaea* (14.6mg/kg) before or after treatment with MMC (2mg/kg).

Based on this finding *P.europaea* extract can be classified as a desmutagen in first order and a bioantimutagen in second order. It is known that MMC requires a bioreductive transformation to form active species that crosslink DNA (Wang *et al.*, 2008). Depending on biotransformation pathway, metabolism of MMC may generate ROS (Gustafson and Pritsos, 1992). A protective effect of *P. zeylanica* root extract in causing significant reduction in CA and reduction in activity of hsp70 (Siddique *et al.*, 2011) was attributed to direct action of compounds present in the extract of against ethinylestradiol by inactivating it enzymatically or chemically. The compound(s) present in *P. europaea* extract may also scavenge electrophiles/ nucleophiles (Serrilli *et al.*, 2010; Sundari *et al.*, 2017). They may also enhance DNA repair system, DNA synthesis or even may prevent bioactivation of certain chemicals (Kuroda *et al.*, 1992).The present investigation was confined only to experiments with crude extract of the aerial parts of plant and not to individual components. Hence, it is not possible to attribute antimutagenic potency to any particular component. Plumbagin itself has been well documented to have antioxidant properties (Kunar *et al.*, 2013).However, it is worthwhile to mention that some of the components like: flavonoids, tannins, coumarins essential oils and polyphenols are present in different parts of this plant (Muhammad *et al.*, 2009; Nile and Khobragade, 2010).

Flavonoids are most likely candidates among the methanol extract for modifying the in vivo mutagenicity of MMC, since they are the known antioxidants, free radical scavengers and chelators of divalent cations (Aqil *et al.*, 2008). Based on in vitro and in vivo studies both in humans and in animals, a number of potentially positive health effects, including anticancer and anticarcinogenic effects, have been ascribed to flavonoids, similar to those found in *P.europaea* extract (Akinboro*et al.*, 2016). Flavonoids present in extract act as blocking agents, thus prevent metabolic activation of promutagens. They can also form adducts or scavenge free radicals and thus prevent tumor formation (Noel *et al.*, 2006).

4. Conclusion

The present results suggest that the extract of *P. europaea* is not cytotoxic at low concentrations, but may exhibit genotoxic effects. However, it shows protective (pretreatment) and reparative (posttreatment) in vivo effects against genotoxic damage induced by the therapeutic drug MMC. The antimutagenic activity showed by the extract provides a scientific validation for the therapeutic consumption of *P. europaea* extract in folk medicine. Caution regarding indiscriminate use by the public of this and other medicinal plants continues to be necessary.

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