Reducing genotoxicity of cyclophosphamide by hydro-coholic leaves extract of *allium porrum* in normal mouse bone marrow stem cells

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Abstract

This paper evaluates percentage of chromosomal aberration (CA) and mitotic index (MI) in mice bone marrow as a method for assessing the ability of hydro-ethanolic leaves extract of leek (*Allium Porrum*) to reduce genotoxicity of Cyclophosphamide (CP). Mice were allocated in to 4 groups, two negative controls, the first group receive (0.2 ml) D.W. and the second group was treated with (0.1 ml) DMSO, the positive control group, (CP) 40mg/kg was given single intraperitoneally dose for 24 hr, while the treatment groups were given different doses of extract (75, 150, 200, and 250 mg/kg) for 5 consecutive days and the same dose of CP. Experimental data was analyzed using one –way ANOVA. The results demonstrated the intermediate dose (150 mg/kg) of extract increased in MI significantly and reduced CA (P<0.01) significantly. This study concluded that *Allium porrum* leaves were able to reduce genotoxicity of CP and protect normal proliferating cells in bone marrow from the effect of CP.
Key words: Allium porrum, Genotoxicity, Cyclophosphamide, Chromosomal Aberrations, mitotic index

Introduction

Allium is a genus which includes approximately 500 species, the most widely used of which are onions (Allium cepa), garlic (Allium sativum), leeks (Allium porrum), chives (Allium schoenoprasum). Allium vegetables are rich in flavonols and organosulfur compounds [1]. Some components of Allium vegetables are reported to block several stages of carcinogenesis, although the underlying mechanisms of action are generally unclear [2]. Associations between consumption of Allium vegetables and risk for cancer has been assessed in several epidemiologic, mainly case-control, studies, these have pointed to lower risks for cancers of the stomach, colon, esophagus, and perhaps breast [3].

Allium porrum L. (Allium ampeloprasum var. porrum), commonly named leek. The leek is a species of the genus Allium and a member of the Alliaceae (Liliaceae) family, closely related to garlic and onion [4]. Leek is a biennial herb and commonly grows as mildly pungent succulent linear leaves and especially its thick cylindrical stack consisting of blanched leaf stalks and a small simple bulb [5]. It is winter-hardiness, long shafts in winter varieties, erectness of the leaves and dark leaf color were the desirable traits. It is adapted to different climates and market demands. It has been developed in many European countries from Bulgaria to Ireland and in other parts of the world (e.g., Middle East) [6].

Many anticancer drugs affect DNA, and the damage to DNA may result as chromosomal aberrations causing chromosomal instability, and may lead to mutagenesis [7, 8]. These mutagenic effects of chemotherapeutic agents can lead to the development of resistance to the chemotherapeutic agents or may lead to the development of secondary tumors and abnormal reproductive outcomes [9]. Hence, it is vital to determine the frequency and severity of mutagenicity following treatment with cancer chemotherapy and to develop strategies to reduce its occurrence in non-tumor cells [10].

Cyclophosphamide (CP) is a common anti-cancer chemotherapeutic agent, also known as Cytosan or Endoxan, it is anitrogen mustad alkylating agent (C<sub>7</sub>H<sub>15</sub>Cl<sub>2</sub>N<sub>2</sub>P.H<sub>2</sub>O) adds an alkyl group (C<sub>n</sub>H<sub>2n+1</sub>) to DNA and attaches the alkyle group to guanine base of DNA, at the number 7 nitrogen atom of the imidazole ring [11]. Cyclophosphamide is widely prescribed antineoplastic drug, especially for the treatment of acute myeloid leukemia and a wide range of cancers including many types of neuroblastomas, adenocarcinomas of the ovary, and certain malignant neoplasm of the lung [12]. It is also used as an immunosuppressant agent for arthritis, scleroderma, glomerulonephritis, chronic hepatitis, multiple sclerosis, and organ transplantation, used alone or in combination with other chemotherapeutic drugs [13].

Bone marrow is a major hematopoietic organ composed of hematopoietic cells in different stages of ripeness, including erythrocytes, leukocytes and platelets [14]. High dose of CP produces acute bone marrow depression by killing hematopoietic progenitor [15]. All of red blood cells, white blood cells and platelets would then be reduced, followed by relatively rapid recovery. Studies show that CP could provide effective treatment of patients with myelodysplastic syndromes...
or leukemia before HSCs transplantation from various donors[16]. The aim of this work was to evaluate the cytogenetic effects of leek (Allium porrum) extract in modulating the action of CP in mouse bone marrow.

**Materials and methods**

**Laboratory animals**

Thirty five albino Swiss male mice were obtained from National Center for Drug Control and research / Ministry of Health / Baghdad. Their age ranged between (8-12) weeks and weighting (25 ±2) gm. They were divided into 4 groups, each group was put in a separated plastic cage under laboratory conditions.

**Preparation of plant extracts**

The plant was collected from beach farms in Al Kut city. The extracted part of the plants were leaves. The extraction was done according to Shakya et al.,(2010). The leaves were air-dried, and then powdered using a coffee grinder, 200 grams of the leaf powder were extracted for 16 hours in 1000 ml of the solvent (distilled water and ethanol) (50/50) using the soxhlet apparatus at(45°C). The leaves extract solution was then evaporated at 40°C using oven, and the resultant crude extract was frozen at -20°C until being used to prepare the required doses and concentrations [17]. To prepare the required doses, the Leek (Allium Porrum) extract was dissolved in Dimethyl sulfoxide (DMSO) and then filtrated by filter paper.

**Cyclophosphamide treatment**

Cyclophosphamide (Endoxan) 500mg, which was the product of (Halle, Germany), it is obtained from Al- Karama Teaching Hospital as vial. For mouse injection, a dose of 40 mg/kg was tested by dissolving it in distilled water to prepare the required dose and concentration, which is equivalent to (1 mg/mouse). Such concentration has been found to be genotoxic of bone marrow of mouse[18].

**Administration of laboratory animals:** The animals were allocated into 4 groups and administered single daily dose intraperitoneally as follows:

**Group 1:** Negative control 1, 5 mice were treated with 0.2ml distilled water for 5 consecutive days.

**Group 2:** Negative control 2, 5 mice were treated with 0.1ml the vehicle DMSO for 5 consecutive days. It’s used for dissolved Allium porrum extract.

**Group 3:** Positive control, 5 mice were treated with 0.2ml CP 40mg/kg for 24hr.

**Groups 4:** The treatment groups were carried out as an interaction between 4 doses of Allium Porrum extract (75, 150, 200 and 250 mg/kg) and single dose of CP (40 mg/kg). Twenty animals used for this treatment allocated to five mice for each dose. The extract was given for 5 consecutive days then single dose of CP given on the fifth day after 2 hrs from last dose and left
24hr. Then mice in these groups were sacrificed on the sixth day, and bone marrow samples were taken for cytogenetic analysis (MI and CA).

Cytogenetic experiments

- **Chromosome preparation from somatic cells of the mouse mone marrow**

  The experiment was done according to Allen et al., (1977)[19]. Cyclophosphamide was injected for 24 hr and colchicine was injected 2 hrs before sacrificed. Mice was sacrificed by cervical dislocation. It was dissected and both of femur bones were excised. Bone marrow was aspirated by flushing with phosphate buffer saline (PBS) in the centrifuge tube. The suspension was flushed in the tube properly to get good cell suspension and centrifuged for 10 min at 2000 rpm. Supernatant was discarded and the Pellet was treated with pre-warmed (37°C) KCl (0.56%) and shaken well. The suspension was put in a water bath (37°C) for 20 min. Pellet was treated with freshly prepared fixative (Methanol: Glacial Acetic Acid, 3:1) and shaken well. Fixative was repeated 3 times to get debris free white pellet. Slides were left overnight and stained with Giemsa's stain and observed under microscope in 40 x and then in 100x magnifications. A total of 100 well spread metaphase plates were scored for chromosomal aberrations (gaps, chromatid breaks, polyploidy a centric fragment, ring and fragmentation) and data of scoring was expressed as percentage chromosomal aberrations. Cells with gaps were not included in this percentage since gaps are not stable aberrations they were excluded from the total number of aberrations[20].

  **Cytogenetic analysis test**

1- **Mitotic index (MI) assay:** The slides were examined under high dry power (40 x) of light microscope, according to the following equation[21]:-

\[ \text{Metaphase Index} \, (\%) = \left( \frac{\text{Number of Metaphase Cells}}{\text{Total Count}} \right) \times 100 \]

2- **Chromosomal aberration (CA) assay:** The prepared slides were examined under the oil immersion lens (100 x) of light microscope for 100 divided cells per each animal.

**Statistical analysis**

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (one–way Anova), using the computer program GenStat discovery Copyright 2011. The difference was considered significant when the probability value was less than p<0.01.

**Results and Discussion**

The results of metaphase test are presented in Table (1). The frequency of MI in negative control group 1 and negative control group 2 showed no significant differences. The single-dose
of CP (positive control) caused a significant reduction in MI (p<0.01) and high damage metaphase after one day, in comparison with two negative controls.

**Table 1:** Percentages of mitotic index in bone marrow of mice after treatment with the CP alone and CP with several doses of leek (*Allium Porrum*) extract (Mean ± SE)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose</th>
<th>Mitotic Index %μ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control 1</td>
<td>0.2ml</td>
<td>6.52± 0.2</td>
</tr>
<tr>
<td>Treated with water only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control 2</td>
<td>0.1ml</td>
<td>a6.12±0.066</td>
</tr>
<tr>
<td>Treated with DMSO only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>40mg /Kg</td>
<td>b**3.7±0.152</td>
</tr>
<tr>
<td>Treated with CP only</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated with several dose of <em>Allium Porrum</em> extracts + CP 40mg/kg</td>
<td>250ml/Kg</td>
<td>4.38±0.12</td>
</tr>
<tr>
<td></td>
<td>200ml/Kg</td>
<td>5.08±0.25</td>
</tr>
<tr>
<td></td>
<td>150ml/Kg</td>
<td>c**6.054±0.48</td>
</tr>
<tr>
<td></td>
<td>75ml/Kg</td>
<td>3.84±0.12</td>
</tr>
</tbody>
</table>

a Negative control 2 vs. negative control 1, b Positive control vs. negative control 1

The result of %MI showed, when positive dose of CP was given together with leek (*Allium Porrum*) extracts, an increased MI, especially at 150 mg/kg (6.054%), and slightly increased at doses (75, 200 and 250 mg/kg) was observed. All these results were statistically significant (p<0.01).

Chromosomal aberrations findings present in Table 2. Animals treated with the(CP) positive control dose (40mg/kg) showed a high frequency of total structural and numerical chromosomal aberrations(7.42%) in mice bone marrow cells, these findings were significant (p<0.01) when compared with two negative controls 1 and 2 (0.1%, 0.11%) respectively. When the same doses of CP were given together with leek (*Allium Porrum*) extract, reduced chromosomal aberrations, especially at 150 mg/kg (1.088%), these results showed significant value (p<0.01), and there was a slightly reduction at these doses (75, 200 and 250mg/kg).
Table 2: Percentages of different types of chromosomal aberrations (CA) in bone marrow of mice after treatment with the CP alone and CP with several doses of leek (*Allium Porrum*) extract (Mean ± SE)

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>The Dose</th>
<th>Chromosomal aberrations</th>
<th>Chromatid aberrations</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acentric Fragment</td>
<td>Chromatid aberrations</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ring</td>
<td>Polyploidy</td>
</tr>
<tr>
<td>Negative control 1</td>
<td>0.2 ml</td>
<td>0.042 ± 0.003</td>
<td>0.00 ± 0</td>
<td>0.0000 ± 0000</td>
</tr>
<tr>
<td>Treated with water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control 2</td>
<td>0.1ml</td>
<td>0.03 ± 0.005</td>
<td>0.002 ± 0.002</td>
<td>0.0000 ± 0</td>
</tr>
<tr>
<td>Treated with DMSO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Positive control</td>
<td>40 mg/kg</td>
<td>0.156 ± 0.017</td>
<td>0.0680 ± 0.009</td>
<td>0.0140 ± 0.006</td>
</tr>
<tr>
<td>Treated with CP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Only</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment groups</td>
<td>250 ml/kg</td>
<td>0.0900 ± 0.021</td>
<td>0.0540 ± 0.012</td>
<td>0.004 ± 0.080</td>
</tr>
<tr>
<td>Treated with Several doses of Leek Extracts + CP 40mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 ml/kg</td>
<td>0.084 ± 0.020</td>
<td>0.0180 ± 0.008</td>
<td>0.008 ± 0.023</td>
<td>0.142 ± 0.023</td>
</tr>
<tr>
<td>150 ml/kg</td>
<td>0.0360 ± 0.012</td>
<td>0.0000 ± 0.000</td>
<td>0.002 ± 0.025</td>
<td>0.080 ± 0.025</td>
</tr>
<tr>
<td>75 ml/kg</td>
<td>0.158 ± 0.018</td>
<td>0.0680 ± 0.009</td>
<td>0.390 ± 0.046</td>
<td>0.390 ± 0.046</td>
</tr>
</tbody>
</table>

*Gap was excluded from total aberration[20]. a Negative control 2 vs. negative control 1, b Positive control vs. negative control 1, c Treatment groups vs. Positive control, ** Significant at P<0.01.

Bone marrow of control group 2 treated with DMSO 0.1ml showed no significant differences in % MI and % CA when compared with control group 1 (Tables 1 and 2). This may be a safe dose of DMSO given to control group [22].

The single - dose of CP (positive control) caused a significant reduction (p<0.01) in MI and high damage metaphase after one day, in comparison with two negative controls. We are consistent with Abdelmegid et al,(2013) who found Endoxan(CP) inhibited the mitotic index significantly[23]. Also these findings are consistent with Shang-Fu Xu et al, (2012) that he noticed the bone marrow cell numbers were significantly decreased in bone marrow suppression mice that were treated with CP[24].

The reduction of MI in positive group may be due to that CP acts through alkylation of DNA bases, crosslinking of two DNA strands occurred by acrolein and phosphoramides are the active compounds of CP. These active compounds of the CP slow down the growth of cells by interfering with the actions of DNA within those cells, which is capable of inducing DNA crosslinks and strand lesions [25], or by attaching to the triphsphate moiety of the sugar-
phosphate DNA backbone. These two reactions of CP with DNA, if unrepaired, may stop the DNA replication machinery leading to double-stranded DNA breaks and hence decreases in metaphase [26]. The results suggest that the increment on metaphase value in dose of (150mg/kg) belonged to the absorption and tissue distribution of leek (Allium Porrum) extract in this dose is higher than high and low doses of extract after intraperitoneally administration in mouse bone marrow. The reason of little changes in MI between low dose of treatment groups (75mg/kg)and positive control group, might be attributed to that leek (Allium porrum) extract had different compounds chlorophylls , alkaloids, flavonoids, glycosides and tannins, these compounds may cause increase MI in concentration depended manner [27].

Table 2 showed the results of CA was significantly different (p<0.01) between positive control and two negative control 1 and 2, we agree with Aditya.M. et al.,(2013) that CP induced chromosomal aberrations in mouse bone marrow cells by inducing free radicals which have the ability to cause damage to DNA and RNA and inhibit some enzymes from reacting with amino acids [28].

In this experiment, the effect of CP was noticed after treatment with single dose for one day and showed a high frequency of total structural and numerical chromosomal aberrations (Figure- 1). It was pointed that the percentage of fragment was higher than other chromosome aberration, which reached to (6.76%) Table 2 from total after treatment with CP, this may be related to the differences in the repair systems for each change, or by clastogenic effects of CP. Bifunctional alkylating nitrogen mustards of CP are generated, which is capable of reacting with the nitrogen-7 atom of purine bases in DNA, especially when they are flanked by adjacent guanines[29]. At alkaline or neutral pH, the nitrogen mustard is converted to chemically reactive carbonium ion through imonium ion. Carbonium ion reacts with the N7 of the guanine residue in DNA to form a covalent linkage. The second arm in phosphoramide mustard can react with a second guanine moiety in an opposite DNA strand or in the same strand to form crosslinks[30]. The O6 atom of guanine may also be a target for oxazaphosphorine [31].

The different intermolecular distance between the chloroethyl groups in CP mustards results in a different range of cross-linked DNA, all of these mechanisms above enhance chromosome aberration.

The results in Table 2 clearly indicated that leek (Allium Porrum) extract modulates the CP induced genotoxicity in a dose 150mg/kg in mouse bone marrow in vivo, we suggest that this intermediate dose of extract may act on the repair systems inside the cells more than the low and high doses. This dose provided protection when given for five days prior the single i.p administration of CP (40 mg/kg). Thus, tested leek (Allium Porrum) extract leaves have preventive potential against CP induced chromosomal aberrations in Swiss mouse bone marrow.

Several studies indicate that CP has a pro-oxidant character, and generation of oxidative stress after CP administration leads to decrease in the activities of antioxidant enzymes and increase in lipid peroxidation in liver, lung and serum of mice and rats [32]. The present investigation was carried out to evaluate the chemoprotective potential of Allium porrm extract against oxidative stress mediated cellular toxicity induced by CP in Swiss albino mice.
Nathalie B. et al. (2012) found from his study that the green leek (*Allium Porrum*) leaves generally have significantly stronger antioxidant properties than the white shaft and the antioxidant activity showed by phenolics and ascorbic acid contribute significantly to the antioxidant activity of leek (*Allium Porrum*), this properties exerts its protective effect against CP-induced cellular toxicity in part by scavenging the free radicals generated by CP reactive metabolites [6].

**Figure (1):** Cells in metaphase stage taken from mice treated with CP the positive control (40mg/ kg), showing: chromatids break (A), ring chromosome (B), polyploidy chromosome (C), and fragment chromatid (D) (100x).
In an interaction treatments, 150mg/kg extract showed a significant efficiency in reducing the genetic effects of CP, and again these actions have been attributed to the chemical constituents of leek (Allium Porrum) such phenols and flavonoids with regard to the forthcoming repair mechanisms. Castillo, C. A. (2012) found the flavonoid of leek enhances the post-replication repair[33]. While others have demonstrated that flavonoids and terpenes stimulate the mechanism of error-free repair, furthermore terpenes can activate recombination repair mechanism, beside their action in activating the detoxification enzymes [34].

Conclusions

We conclude that leek (Allium Porrum) extract exerts its chemo protective effect against CP-induced cellular toxicity in part by scavenging the free radicals generated by CP reactive metabolites. Because CP does not act through oxidative stress mechanism, it is expected that the leek compound would be beneficial to the host but without reducing the efficiency of the treatment. We here predict that this finding gives the directions for the future research possibilities for the design and development of leek (Allium Porrum) extract related modulatory drugs in combination with the CP. Such drugs might minimize the side effects caused by the widely used chemotherapeutic agent CP.

References


