Efficacy of Carob Leaves and Pods in Reducing Cyclophosphamide-Induced Toxicity on Bone Marrow and Peripheral Blood Leukocytes

Mohammad Y. Abajy, Ream Nayal*, Mahassen Alqubaji and Yara Abdрабbo

1Department of Biochemistry and Microbiology, Faculty of Pharmacy, University of Aleppo, Aleppo, Syria.
2Department of Pharmacognosy, Faculty of Pharmacy, University of Aleppo, Aleppo, Syria.

Abstract: Cyclophosphamide is one of the chemotherapeutics for cancer, however this drug has severe side effects in multiple organs and is a well-known mutagenic agent. In this work the protective activity of antioxidant-rich Carob (Ceratonia siliqua L.) pods and leaves extracts against cyclophosphamide induced genotoxicity was studied. Safety of these extracts was evaluated in acute and subacute toxicity tests at a dose of 1500 mg/kg/day in albino rats. Both extracts exhibited no mortality or significant changes in rats' general behavior. Moreover, organ weights and blood chemistry revealed no toxic effects of these extracts (p>0.05). The anti-mutagenic potential of carob pods and leaves extracts were evaluated using in vivo bone marrow chromosomal aberration and micronucleus assays and in vitro comet assay for peripheral blood white cells. Results showed that rats that were treated with cyclophosphamide along with carob pods had a significant decrease in chromosomal aberrations when compared with cyclophosphamide-treated rats (p<0.05). Pods extract also showed significant reduction in cyclophosphamide-induced DNA migration (p<0.05). There were also highly statistically significant differences in the means of micronuclei (MNs) in immature red blood cells between cyclophosphamide group and carob leaves group (p<0.01). It could be concluded that Carob extracts had protective effect against cyclophosphamide- induced genotoxicity and may be considered as anti-mutagenic agent after conducting more studies.

Keywords: Ceratonia siliqua, cyclophosphamide, acute and subacute toxicity, bone marrow, chromosomal aberration, comet assay.

Funding: This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.


This article is under the CC BY-NC-ND Licence (https://creativecommons.org/licenses/by-nc-nd/4.0)
1. INTRODUCTION

Cyclophosphamide (CP) is an anti-cancer agent and is widely used for its anti-tumor effects against a wide variety of human neoplasms. More recently, its immunosuppressive properties have been exploited successfully in the treatment of non-neoplastic diseases such as rheumatoid arthritis, lupus erythematosus, and Wegener’s granulomatosis. In the liver, cyclophosphamide is converted by hydroxylation into 4-OH-CP, which equilibrates with its tautomeric form aldophosphamide (ALDO). β-elimination of acrolein from ALDO leads to the formation of the alkylating agent phosphoramide mustard (PAM), which suppresses cell growth through DNA alkylation. Additionally, the reactions caused by acrolein and the by-product chloroacetalddehyde contribute to the toxicity of cyclophosphamide. It was shown that free radicals and reactive oxygen species (ROS) are formed during the activation of cyclophosphamide and produce tissue injury. Toxic effects of cyclophosphamide are the suppression of white blood cells (WBC) production, nausea, vomiting, gonadal atrophy, and renal and bladder injury. In addition, CP is a derivative of the alkylating agent nor nitrogen mustard, a compound chemically related to the first substances known to induce chromosome rearrangements and gene mutations in germ cells of experimental animals. To overcome these toxic effects induced by CP, safe cytoprotective antioxidants are necessary and should be administered to patients before or during receiving CP. Many studies have focused on investigating the protective activity of plants as natural antioxidants against CP induced toxicity and have shown a preventive activity of many plants such as tea tree, ginseng, and ginkgo. Carob (Ceratonia siliqua) is an evergreen tree that belongs to the Fabaceae family. Its original habitats are the western parts of Asia, but after its domestication, it spreads to all Mediterranean basins and then to the western shores of the Americas, South Africa and southern regions of Australia. Traditionally, barks and leaves of carob have been used as an antidiarrheal and diuretic, while its fruits were used to relieve cough and to treat warts. Most commercially popular carob products are: Pods, bean flour, seed gums, carob chocolate and syrup. Carob contains polyphenols, tannins, proteins, fats and carbohydrates, its seeds and pods are particularly rich sources of condensed tannins (proanthocyanidin), ellagitannin and gallotannin. These phytochemicals exhibited scavenging activities against numerous diseases caused as a result of free radical attack. Furthermore, water extracts from carob pods showed a high antioxidative activity in different in vitro tests because of the presence of proanthocyanidins, gallic acid, (+)-catechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate and quercetin glycosides. In addition, the presence of carotenoids in the pods extract enhances the antioxidant capacity, which may suggest a synergistic effect between polyphenols and carotenoid compounds. Therefore, the aim of the present study was to investigate the protective activity of antioxidant-rich Carob pods and leaves against cyclophosphamide induced genotoxicity.

2. MATERIALS AND METHODS

2.1. Preparation of the aqueous-ethanol extracts

Carob leaves were obtained from Aleppo University campus in June 2020 and the pods were collected in September 2020. Plant parts were authenticated by Dr. R. Khatib, department of pharmacognosy, Damascus University, Syria. Voucher specimens with the number CSL-V31 and CSP-V30 were deposited at the pharmacognosy and phytochemistry department, faculty of pharmacy, Aleppo university, Syria. Leaves and pods were shade dried at room temperature and powdered using an electrical grinder. To 40 g pods or leaves 200 ml of 70% ethanol were added and the mixture was incubated overnight under continuous stirring at 100 rpm. After filtration, the residue was reextracted with 200 ml ethanol 70% at the same conditions. The combined extracts were dried in a rotary evaporator at a temperature of 42 °C. Extraction yield was calculated and extracts were stored at -20 °C.

2.2. Evaluating the subacute and acute toxicities of carob extracts

2.2.1. Experimental animals

Adult albino rats (90-140 g) were obtained from the animal house of the faculty of Pharmacy. Rats were maintained at a normal room temperature (22±1)°C with a 12 h light: 12h dark cycle and allowed free access to bottles of water and pelleted diet feed and maintained in aerated plastic cages. This study was conducted as per the guidelines of Care and Use of Laboratory Animals (2011) and the protocol was approved by Animal Ethics Committee of Aleppo university, (No: 873/l, 2021).

2.2.2. Acute toxicity study

In this study 18 Wistar rats were divided into 3 groups: Group 1 was orally administered with a single dose of carob pod extract 1500 mg/kg body weight. Group 2 was orally administered with a single dose of carob leaves extract 1500 mg/kg. Group 3 was given distilled water (control group). Signs of toxicity were observed within 1-4 hrs after treatment and periodically for 24 hrs. Changes in the skin, eyes, mucus membrane, body weight and behavioral patterns were noted during the test period (2 weeks).

2.2.3. Subacute toxicity study

This investigation was completed in 14 days. Experimental animals were divided into three groups of six rats each. The first Group served as control (rats were administered with distilled water), while the second and third groups were administered with pods extract and leaves extract, respectively, at a daily dose of 1500 mg/kg for 14 days. On day 14, rats were anesthetized using chloroform and blood samples were taken (after overnight fasting) for biochemical and hematological analyses using both EDTA and heparin tubes.

1. Relative organ and body weights

On day 14 the body weight of each rat was measured. Several organs (lungs, spleen, heart, liver and kidneys) were weighed and relative organ weight was calculated as following:
2. Blood analysis

The following blood indices were analyzed: Complete blood count (CBC) includes: hematocrit (HCT), corpuscular volume (CV), count of red blood cells, count of white blood cells (WBC), lymphocytes (LYM), neutrophils (NEU), monocytes (MONO), basophils (BASO). Additionally, serum creatinine & urea (kidney function) and the liver enzyme ALT were also analyzed.

2.3. Chromosomal aberrations test

18 rats were divided into three groups as following: Group 1 did not subject to any treatment (negative control), group 2 was treated with cyclophosphamide (50 mg/kg, i.p) and group 3 was treated with carob pods extract (500 mg/kg, p.o.) for ten days followed by a single dose of cyclophosphamide (50 mg/kg, i.p.) as a challenge.

Preparation of mitotic chromosomes

Each rat was injected intraperitoneally (i.p.) with 10 mg/kg of Colchicine for mitotic arrest. After 2 hours, rats were anesthetized with an overdose of chloroform and the bone marrow was aspirated from the femur into a suspending medium (0.075 M KCl) for 20 min. After centrifugation the residue was fixed in a mixture of methanol and glacial acetic acid (3:1). Chromosomes samples were prepared by dropping the cell suspension onto an ice cold slide. Slides were dried and stained for 15 - 20 minutes with 10% Giemsa solution at pH 6.8. Slides were screened for different types of chromosomal abnormalities (rings, breaks, exchanges and numerical abnormalities).5,16,17

2.4. Bone marrow micronucleus assay

15 Rats were divided into three groups as follows:

Group 1: Rats were given orally carob leaves extract at a daily dose of 500 mg/kg for ten days.

Group 2 : Rats were given orally distilled water for ten days then treated i.p. with 100 mg/kg cyclophosphamide (positive control).

Group 3: Rats were treated with carob leaves extract for ten days followed by cyclophosphamide as a challenge.

72 hours after cyclophosphamide injection peripheral blood samples were drawn on Heparin tubes for blood glucose testing. After that animals were sacrificed and the bone marrow was aspirated from femur into 5% bovine serum albumin in phosphate buffered saline (pH 7.2). Cell suspension was centrifuged (1000 rpm for 5 min). Smears were prepared on glass slide, fixed with methanol (100%) and stained with Giemsa dye. Smears were analyzed under light microscopy (1000X) for the presence of micronuclei (MNs) in immature erythrocyte [polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs)]. 1000 immature red cells were counted per sample.18

2.5. In vitro Comet assay

a. Preparation of Cells: (500 µl) Heparinized peripheral blood from human volunteers were mixed with 5 ml RPMI-1640 media in microcentrifuge tube and incubated for 24 hours at 37°C under 5% CO₂ atmosphere. Informed consent was obtained from volunteers who donated blood samples. The mixture was divided into four tubes. To three tubes either 25 µg of cyclophosphamide or 25 µg carob pods extract or 25 µg cyclophosphamide with 25 µg carob pods extract was added and the last tube served as a negative control. After 4 hours incubation at 37°C tubes were centrifuged at 2500 rpm for 10 minutes. The top layer was removed and 400 µl from each tube was mixed with 400 µl of low melting agarose (1% in distilled water).19,20

b. Preparation of Slides: Ethanol cleaned slides were dipped into hot agarose (>60°C), then they were left to solidify for 2 min in a refrigerator. From each of the above mentioned mixture 400 µl of cell suspension were added onto agarose pre-coated slide.19

c. Alkaline lysis and electrophoresis: Slides were submerged in a lysis solution A1 (1.2 M NaCl, 100 mM Na₂EDTA, 0.1% SDS, 0.26 M NaOH) for 24 h at 4°C in the dark, after that slides were rinsed 3 times with solution A2 (0.03 M NaOH, 2 mM Na₂EDTA) for 20 min. at temperature of (18–25°C). Electrophoresis was conducted in an A2 solution for 25 min. at 40 mA and 20 V.21

d. Slides staining: Slides were rinsed and neutralized in distilled water, then they were placed in ethidium bromide stain solution for 20 min. and examined using a fluorescence microscope. For each slide, 10 microscopic fields were observed, and cells were classified into two categories; undamaged cells and comet shaped cells.

3. STATISTICAL ANALYSIS

Results were analyzed using Independent Samples t-Test, One Way ANOVA, Tukey and Z Proportions test. Statistical analysis was performed on SPSS software (IBM® SPSS® Statistics 26). Significance was considered when P values were less than 0.05.

4. RESULTS AND DISCUSSION

4.1. Acute and sub-acute toxicity of carob ethanolic leaves and pods extracts

The recent worldwide increasing interest at using herbal medicine for illness treatment and prevention is associated with increasing concern about the safety and toxicity of medicinal plants.22 One of the methods used for testing the toxicity of plants is the assessment of in vivo toxicity. Therefore, in this study acute and subacute toxicity of carob extract was investigated in rats as experimental animals. Results of an acute toxicity study showed that the LD₅₀ was more than 1500mg/kg because at this dose no signs of
hypoactivity, morbidity or lethality were observed in animals. Acute toxicity methods are utilized to detect the harmful effects of a substance to the organism as a result of short-term exposure. These methods evaluate the mortality or the changes in body weight, behavior and overall well-being of the animals.\textsuperscript{3-5, 12, 13} In the sub-acute toxicity study, the 14 days administration of carob pods and leaves extracts did not cause morphological or behavioral changes in the treated rats compared to the control group. Results of organ weights showed that there were none-significantly different between carob pod group or leaves groups and control group (Table 1). Moreover, no deaths occurred during this period. As shown in (Table 2 & 3), there were also no significant changes in blood parameters, in serum creatinine, urea and in the liver enzyme ALT among the three groups (p>0.05).

Results of relative organ weight (%) after administration of carob extract for 14 days are expressed as Mean±SD of six rats per group. There were no statistically significant differences in the relative organ weight % among groups treated with either carob pods extract or carob leaves extract or distilled water, p>0.05.

### Table 1. Relative organ weights of rats treated with carob pod or leaves extract or distilled water

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Carob pods group</th>
<th>Carob leaves group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lungs weight %</td>
<td>0.0070±0.0002</td>
<td>0.0071±0.0013</td>
<td>0.0082±0.0020</td>
</tr>
<tr>
<td>Spleen weight %</td>
<td>0.0029±0.0005</td>
<td>0.0025±0.0006</td>
<td>0.0081±0.0097</td>
</tr>
<tr>
<td>Heart weight %</td>
<td>0.0044±0.0003</td>
<td>0.0039±0.0007</td>
<td>0.0051±0.0015</td>
</tr>
<tr>
<td>Liver weight %</td>
<td>0.0336±0.0026</td>
<td>0.0341±0.0088</td>
<td>0.0450±0.0136</td>
</tr>
<tr>
<td>Kidneys weight %</td>
<td>0.0083±0.0001</td>
<td>0.0079±0.0019</td>
<td>0.0091±0.0025</td>
</tr>
</tbody>
</table>

Results of hematological parameters in different rat groups after daily administration of carob extract for 14 days are expressed as Mean±SD of six rats per group. The values in the control group were not statistically different from those in groups treated either with carob pods or carob leaves, p>0.05.

### Table 2. Effects of carob extracts on hematological indices

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Carob pods group</th>
<th>Carob leaves group</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC mm(^3)</td>
<td>1600.00±242.264</td>
<td>1075.00±485.627</td>
<td>2200.00±336.650</td>
</tr>
<tr>
<td>RBC *10(^3)/mm(^3)</td>
<td>7.425±0.70415</td>
<td>7.625±0.85391</td>
<td>7.625±2.16083</td>
</tr>
<tr>
<td>HGB g/dl</td>
<td>14.400±1.0801</td>
<td>14.725±1.4592</td>
<td>16.275±4.1652</td>
</tr>
<tr>
<td>HCT %</td>
<td>44.100±3.9632</td>
<td>43.500±5.4705</td>
<td>46.775±12.6803</td>
</tr>
<tr>
<td>MCV fl</td>
<td>50.800±11.6662</td>
<td>57.000±1.1136</td>
<td>50.950±17.0375</td>
</tr>
<tr>
<td>MCH pg</td>
<td>19.375±0.9465</td>
<td>19.325±0.4349</td>
<td>21.125±2.9421</td>
</tr>
<tr>
<td>MCHC %</td>
<td>32.675±1.6978</td>
<td>33.925±0.8421</td>
<td>34.175±2.3902</td>
</tr>
<tr>
<td>RDW %</td>
<td>17.175±1.2420</td>
<td>16.000±0.9695</td>
<td>15.900±0.9201</td>
</tr>
<tr>
<td>PLT *10(^3)/mm(^3)</td>
<td>727.75±133.752</td>
<td>625.75±68.417</td>
<td>574.00±174.921</td>
</tr>
<tr>
<td>Neutrophils %</td>
<td>13.75±1.258</td>
<td>11.25±6.131</td>
<td>10.75±9.032</td>
</tr>
<tr>
<td>Lymphocytes %</td>
<td>76.00±2.944</td>
<td>74.75±7.848</td>
<td>81.50±8.347</td>
</tr>
<tr>
<td>Monocytes %</td>
<td>6.75±0.957</td>
<td>11.25±5.852</td>
<td>5.75±2.500</td>
</tr>
</tbody>
</table>

### Table 3. Effects of carob extracts on kidney and liver functions in rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>Carob pods group</th>
<th>Carob leaves group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea mg/dl</td>
<td>22.00±4.546</td>
<td>26.00±3.916</td>
<td>21.00±1.414</td>
</tr>
<tr>
<td>Creatinin mg/dl</td>
<td>0.6325±0.06602</td>
<td>0.5775±0.10905</td>
<td>0.5900±0.05354</td>
</tr>
<tr>
<td>ALT u/l</td>
<td>33.25±5.737</td>
<td>34.25±7.411</td>
<td>36.50±8.963</td>
</tr>
</tbody>
</table>

Results are expressed as Mean±SD of six rats. After 14 days of administration of carob pods or leaves extract neither carob pods nor carob leaves extract showed toxic effects on kidney or liver functions in comparison to the negative control group, p>0.05.

Previous studies have shown that hematological parameters were very sensitive and could be used as reliable indicators for determining the intrusion of toxic substances.\textsuperscript{24,25} Since liver and kidneys are very crucial organs for metabolism and excretion, respectively,\textsuperscript{24} it is very important to evaluate the toxicity of any new drug on these two vital organs, and this can be verified by several biochemical assays.\textsuperscript{27} Previous studies show that cytotoxicity of carob extract against cancer and normal cells is concentration dependent, although different doses were highly toxic against cancer cells and less toxic against normal cells.\textsuperscript{28,29}

### 4.2. Chromosomal aberrations test

Results of this study showed that cyclophosphamide can potentially induce chromosomal aberrations in the bone marrow cells of Wistar albino rats. However, rats treated with cyclophosphamide along with carob pods exhibited significant decrease in chromosomal aberrations compared to cyclophosphamide-treated rats (p<0.05). The means of aberrations, rings and normal cells in 50 counted cells are presented in table 4.
Carob pods + Cyclophosphamide

Cyclophosphamide

Treatment

genotoxicity validated include either an analysis of chromosome number, ring shape, chromatid deletions and chromosomal fragmentation) (Fig.1) than those taken carob pods with CP.

Moreover, other phenolic compounds (Caffeic acid, ferulic acid, gallic acid, and protocatechuic acid) showed activity against various types of cancer cells such as breast, gastric and lung cancer cells.33,34 Polyphenols exhibit many biologically significant functions such as protection against oxidative stress and degenerative diseases. Experimental data indicate that most of these biological actions can be attributed to their intrinsic antioxidant capabilities. Dietary polyphenols may offer an indirect protection by activating endogenous defense systems and by modulating cellular signaling processes such as NF-κB activation, AP-1 DNA binding, glutathione biosynthesis, PI3-kinase/Akt pathway, MAPK proteins (ERK, JNK and P38) activation and the translocation into the nucleus of Nrf2.34 Previous studies show that chromosomal aberrations, induced by alkylating agents, are a contributing factor to malignant transformation and it has been well established that the carcinogenic activity of alkylating agents correlates with their efficacy in inducing several mutations.35 There are several in vitro and in vivo genotoxicity tests which can detect substances that may induce genetic damage directly or indirectly by various mechanisms. Since, there is no one test which is able to detect all mutagenic agents, the usual strategy is to carry out a set of tests.37,38 The few in vivo assays that have been validated include either an analysis of chromosome aberrations in bone marrow cells or micronuclei in bone marrow or peripheral blood erythrocytes.37 Studies show that cyclophosphamide exerted a micronucleus response in rat bone marrow after single i.p. treatment because of its strong alkylating activity that interferes with DNA replication in cancer cells and normal cells.39 Another mechanism of cyclophosphamide-induced genotoxicity is its association with the induction of increased intracellular reactive oxygen species (ROS) formation and depletion of antioxidants in cells and plasma.40

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal cells &amp; Percentage (%) within type</th>
<th>Aberration cells &amp; Percentage (%)</th>
<th>ring cells &amp; Percentage (%)</th>
<th>Total &amp; Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide</td>
<td>12 (24.0%)</td>
<td>38 (76.0%)</td>
<td>1.74 ± 1.74</td>
<td>50 (100.0%)</td>
</tr>
<tr>
<td>Carob pods + Cyclophosphamide</td>
<td>24 (48.0%)</td>
<td>26 (52.0%)</td>
<td>0.24 ± 0.51</td>
<td>50 (100.0%)</td>
</tr>
</tbody>
</table>

Table 4 showed chromosomal aberrations in the bone marrow cells of albino rats. The number of aberrations and ring cells in the carob pods+cyclophosphamide group was less than those of cyclophosphamide group with statistical difference. Group treated with Cyclophosphamide alone was three times more likely to develop chromosomal aberrations (mitotic chromosomal number, ring shape, chromatid deletions and chromosomal fragmentation) (Fig.1) than those taken carob pods with CP.

These results indicated that pre-treatment with pods extract can effectively prevent the incidence of aberrant cells with multiple aberrations induced by CP. This could be due to the effectiveness of many phytochemicals in extract, such as caffeic acid and chlorogenic acid which have antioxidant activity and can inhibit the formation of mutagenic and carcinogenic N-nitroso derivatives in vitro.33,34 Moreover, other phenolic compounds (Caffeic acid, ferulic acid, gallic acid, and protocatechuic acid) showed activity against various types of cancer cells such as breast, gastric and lung cancer cells.33,34 Polyphenols exhibit many biologically significant functions such as protection against oxidative stress and degenerative diseases. Experimental data indicate that most of these biological actions can be attributed to their intrinsic antioxidant capabilities. Dietary polyphenols may offer an indirect protection by activating endogenous defense systems and by modulating cellular signaling processes such as NF-κB activation, AP-1 DNA binding, glutathione biosynthesis, PI3-kinase/Akt pathway, MAPK proteins (ERK, JNK and P38) activation and the translocation into the nucleus of Nrf2.34 Previous studies show that chromosomal aberrations, induced by alkylating agents, are a contributing factor to malignant transformation and it has been well established that the carcinogenic activity of alkylating agents correlates with their efficacy in inducing several mutations.35 There are several in vitro and in vivo genotoxicity tests which can detect substances that may induce genetic damage directly or indirectly by various mechanisms. Since, there is no one test which is able to detect all mutagenic agents, the usual strategy is to carry out a set of tests.37,38 The few in vivo assays that have been validated include either an analysis of chromosome aberrations in bone marrow cells or micronuclei in bone marrow or peripheral blood erythrocytes.37 Studies show that cyclophosphamide exerted a micronucleus response in rat bone marrow after single i.p. treatment because of its strong alkylating activity that interferes with DNA replication in cancer cells and normal cells.39 Another mechanism of cyclophosphamide-induced genotoxicity is its association with the induction of increased intracellular reactive oxygen species (ROS) formation and depletion of antioxidants in cells and plasma.40

4.3. Bone marrow micronucleus assay

The bone marrow micronucleus test is one of the most important genotoxicity tests to verify the safety of many chemicals.41,42 In a previous study, the effectiveness of carob pods in reducing cyclophosphamide-induced genotoxicity was studied using micronucleus assay in rats and the results demonstrate significant reduction in micronucleus formation in pod extract group.18 In this study the effect of carob leaves extract on the micronuclei in immature erythrocytes was studied using micronucleus assay in rats and the results demonstrate significant reduction in micronucleus formation in pod extract group.18 In this study the effect of carob leaves extract on the micronuclei in immature erythrocytes was studied. The number of micronuclei (MNs) in 1000 immature erythrocytes (Fig.2) was 185.6±48.83 in the CP group and 66.8±21.33 in CP+carob leaves group. Statistically, there was a highly significant difference between both groups (p<0.01). The decrease in the formation of micronuclei in leaves extract group may be due to the antioxidant properties of the leaves and their ability to reduce the formation of reactive oxygen species (ROS) within cells.43 Polyphenols in carob leaves can scavenge free radicals and protect from external sources of oxidation.42-46
A previous study in 1989 showed that cyclophosphamide promotes the emergence of type 1 diabetes in non-diabetic mice because of the destruction of beta cells in the pancreas by an immune mechanism. In our research, CP caused elevation of glucose levels (385 ±41.24 mg/dl), but carob leaves extract was able to reduce glucose levels to the normal range (98.75±18.51 mg/dl) without statistically significant difference with control group (88.50±14.64 mg/dl). A previous study was conducted in 2017 to investigate the importance of carob leaves in reducing blood sugar in alloxan-induced diabetes rats. It was found that leaves extract was highly effective in reducing blood glucose.

Also, the histological study of rats’ pancreas proved the effectiveness of this extract in the regeneration of pancreatic cells that were damaged as a result of the use of alloxan. This is consistent with the results of our study, which indicates that carob leaves may be a promising herb in the treatment of diabetes.

### 4.4. Comet assay

Results of comet and normal cells in 10 microscopic field for each sample are shown in table 5:

<table>
<thead>
<tr>
<th></th>
<th>Normal cells</th>
<th>Comet cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide</td>
<td>22</td>
<td>75</td>
</tr>
<tr>
<td>Cyclophosphamide + Carob pods</td>
<td>40</td>
<td>14</td>
</tr>
<tr>
<td>Carob pods</td>
<td>50</td>
<td>5</td>
</tr>
</tbody>
</table>

The number of comet cells in heparinized peripheral human blood treated with cyclophosphamide was more than that in peripheral blood treated either with carob pods extract or cyclophosphamide+carob pods extract. An increase in DNA damage was indicated by an increase in DNA migration in cyclophosphamide treated cells (Fig.3) and the percentage of comet cells was 77%. However, a significant reduction in cyclophosphamide-induced DNA damage was seen when pods extract was added and the percentage of comet cells became 25%, while the percentage of comet cells using the extract alone was 9%.

Previous comet assay study in experimental animals shows that cyclophosphamide caused DNA damage in the stomach, colon, liver, urinary bladder and bone marrow. In this study cyclophosphamide induced DNA damage in the peripheral blood cells and a significant reduction in cyclophosphamide-induced DNA damage was observed when using carob pods extract (p<0.05). The potential anti-mutagenic activity of carob pods may be due to the presence of polyphenols which have antioxidant activities, as well as polysaccharides which can stimulate the immune system. Results of one of the previous studies, that investigated whether carob supplementation could prevent...
CCl₄ toxic damages on rats liver and kidney and its efficacy on the antioxidant defense system, show that CCl₄ caused a significant increase in the AST, ALT, GGT and LDH levels in comparison to those of control rats, whereas carob supplementation caused a significant decrease in these serum marker enzymes in comparison to those of CCl₄ treated rats. Results showed also that CCl₄ caused severe damage in vital organs like liver because of the excessive generation of free radicals and carob showed protective feature and antioxidant activity.⁵⁰,⁵¹ However, this is consistent with the results of our study, which showed the importance of antioxidants in carob extracts.

5. CONCLUSION

Results of chromosomal aberration, micronucleus and comet assays showed that carob pods and leaves extracts inhibited the genotoxic effect of cyclophosphamide (CP) which might be attributable to the presence of flavonoids and other phenolic constituents. Therefore, it could be concluded that carob pods and leaves extracts were safe in acute and subacute tests and had a protective effect against CP-induced genotoxicity. Further studies will be conducted in order to verify the effect of extracts administration on reducing the toxic effects of CP before or during patients receiving this drug.

10. REFERENCES

17. Shukla Y, Arora A, Tanje P. Antimutagenic potential of curcumin on chromosomal aberrations in Wistar...


38. Hussein A, Shedeed N, Abdel-Kaleh K, Shams El-Din M. Antioxidative, antibacterial and antifungal activities...


