Ameliorative effect of *Cymbopogon citratus* extract on cisplatin-induced genotoxicity in human leukocytes

Islam M. El-Garawani
Department of Zoology, Faculty of Science, Menofia University, Menofia, Egypt.
Email: dr.garawani@yahoo.com

Abstract

Cisplatin (Cis) as anticancer drug has many side effects and non-selective targeting of cancerous cells. In this work, the antigenotoxic potential of lemon grass (*Cymbopogon citratus*) leaves aqueous extract (CC) against the cisplatin-induced genotoxicity in human peripheral leukocytes was investigated in vitro. Firstly, the antioxidant activity of plant extract was investigated by DPPH method which proved a good antioxidant potential of CC extract. CC enhanced the proliferation and increased cell count when compared to the control and Cis-treated groups. Furthermore, the expression of nuclear protein Ki-67 as a marker of cell proliferation was tested immunocytochemically and the results supported the ameliorative effect of CC against the genotoxic effect of Cis. The nuclear dual acridine orange/ethidium bromide fluorescent staining of cells showed the distinct morphological features of apoptosis and cell death among Cis-treated groups. The reduction of total genomic DNA fragmentation on agarose gel was noted among CC-treated groups when compared to Cis-treated groups.

Keywords: Cisplatin, *Cymbopogon citratus*, genotoxicity, leukocytes and proliferation in vitro.

1. Introduction

Cisplatin (cis-diamminedichloroplatinum), is one of many anti-cancer drugs that causing DNA damage. Although cisplatin is a powerful chemotherapeutic drug in the solid cancers treatment, it also has great side effects such as reducing the levels of plasma antioxidants and generating free radicals in normal cells (Weijl et al., 1998 and Yoshida et al., 2003). It also induces chromosomal aberrations in mammalian cells and human peripheral blood lymphocytes in vitro (Osanto et al., 1991; Nefic and Ibrulj, 1999 and Khabour et al., 2014), and sister-chromatid exchanges in human lymphocytes in vitro (Bradley et al., 1979 and Khabour et al., 2014). It increases the micronuclei frequency in cisplatin -treated human lymphocytes in vitro (Gebel et al., 1997).

A considerable attention is currently focuses on preventing genotoxic effects of anticancer drugs by using the antioxidants of natural origin. *Cymbopogon citratus* which is belonging to Poaceae family is a tall aromatic grass of approximately 1.5 m high. It is a monocotyledonous perennial grass which has slender sharp edged leaves. CC has no toxic effects in Brazilian traditional folk medicine (Souza-Formigoni et al., 1986). Its leaves tea made is used in Brazil as antispasmodic, analgesic, anti-inflammatory, antipyretic, diuretic and sedative (Carlini et al., 1986). Lemon grass leaves aqueous extract exert cytoprotective and antioxidant properties (Lee et al., 2008; Figueirinha et al., 2010 and Tiwari et al. 2010). It is also reported that lemon grass inhibited human lymphocytes damage induced by mitomycin-C (Meevatee et al., 1993). Hepatic oxidative stress and toxicity induced by cisplatin in albino rats can be alleviated by lemon grass aqueous extract (Arhogho et al., 2014).
The present study was conducted to investigate the potent ameliorative effect of lemon grass aqueous leaves extract against the possible genotoxic effect of cisplatin on human peripheral leukocytes in vitro.

2. Materials and Methods

A. Chemicals

I. Cis - diamminedichloroplatinum, Cisplatin, (Unistin, EIMC, Egypt).

II. Ascorbic acid (vitamin C), Sigma pharmaceuticals (assay: 99.1%), was prepared fresh in methanol as a standard stock solution.

III. 2,2-diphenyl-1-picrylhydrazylic free radicals (DPPH), Sigma pharmaceuticals, was freshly prepared as a 0.3 mM stock solution in methanol.

IV. Medicinal plant: lemon grass (Cymbopogon citratus) was obtained freshly from Applied Research Center of Medicinal Plant (ARCMP), Kafr El Gabal, Giza, Egypt.

All chemicals used in this work were of high analytical grade.

B. Methods

Preparation of Cymbopogon citratus aqueous extracts

Fresh leaves were collected and minced into fine pieces. 200g of leaves was extracted with 40X (w/v) of hot distilled water at 55°C for 6 hours. The extract was then filtered using Whatman filter paper (No. 1). The filtrate was lyophilized by a freeze-dryer (Sniffers scientific, Holland), and then it was stored at -20°C until use (Kumazawa et al., 2002).

Determination of free radical scavenging potential of Cymbopogon citratus extracts by DPPH assay

The 2, 2-diphenyl-1-picrylhydrazyl free radical scavenging assay was done to determine the antioxidant activity of the Cymbopogon citratus leaves aqueous extract. The changes in color from violet to yellow which caused by the scavenging reaction were evaluated colorimetrically at 517 nm using a UV-VIS spectrophotometer (Hewlett Packard, USA) (Blois, 1958). The % antioxidant activities (AA %) was calculated according to the following equation:

\[ AA\% = 100-\frac{[Ahs_{sample}-Ahs_{blank}]}{Ahs_{control}} \times 100 \]

The test was carried out three times independently.

Study design

Human peripheral blood cultures were divided into nine groups as follow:

Normal (untreated), Cisplatin (5µg/ml), Cisplatin (10µg/ml), Cymbopogon citratus (5µg/ml), Cymbopogon citratus (10µg/ml), Cisplatin (5µg/ml) + Cymbopogon citratus (5µg/ml), Cisplatin (5µg/ml) + Cymbopogon citratus (10µg/ml), Cisplatin (10µg/ml) + Cymbopogon citratus (5µg/ml) and Cisplatin (10µg/ml) + Cymbopogon citratus (10µg/ml).

Each group was processed as three replicates.

Cell culturing and treatments

Peripheral blood was collected from normal healthy non-smoker male volunteer under sterile conditions and EDTA-anticoagulant. One milliliter of whole peripheral blood was cultured in 5ml RPMI-1640 supplemented with 15% heat-inactivated fetal bovine serum, penicillin (100 U/ml)-streptomycin (100 g/ml) and (10 g/ml) phytohemagglutinin, then the tubes were kept at 37°C. All components were (GIBCO-BRL). Treatments were administered after 48h of incubation, then left to complete 72h before harvest. Simultaneous treatment was carried out among the groups of cisplatin and Cymbopogon citratus combination.

Peripheral leukocytes isolation

Leukocytes were isolated from treated and control cultured blood by incubation with 8ml erythrocyte lysing buffer (0.015M NH₄Cl, 1mM NaHCO₃, 0.1 mM EDTA). Then, they were centrifuged for 5 minutes at 100 rpm using cooling centrifuge (Sigma 3K 30, Germany). These steps were repeated until a white pellet appeared (Punareewattana et al., 2001).

Cell viability and count assay

The viability test was carried out for the harvested cells according to (Darzynkiewie et al., 1994). Briefly, 10µl of 0.4% trypan blue stain were added to 10µl of cell suspension and the total of 500 cells was recorded for viable cells against dead cells. The total count per 1ml was also evaluated using haemocytometer under a light microscope (Olympus, Japan).

The viability percentage was calculated as follow:

\[ %\text{Viability} = \frac{\text{Number of viable cells}}{\text{Number of viable and non-viable cells}} \times 100 \]

Evaluation of cell cycle arrest by Ki-67 expression

The nuclear antigen Ki-67, which expressed during most phases of the cell cycle, a marker of proliferating cells, was detected using monoclonal antibodies Ki-67. The functional capacity of leukocytes was evaluated by the amount of visualized Ki-67 stained positive cells. For immunochemical localization of Ki-67, cells were stained using avidin-biotin complex immunoperoxidase
technique (Cordone et al., 1992). The mouse anti human Ki-67 monoclonal antibody was used for the detection of nuclear Ki-67 (DakoCytomation, Glostrup, Denmark). Counterstaining was performed using Mayer's hematoxylin. The mean percentage of Ki-67 positively stained cells under × 100 magnifications was calculated using light microscope (Olympus, Japan) and representative images were captured. Scoring of Ki-67 expression was performed as negative (blue nuclei) and positive (brown stained nuclei) of 500 cells per sample.

**Dual acridine orange/ethidium bromide fluorescent staining**

Ethidium bromide/acridine orange staining was carried out to detect the morphological evidence of apoptosis and viability of treated and control groups, 10µl of cells were incubated for 1 minutes with 5 µl (10µg/ml) of a solution of acridine orange/ethidium bromide 1:1 ratio of (100 µg/ml) in PBS. Stained cells were visualized under (Olympus fluorescence microscopes, Japan) immediately and the images were digitally photographed. The apoptotic cells, with their shrunken, nuclear fragmentation, bright orange fluorescent and condensed chromatin were scored and the percentage of apoptotic cells were calculated, while the viable normal cells appeared green in color.

**Detection of DNA fragmentation by agarose gel electrophoresis**

DNA extraction and detection of fragmentation were done according to "salting out extraction method" of Aljanabi and Martinez (1997) with some modifications by Hassab El-Nabi and Elhassaneen (2008). Harvested cells were lysed in 300 µl lysing buffer (50 mM NaCl, 1 mM Na2EDTA, 0.5% sodium dodecyl sulfate, pH 8.3) overnight at 37°C then; 100 µl of 4M NaCl was added to the samples. Centrifuge the mixture at 10,000 rpm for 10 minutes. The supernatant was transferred to a new tube then DNA was precipitated by 1 ml cold isopropanol by centrifugation for 10 min at 12,000 rpm. Wash the pellets with 1 ml 70% ethyl alcohol twice. Resuspend the pellets in TE buffer (10 mM Tris, 1mM EDTA, pH 8). Incubate for 30 - 60 minutes with loading mixture (0.1% RNase + loading buffer), and then samples were loaded directly into agarose gel wells. All centrifugation steps were done at 4°C using (Sigma 3K30, Germany). The extracted DNA samples were estimated spectrophotometrically for purity at optical density (OD) 260/280 using (Hewlett Packard 8453, USA) spectrophotometer (Surzycki, 2000).

Gels were prepared using 1.8% normal melting electrophoretic grade agarose in 1X Tris borate EDTA buffer and electrophoresed for 1 h at 50 volts. The fragmented DNA appeared and located throughout the lanes against thirteen bands of DNA marker (100–3000 bp). The intensities of fragmented DNA molecules were measured by (Biogene software, France) as a maximum optical density values.

**Statistical analysis**

Results were expressed as the Mean ± Standard Deviation (M ± SD). Student's t-test, for normally distributed data (McClave and Dietrich, 1991), was used to calculate the significance of differences observed between mean values of treated and control groups at a level of significance of (P < 0.05).

**3. Results**

**Determination of free radical scavenging activity of the extract by 2, 2-diphenyl-1-picrylhydrazyl assay (DPPH)**

The results revealed the presence of an increase in the percentage of antioxidant activities of CC extract in a concentration-dependent manner which reached about 100% at a concentration of 89.86 µg/ml to be in parallel with ascorbic acid value at 97.14 µg/ml (Figure 1).

![Figure (1): Free radical scavenging activity of CC (LG) extract using a stable DPPH radical against vitamin C (standard).](image)

**Cell viability and count**

The standard trypan blue viability test revealed that all tested groups have about 95% viable cells after 48 hours of culturing before treatments administration.

Cells count was carried out after cells harvest at the end of the experiment (48 hours of culturing then 24 hours of treatment administration) and the results were expressed in (Table 1) which shows the decrease in proliferation capacity due to Cis treatments in concentration dependent manner and the modulation of this inhibition by CC (10µg/ml) simultaneous treatments. While low dose of CC (5µg/ml) fail to ameliorate that inhibition. In addition, the
enhancement of proliferation was countered among the CC groups when compared to the untreated groups.

**Evaluation of cell cycle arrest by Ki-67 expression**

The nuclear antigen Ki-67 expression which evaluated as a mean percentage of positive stained (brown) cells counting revealed the antiproliferative effect of Cis which was noted by the decrease of Ki-67 expression with respect to untreated groups shifting cells towards G0 resting state. Moreover, the successful ameliorative potential of simultaneous CC (10µg/ml) treatment against Cis (5 and 10µg/ml) toxicity. While low dose of CC (5µg/ml) fail to ameliorate the Cis antiproliferative effect. Additionally, CC treatment alone enhanced the proliferation capacity of cells which obviously noted by the increase of Ki-67 expression when compared to untreated and Cis-treated groups (Table 1).

**Detection of total genomic DNA fragmentation**

The obtained results (Figure 4) revealed the powerful protection of CC extract against the total genomic DNA damage which has been induced by Cis treatment high dose more than Cis low dose. The DNA fragmentation was obviously countered as optical densities of fragmented DNA molecules at Cis low and high dose groups as (53.2±3.74 and 77.1±2.5) respectively, which were ameliorated by CC high dose treatment more than that of CC low dose. While no fragmentation can be observed among other groups when compared with untreated groups (Table 1).

**4. Discussion**

Although cisplatin is a powerful and widely used anti-neoplastic drug in adults and many pediatric cases, its clinical use is highly limited due to its remarkable side effects. The major suggested mechanism by which cisplatin exerts its genotoxic effect is by its ability to induce oxidative stress inside cells (Khabour et al., 2014) and free radical generation (Yoshida et al., 2003). The cytotoxicity of the drug is also thought to be due to its ability to bind DNA to form cisplatin–DNA adducts, beside that, the intracellular accumulation of cisplatin, impaired DNA-repair processes, and decreased levels of cisplatin-inactivating factors, such as glutathione and metallothioneins (Perez, 1998; Zhang et al., 2001; Rajewsky and Müller, 2002). Due to the non-selectivity of cytotoxic drugs in targeting normal and cancerous cells (Thomas et al., 2000), the alternative cancer therapy is valuable and has to be established.

Recently, the modulation of toxic effect by natural origin extract has been paid a great attention. Plants have the
ability to synthesize aromatic substances such as polyphenolic compounds, mainly flavonoids and phenolic acids, which have potent antioxidant activities due to their hydrogen-donating and metal-chelating capacities. These flavonoids and polyphenolics have been proved to possess the ability to scavenge several oxidizing species such as hydroxyl radicals (Shimoi et al., 1996, 2000; Benavente-García et al., 2002). In this work, the DPPH assay results proved the antioxidant activities of CC leaves aqueous extract (Kanatt et al., 2014) which may be due to its contents of phenolic acids, flavonoids and tannins (Olorunnisola et al. 2014).

The nuclear antigen Ki-67 is a protein detected in G1, S, G2 and M phases of the cell cycle, but not in G0 (Diop et al., 2005). It is used to recognize proliferating cells while it is absent in resting cells (Gerdes et al., 1983). In this study, the results of cell count and Ki-67 nuclear expression revealed that Cis caused cell cycle arrest which has been evaluated by cell count and Ki-67 down expression. While CC treatment enhanced cell proliferation and modulated Cis toxic effects, which may be due to the antioxidant activities of CC leaves extract (Kanatt et al., 2014). On the other hand, the results of total genomic DNA fragmentation and morphological changes by dual-fluorescent assay supported the previous results as the effective CC protection, specially high dose (10 µg/ml), against the genotoxic effect of Cis (De et al., 2015) and enhancement of cells proliferation which also an evidence of the antioxidant potential of CC extract.

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Table (1): shows the % of cells count variation with respect to control, % of morphological evidence of apoptosis evaluated by acridine orange/ethidium bromide dual fluorescent staining method, the mean optical densities of fragmented DNA on agarose gel and % of Ki-67 +ve cells for treated and untreated groups.

<table>
<thead>
<tr>
<th>Treatments (µg/ml)</th>
<th>Control</th>
<th>5 Cis</th>
<th>10 Cis</th>
<th>5 CC</th>
<th>10 CC</th>
<th>5 Cis + 5CC</th>
<th>5Cis + 10CC</th>
<th>10Cis + 5CC</th>
<th>10 Cis + 10CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>% cells count</td>
<td>-</td>
<td>-20.6±0.47</td>
<td>-32.2±0.25</td>
<td>+45.6±0.60</td>
<td>+47.5±1.41</td>
<td>+30.2±0.96</td>
<td>+44.6±1.40</td>
<td>-58.3±1.38</td>
<td>-15.9±1.37</td>
</tr>
<tr>
<td>% Ki-67 +ve cells</td>
<td>72.3±2.52</td>
<td>27.7±1.35</td>
<td>21.7±1.10</td>
<td>82.7±1.51</td>
<td>85.0±1.25</td>
<td>63.3±2.40</td>
<td>83.8±1.37</td>
<td>21.8±0.61</td>
<td>35.0±1.15</td>
</tr>
<tr>
<td>% Apoptotic cells</td>
<td>4.1±1.0</td>
<td>31.0±0.15a</td>
<td>41.1±0.96a</td>
<td>5.4±0.26</td>
<td>14.7±0.74a</td>
<td>5.6±0.35</td>
<td>23.7±0.89a</td>
<td>32.9±0.95a</td>
<td>42.1±2.61a</td>
</tr>
<tr>
<td>Fragmented DNA</td>
<td>4.5±1.43</td>
<td>53.2±3.74</td>
<td>77.1±2.56</td>
<td>5.3±1.91</td>
<td>4.0±1.99</td>
<td>30.6±3.43</td>
<td>5.9±1.65</td>
<td>42.9±2.70</td>
<td>7.5±1.45</td>
</tr>
</tbody>
</table>

*p < 0.05: significance with respect to the control group for fluorescent apoptotic cells percentages and DNA fragmentation optical densities. Cis: Cisplatin and CC: Cymbopogon citratus

5. References


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