

Evaluation of the Possible Immuno-Protective Effect of *Nigella Sativa* Seed Oil on Cyclophosphamide- Induced Myelosuppression in Mice

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Abstract

Myelosuppression is one of the serious adverse effects of cancer chemotherapy that lead to life threatening febrile neutropenia and considered a limiting factor for successful therapy. Cyclophosphamide a widely used anticancer drugs, induces severe bone marrow suppression by damaging hematopoietic stem cells. As cancer incidence expands globally, the demand for an effective myeloprotective therapy during cancer treatment is also increasing.

Nigella sativa seed oil, a well-known plant extract that widely used for various health conditions. This study aims to evaluate the myeloprotective activity of *Nigella sativa* seed oil in cyclophosphamide-induced myelosuppression mice model. Myelosuppression induced by single intraperitoneal injection of cyclophosphamide (200 mg/kg). Animals were divided into 4 groups each with 6 mice. First group served as negative control group received only normal saline. A second group served as experimental myelosuppression model group achieved by cyclophosphamide. Additional 2 groups were mice received *Nigella sativa* seed oil (1ml/Kg/day) or (2ml/Kg/day) orally for 6 consecutive days starting day 1 with cyclophosphamide on day 3. Blood were collected from retro-orbital area on day 7 for total and differential leukocytes counts. Compared to model group, it is reported that *Nigella sativa* seed oil (1ml/Kg) significantly ($P<0.05$) increases total leukocytes count (1000 ± 73 versus 700 ± 36 cell/ μ L) and bone marrow cells viability (50.17 ± 5.36 versus $9.5\pm0.76\%$). Furthermore, increasing *Nigella sativa* seed oil dose to (2ml/Kg) resulted in further improvement, where we reported a significant ($P<0.05$) increase in total leukocytes count (1267 ± 352 versus 700 ± 36 cell/ μ L) and bone marrow cells viability (67.67 ± 5.49 versus $9.5\pm0.76\%$). The study concluded that *Nigella sativa* seed oil has a promising strong myeloprotective and immunomodulatory effects against cyclophosphamide-induced myelosuppression.

Keywords: Cyclophosphamide, *Nigella sativa* seed oil, Total leukocytes count, Bone marrow cells viability.

تقييم الوقاية المناعية المحتملة لزيت بذور حبة البركة على تثبيط نخاع العظم المحفز بواسطة عقار السايكلوفوسفاميد في الفئران

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الخلاصة

يعتبر تثبيط نخاع العظم واحداً من ابرز التأثيرات الجانبية للعلاج الكيميائي والذي يؤدي الى نقص الخلايا المتعادلة المهمد للحياة ويعتبر ذلك من اهم العوامل المحددة لنجاح هذا النوع من العلاج. ويعتبر عقار سايلكوفوسفاميد واحداً من اكثر الادوية المضادة للسرطان استخداماً والذي بدوره يحفز تثبيط نخاع العظم الحاد وذلك بواسطة تحطيم الخلايا الجذعية المكونة للدم. إن نسبة حدوث السرطان اصبحت واسعة على الصعيد العالمي لذلك فإن الطلب على العلاجات التي تحمي نخاع العظم خلال فترة العلاج الكيميائي اصبح في تزايد أيضاً. إن زيت بذور حبة البركة هو مستخلص معروف وكثير الاستخدام في حالات صحية كثيرة تهدف هذه الدراسة الى تقييم فعالية زيت بذور حبة البركة في حماية نخاع العظم على نموذج الفئران. تم تحفيز تثبيط نخاع العظم بواسطة حقن جرعة منفردة من عقار السايكلوفوسفاميد بجرعة (٢٠٠ ملغم/كغم). تم تقسيم الحيوانات الى اربعة مجاميع تحتوي كل مجموعة على ستة فئران.

اعتبرت المجموعة الاولى مجموعة السيطرة وحقنت الفئران بمحلول ملحي عادي.

المجموعة الثانية اعتبرت نموذج تثبيط نخاع العظم وحقنت بعقار السايكلوفوسفاميد. وفي المجموعتين الاضافيتين اعطيت الفئران زيت بذور حبة البركة (١ مل/كغم/يوم) او (٢ مل/كغم/يوم) عن طريق الفم لمدة ستة ايام متتابعة تبدأ من اليوم الاول وحقنت الفئران بعقار السايكلوفوسفاميد في اليوم الثالث بجرعة منفردة. في اليوم السابع تم جمع الدم من منطقة مدار محجر العين لغرض احصاء عدد خلايا الدم البيضاء. بالمقارنة مع مجموعة النموذج، اظهرت النتائج انه زيت بذور حبة البركة بجرعة ١ مل/كغم تزيد من عدد خلايا الدم البيضاء بصورة معنوية (1000 ± 73 مقارنة مع 700 ± 36 خلية/مايكروليتر) وتزيد من قابلية نمو خلايا نقي العظم (50.17 ± 5.36 مقارنة مع $9.5\pm0.76\%$). بالإضافة الى ذلك فإن زيادة جرعة زيت بذور حبة البركة الى (٢ مل/كغم) ادى الى زيادة اضافية في عدد خلايا الدم البيضاء بصورة معنوية (1267 ± 352 مقارنة مع 700 ± 36 خلية/مايكروليتر) ونمو في خلايا نقي العظم (67.67 ± 5.49 مقارنة مع $9.5\pm0.76\%$). ويمكن الاستنتاج بأنه زيت بذور حبة البركة يمكن ان يكون لديه تأثير قوي في حماية نخاع العظم وتأثيرات مناعية ضد تثبيط نخاع العظم المحفز بواسطة عقار السايكلوفوسفاميد.

الكلمات المفتاحية: السايكلوفوسفاميد، زيت بذور حبة البركة، عدد خلايا الدم البيضاء، حيوية خلايا نخاع العظم.

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Introduction

Bone marrow is the major site that responsible for hematopoiesis by hematopoietic stem cells (HSC) which are continuously proliferating cells that can be differentiated to form different mature blood cells that include red blood cells, white blood cells (granulocytes, monocytes and lymphocytes) and platelets⁽¹⁾. White blood cells (leukocytes) are major part of the immune system that protect the body and defend against pathogens and fight infections. Normal level of total leukocyte count in adult human is (4000 – 10000 cells/mm³)⁽²⁾, while in mice is (2000-10000 cells/mm³)⁽³⁾.

Myelosuppression is the most common and popular adverse effect of several chemotherapy which are the most effective therapy for cancer⁽⁴⁾. The myelotoxicity caused by cytotoxic drugs lead to dose reductions of these drugs and delays in therapy, these can compromise the outcomes of chemotherapy and reduces overall survival^(5,6). Cyclophosphamide (CP) is one of the oldest and the most successful anticancer drugs, it acts as alkylating agent that belong to oxazaphosphorines group⁽⁷⁾. The initial clinical trials of cyclophosphamide for cancer treatment were performed in 1958, and in 1959 approved by the FDA as a cytotoxic agent. In fact, it is well established that cyclophosphamide is a prodrug metabolize to generate active alkylating metabolites that include 4-hydroxy cyclophosphamide, aldophosphamide mustard, which can interfere with DNA synthesis in rapidly dividing cells and can lead to apoptosis. Cyclophosphamide has toxic effects on different organs that include bone marrow, heart, gonadal and bladder. The main cyclophosphamide toxicity is on bone marrow and bladder⁽⁸⁾, the cyclophosphamide myelotoxicity as a result of lack specificity in anti-tumour activity. When killing tumour cells, It also cause serious damage on normal cells, for instance, hematopoietic stem cells which are rapidly dividing cells in the bone marrow, this leads to decrease the ability of these cells to proliferate and differentiate with reduction in the formation of different blood cells. Myelosuppression can lead to anaemia, leukopenia, and thrombocytopenia. Leukopenia is a life-threatening condition that lead to febrile neutropenia combined with bacterial and fungal infections⁽⁹⁾.

Different plants extract found to have a protective effect against a variety of toxicity that induced by chemotherapy⁽¹⁰⁾. *Nigella sativa* is an annual herb belonging to the Ranunculaceae family. It is also known as (black cumin), the seed have been used as a seasoning spice and food additive in the Middle East and Mediterranean areas⁽¹¹⁾. *Nigella sativa* seeds contain proteins, saponins, alkaloids, fixed oil, and essential oil. The biological effects of *Nigella sativa* are attributed to the various characterized constituents. Thymoquinone (TQ), the main bioactive constituent of the essential oil may

be responsible for major therapeutic effects of *Nigella sativa*⁽¹²⁾. In addition to the seed, its cold pressed oil is utilized as natural dietary supplements and therapeutic agents to support the immune system, treat asthma, allergic rhinitis, diabetes, gastrointestinal disturbance and other conditions in the European Union and other developed countries⁽¹³⁾. *Nigella sativa* seed oil (NG oil) used to evaluate its immune-protective and immunomodulatory effect on myelosuppression induced by cyclophosphamide. The aim of this study is to evaluate the immune-protective and immunomodulatory effect of *Nigella sativa* seed oil on cyclophosphamide-induced myelosuppression in mice.

Materials and Methods

Animals

Forty two adults' albino male mice weighing (20-25) gram were brought from and maintained in the Animal House of College of Pharmacy/University of Baghdad under normal conditions of temperature, humidity and light/dark cycle. The animals were fed commercial pellets and tap water *ad libitum* throughout the experimental period. The study was approved by the Scientific and Ethical-committees of the College of Pharmacy/University of Baghdad.

Drugs

Cyclophosphamide as monohydrate (1000mg vial) was purchased from (Baxter health care Ltd, Germany).

***Nigella sativa* seed Oil 100% pure was purchased from Toroslar company (Turkey), which was prepared by cold pressing from fresh *Nigella sativa* seeds and packaged in amber glass bottle to protect it from sunlight exposure.**

Experimental protocol

Mice were randomly allocated into four groups, each containing 6 mice as follow:

Group I: Mice were received a single intraperitoneal injection of 0.15 ml 0.9 % normal saline at day 3. This group served as a negative control.

Group II: Mice were received a single dose of intraperitoneal cyclophosphamide (200mg/kg body weight) on day 3. This group served as experimental model⁽¹⁴⁾.

Group III: Mice were administered a dose of *Nigella sativa* seed oil (1ml/Kg body weight/day) orally by oral gavage for 6 consecutive days starting day 1, with a single dose of intraperitoneal Cyclophosphamide (200mg/Kg body weight) on day 3.

Group IV: mice were orally administered *Nigella sativa* seed oil at a dose of (2ml/Kg body weight/day) by oral gavage for 6 consecutive days starting day 1, with a single dose of intraperitoneal Cyclophosphamide (200mg/Kg body weight) on day 3.

In groups (III and IV) animals, each dose of *Nigella sativa* seed oil was administered once daily for 6 consecutive days starting day 1, and on day 3, they received a single dose of cyclophosphamide (200mg/Kg body weight) by IP injection. Twenty-four hours after the end of the treatment duration (day 7), the animals were euthanized by diethyl ether (BDH chemicals, England) and cervical dislocation⁽¹⁵⁾.

Samples collection

1- Blood collection

After animals have been euthanized on day 7, 0.5 ml of blood was drawn from retro-orbital area of mice eyes and collected in EDTA tube. Samples were then prepared for the analysis of total Leukocyte Count⁽¹⁶⁾.

2- Bone marrow extraction.

After mice euthanized, femur bone was extracted and used to extract bone marrow cells by flushing the marrow cavity with phosphate buffered saline (PBS) PH 7.4 and collected as cell suspension, that was used immediately for bone marrow cell viability test^(17,18).

Analysis

Estimation of total leukocytes count in blood:

Total white blood cells counts were performed on an automated hematology analyzer (XP - 300, sysmex / Japan) using direct detection method⁽¹⁹⁾.

Bone marrow cells viability test

This test was used to determine the number of viable bone marrow cells present in a cell suspension to the total cell count⁽²⁰⁾. Equal parts of 0.04% Trypan blue solution and bone marrow cell suspension were mixed to form a mixture that utilized for counting of the viable cells (clear) and nonviable cells (blue) immediately (within 3-5 minute after mixing) using Neubaur hemocytometer under light microscopy.

Viable bone marrow cells calculated as percentage of total cells. Calculation performed by the following equation:

$$\text{Viable cells (\%)} = [\text{number of viable cells} / \text{total number of cells}] * 100$$

Statistical analysis

The numeric data presented in the study expressed as mean±standard error of the mean (SE). All statistical analyses were carried out using the Statistical Package of Social Science (SPSS) software version 25. Intergroup comparisons were made using nonparametric tests (Kruskal- Wallis H test, Mann-Whitney U test). Differences were considered significant at $P < 0.05$ ⁽²¹⁾.

Results

Effect of two doses of *Nigella sativa* seed oil on total leukocyte count in cyclophosphamide - induced myelosuppression.

The data presented in (table 1) and (figure 1) showed that administration of a single dose of intraperitoneal cyclophosphamide (200mg/kg) to mice on day 3 (group II, experimental model) resulted in the significant ($P < 0.05$) reduction of total leukocyte count (700 ± 36) compared to the negative control animals (group I, 5800 ± 520), that lead to the severe suppression of bone marrow function. However, in group III mice, which administrated *Nigella sativa* seed oil at a dose of (1ml/Kg/day) 3 days prior to a single intraperitoneal dose of cyclophosphamide (200mg/kg) on day 3 resulted in significant attenuation of the cyclophosphamide myelosuppressing effect (table1) (figure 1), the total leukocyte count in those animals (group III) increased (1000 ± 73) significantly ($P < 0.05$) compared to animals that received cyclophosphamide only (group II, experimental model). Furthermore, increasing the dose of *Nigella sativa* seed oil to 2ml/Kg/day administered 3 days before cyclophosphamide treatment resulted in further improvement of bone marrow function (table1) (figure 1). Data showed that animals received (2 ml/Kg/day) of *Nigella sativa* seed oil (group IV) resulted in significant rise ($P < 0.05$) in total leukocyte count (1267 ± 352) compared to the experimental model group (group II).

Effect of two doses of *Nigella sativa* seed oil on bone marrow cells viability in cyclophosphamide-induced myelosuppression.

The data presented in table 1 and figure 2 showed that administration of single intraperitoneal dose of cyclophosphamide (200mg/Kg) causes aggressive suppression of bone marrow function manifested as highly significant reduction ($P < 0.05$) in bone marrow cells viability (9.5 ± 0.76) in the experimental model (group II) compared to the negative control animals (92 ± 0.58) (group I). However, in group III mice received *Nigella sativa* seed oil at dose of (1ml/Kg/day) 3 days prior to (200mg/Kg) cyclophosphamide therapy resulted in significant ($P < 0.05$) increase in bone marrow cell viability (50.17 ± 5.36) compared to model group (group II) (Table 1) (Figure 2). furthermore, Increasing the dose of *Nigella sativa* seed oil to (2ml/Kg/day) administered 3 days before cyclophosphamide treatment (group IV) resulted in further increase in bone marrow cells viability (67.67 ± 5.49) and the results appear a significant increase in the viability of bone marrow cells ($P < 0.05$) (group IV) compared to the experimental model group (group II) (Table 1) (Figure2).

Table 1. Effects of *Nigella sativa* seed oil on total leukocytes count and bone marrow cells viability in cyclophosphamide-induced myelosuppression in mice.

Groups	Type of treatment	Total leukocyte count (cell/ μ L) (Mean \pm S.E.M)	Bone marrow Cell viability (%) (Mean \pm S.E.M)
Group I (negative control)	Normal saline	5800 \pm 520 *	92 \pm 0.58 *
Group II (experimental model)	Cyclophosphamide (200mg/Kg)	700 \pm 36	9.5 \pm 0.76
Group III	Cyclophosphamide (200mg/Kg) + <i>Nigella sativa</i> seed oil (1ml/Kg)	1000 \pm 73 *	50.17 \pm 5.36 *
Group IV	Cyclophosphamide (200mg/Kg) + <i>Nigella sativa</i> seed oil (2ml/Kg)	1267 \pm 352 *	67.67 \pm 5.49 *

Data are expressed as mean \pm standard error of means (SEM).

* Significantly different ($P < 0.05$) with respect to the experimental model group.

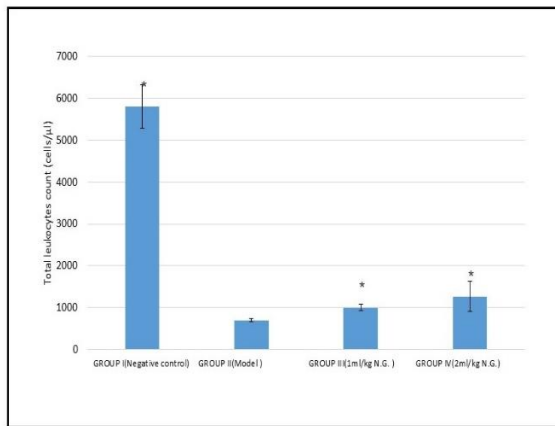


Figure 1. Bar chart showing total leukocytes count in various experimental mice groups.

* Significantly different ($P < 0.05$) with respect to the model group.

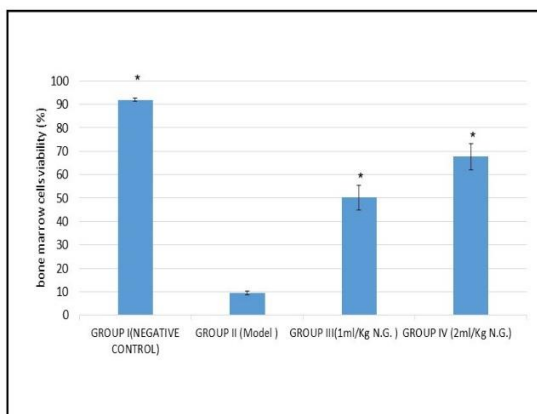


Figure 2. Bar chart showing bone marrow cells viability test in various experimental mice groups.

* Significantly different ($P < 0.05$) with respect to the model group.

Discussion

Myelosuppression is commonly seen with chemotherapy through interfering with normal cell production in bone marrow leading to myelotoxicity that manifested as a reduction in total white blood cells causing a serious life threatening leukopenia combined with secondary infections⁽²²⁾, so the clinical outcome of treatments with chemotherapy is severely limited, owing to their toxicity to normal tissues. Therefore, there is a necessity to discover adjuvant therapy which may be used in conjunction with anticancer drugs to reduce their associated toxic adverse effect and improve the efficacy of the therapy⁽²³⁾.

The main dose-limiting toxicities for cyclophosphamide are febrile neutropenia combined with secondary infections, often result in dose reductions, therapy delays and lowers overall survival⁽²⁴⁾.

The current study evaluates the immunoprotective effect of *Nigella sativa* seed oil on cyclophosphamide-induced myelosuppression. The results obtained from this study revealed a beneficial modulating effect on peripheral total leukocytes count and bone marrow cells viability (table 1).

The present study revealed that cyclophosphamide induced myelosuppression, which was evident by the high reduction of total leukocytes count and bone marrow cells viability in animals treated with cyclophosphamide only compared to the negative control animals (Table 1) (Figure 1 and 2) that resulted in aggressive suppression of bone marrow function. The most obviously change in white blood cells count due to their short life cycles; the results are in agreement with studies of other researchers, who observed the myelosuppressive effect of cyclophosphamide administration to animals manifested as reduction of the total leukocyte count after 3-4 days from last cyclophosphamide dosing^(25, 14). This experimental

model of cyclophosphamide-induced myelosuppression is a well validated and widely used animal model to study the effects of chemotherapy-induced myelosuppression in terms of pathogenesis and therapy^(18,26). This study was in line with study of others in cyclophosphamide induced bone marrow suppression by decreasing bone marrow cells numbers in animals treated with cyclophosphamide⁽²⁷⁾. Data obtained from this study revealed an interesting dose-dependent myeloprotective and immunostimulatory effects of NG oil on bone marrow function. This beneficial effect obtained from the reported increase in total leukocyte count and bone marrow cell viability (table 1), which imply an improvement in bone marrow function after chemotherapy-induced myelosuppression. This is an important result and may open the way for a therapeutic application of NG oil.

This is the first study that investigates the effect of NG oil on bone marrow function in chemotherapy-induced myelosuppression and there are little studies in this regard. However, NG oil used in other studied showed immunostimulatory actions in experiments with different settings. A study showed increase in the total white blood cells, lymphocyte count and neutrophil count in mice treated with NG oil⁽²⁸⁾. In addition, other study showed that NG oil has an immunostimulatory effect on *Trypanosoma brucei* infected animals and revealed a significant rise in total white blood cells of infected animals⁽²⁹⁾. Another study revealed the immune-potential effect of NG oil in immunocompromised animals by stimulation of macrophage phagocytic activity either directly or via lymphocytes activation⁽³⁰⁾. Furthermore, the immuno-protective effect of NG oil was observed in gamma-irradiation induced immunosuppression in animals, which considerably normalized leukopenia and produced significant regeneration in spleen and thymus lymphoid follicles⁽³¹⁾. The study showed the effect of thymoquinone, the major constituent of NG oil, resulted in increase of the total count of white blood cells in healthy animals which are received specific dose from thymoquinone⁽³²⁾, specific concentration of thymoquinone may regulate self-renewal and immunomodulatory potential of mice bone marrow mesenchymal stem cells *in vitro*⁽³³⁾. Another study revealed the treatment of animals with NG extract revealed a significant enhancement in the bone marrow cellularity, total leukocyte count and spleen weight compared to normal control animals⁽³⁴⁾. All the above mentioned studies and results are consistent with the results obtained from this study although each study has its own experimental settings.

On the other hand, some studies showed results in contrast to the results of the current study. In this regard, a study in which type 2 diabetes mellitus patients who received NG oil for 40 days,

total leukocyte count remain statistically unchanged⁽³⁵⁾. Additional study observed a decrease in leukocyte count after chronic treatment of healthy animals with NG oil for 12 weeks⁽³⁶⁾. The difference in the results of these two studies may be attributed to the longer duration of treatment with NG oil if compared with current study.

Nigella sativa seed oil pre-treatment was able to protect bone marrow, since it improves bone marrow cells viability and peripheral leukocyte count in cyclophosphamide induced myelosuppression.

Conclusions

According to the results obtained from this study, it could be concluded that the *Nigella sativa* seed oil has a dose-dependent myeloprotective effect on cyclophosphamide-induced myelosuppression in terms of total leukocyte count and cell viability tests. Therefore, *Nigella sativa* seed oil may have a potential therapeutic value against chemotherapy-induced myelosuppression.

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