

EVALUATING THE ABILITY OF *PLEUROTUS OSTREATUS* AQUEOUS EXTRACT TO MODULATE GENOTOXICITY INDUCED BY CYCLOPHOSPHAMIDE IN MICE BONE MARROW CELLS

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ABSTRACT

This study was aimed to evaluate the ability of local oyster mushroom (*Pleurotus ostreatus*; ID:MF065715.1; cultivated until maturation in growth medium containing cumin extract) to modulate the genotoxicity induced by cyclophosphamid (CP) in bone marrow cells of mice. *In vivo* genotoxicity was assessed by quantifying the incidence of micronucleated polychromatic erythrocytes in the bone marrow cells of mice that were administered different doses of *P.ostreatus* extract (150, 200,250, and 300 mg/kg/day) respectively, individually, and in combination with CP (40 mg/kg body weight) according to the following three experimental protocols (pre 2h, post 2h, and concomitant treatment for 14 days , respectively). Analysis and microscopic examination of micronuclei (MN) revealed no mutagenic effect of *P.ostreatus* extract alone at all the doses evaluated. By contrast, CP administration significantly increased ($P<0.05$) incidence of MN. Importantly, co-administration of *P.ostreatus* extract with CP caused a significant and dose-dependent reduction in MN induced by CP in the murine bone marrow cells. These data suggest that *P.ostreatus* extract administration has a protective effect against genotoxic damage inflicted by CP. The dietary cumin may serve as a scavenger for free radicals generated by CP and may augment the antioxidant activity of *P. ostreatus* extract. These findings open up new avenues for the use of oyster mushroom in many applications, including pharmacological preparations and food supplements.

Key words: Iraqi strain, ID: MF065715.1, wild mushroom, micronucleus assay, *cuminum cyminum*

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تقييم قدرة المستخلص المائي للفطر المحاري *Pleurotus ostreatus* في تعديل السمية الوراثية الناجمة عن عقار سيكلوفوسفاميد في خلايا نخاع عظام الفئران

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وزارة العلوم والتكنولوجيا

العراق - بغداد

المستخلص

تهدف الدراسة لتقييم المستخلص المائي للفطر المحاري المحلي (*Pleurotus ostreatus* ID: MF065715.1) والذي تم تغذيته بالمستخلص المائي للكمون) ضد السمية الوراثية للعقار الدوائي سيكلوفوسفاميد (CP) في خلايا نخاع عظام الفئران. من خلال اختبار النوى الدقيقة (MN) ، حيث تم إعطاء الفئران جرعات مختلفة من المستخلص المائي للفطر (*Pleurotus ostreatus* 150 , 200, 250, 300 mg / kg / يوم) بالتتابع ، مع عقار CP (40 mg / kg من وزن الجسم) عند ثلاث اوقات مختلفة (قبل ساعتين ، بعد ساعتين ، والمعالجة المتزامنة لمدة 14 يوماً متتالياً). كشف تحليل وفحص النوى الدقيقة (MN) أنه لا يوجد أي تأثير مفرّ للمستخلص المائي للفطر المحاري عند كل الجرعات قيد الفحص. بالمقابل عمل CP على زيادة كبيرة ($P > 0.05$) في MN كما أظهرت النتائج أن الفطر *P.ostreatus* دور كبير في خفض MN الناجم عن CP في خلايا نخاع عظم الفئران بالاعتماد على نوع الجرعة. وبناء على البيانات التي تم التوصل اليها اقترحت الدراسة الحالية أن للفطر *P. ostreatus* دور وقائي ضد الأضرار التي يسببها العقار CP ، في حين اظهر أستعمال الكمون في تغذية *P. ostreatus* دور فعال في زيادة نشاط مضادات الأكسدة في الفطر المحارى ، مما يرفع من قدرته على التخلص من الاثار السلبية للعقار CP وبالتالي يفتح مجال اوسع لاستعمال فطر المحارى الناتج من التغذية بالمستخلص المائي للكمون في العديد من التطبيقات، على سبيل المثال، المستحضرات الصيدلانية والمكملات الغذائية.

الكلمات الافتتاحية: السلالة العراقي للفطر، الفطر البري، ID: MF065715.1، فحص النواة الصغيرى، الكمون .

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INTRODUCTION

Cancer is one of the leading causes of death worldwide. Commonly-used anti-neoplastic treatments either prevent the proliferation of or kill tumor cells. Cyclophosphamide (CP), the most frequently used anti-cancer chemotherapeutic agent, is an alkylating agent used to treat a wide range of malignancies. CP's cytotoxicity results from its chemically reactive metabolites that alkylate DNA and proteins, and thereby generate cross-links (3). Damage to healthy somatic tissues and immunosuppression are the common side-effects that limit the use of CP (17). The creation of free radicals and other reactive oxygen species, in addition to lipid peroxidation, have been identified as the main mechanisms of CP-induced toxicity (19). CP is generally used as a genotoxic agent since CP and its metabolites can bind to and damage DNA, leading to chromosome breaks, micronuclei (MN) formation, and finally, cell death (14). Thus, co-administration of antioxidants and free radical scavengers may mitigate some of the adverse side-effects associated with CP treatment (22). Herbal antioxidants containing high concentrations of phenolic and flavonoid compounds, have been shown to have a strong chemo-protective influence against CP-induced DNA damage and oxidative stress in the bone marrow cells of mice (5,27). Numerous studies have demonstrated that high concentrations of antioxidants can alleviate oxidative stress that causes cellular damage, leading to the generation of malignant cells or accelerating aging (30). Oxidative stress stems from an imbalance between the production of free radicals in our bodies (due to a range of factors such as radiation exposure, food intake, and inhalation of certain substances from our environments), and the inability of our bodies to adequately counteract their damaging effects (6). One of the chemo-preventive strategies that can be readily incorporated into our daily lives to avert cancer is the utilization of natural compounds such as bioactive botanicals, food constituents, or functional foods (5). Mushrooms form an essential part of the normal human diet; they are a good source of a lot of minerals, digestible proteins, vitamins, and polysaccharides (12)

.Mushrooms are a good source of several interesting biologically effective compounds that provide medical or health benefits such as disease prevention and treatment (12). Bioactive compounds of the mushroom have been found in cell wall polysaccharides (e.g., β -glucans), among proteins, or the minor secondary metabolites including phenolic compounds, steroids, and terpenes. The efficiency and concentration of bioactive compounds differ among different mushroom types, depending upon conditions of fruiting (if cultivated), substrate, development phase/age, storage conditions, cooking procedures, and age of the fresh mushroom (10). Oyster mushrooms comprise the most commonly consumed *Pleurotus* species, that are often described as oyster mushroom king. Oyster mushroom possess several biological functions due to the unique bioactive compounds they harbor, such as peptides, lipids, monoterpenes, polysaccharides, sterols, dietary fiber, and antioxidant components such as ascorbic acid, β -carotene, and α -tocopherol (20). These bioactive compounds have been reported to diminish the incidence of a variety of human diseases including cancers, inflammatory diseases, atherosclerosis, diabetes, and liver damage (2). Numerous studies have reported the medicinal properties of mushrooms, such as their anti-carcinogenic, anti-inflammatory, anti-viral, anti-bacterial, hypo-glycemic, anti-fungal, hepatoprotective, anti-neurodegenerative, anti-diabetic, anti-angiogenic, and antioxidant properties (1,4). The objective of this study was to evaluate the ability of *pleurotus ostreatus* extract to modulate genotoxicity induced by cyclophosphamide in mice bone marrow cells.

MATERIALS AND METHODS

Direct biochemical analysis of *P.ostreatus* mushroom

The nutritional components, including protein, over all fat, dietary fiber, and total phenolic content (TPC), freeze-dried powder of *P. ostreatus* (ID:MF065715.1) were analyzed using the "American Oil Chemists' Society (AOCS)" standards (8). The TPC, stated as gallic acid equivalents (GAEs) was calculated according to Folin-Ciocalteu method (19).

Laboratory animals: 108 mice were obtained from the National Center for Drug Control and

Research, Ministry of Health, Baghdad. Their weight ranged from 25 ± 2 g. They were assigned into six separate groups (as described in the “Experimental Design” section) under laboratory conditions. These mice were housed in polypropylene cages and were provided food in the form of commercial mouse food pellets, and water ad libitum throughout the experiment

Cyclophosphamide treatment

Cyclophosphamide (Endoxan; 500 mg) was intra-peritoneal injection into mice at a dose equivalent to 40 mg/kg defined as a genotoxic dose for mouse bone marrow cells (4).

Preparation of Cuminum cyminum extract

The cumin extract was prepared by mixing 1 kg of cumin seeds with 4 liters of distilled water in a glass beaker and then placed in ultrasound for 60 minutes at 40°C . The aqueous extract was filtered then concentrated by using a rotary evaporator. A concentration of 20% was attained and was used in the treatment of mushroom medium

Preparation of *P. ostreatus* extract

The *P. ostreatus* mushroom (ID: MF065715.1) was cultivated in growth medium that contained cumin extract at (20%) concentration for 14 days, until mushroom maturation was complete (28). Whole mature mushrooms were dried and then powdered. The (5 g) powder was extracted by stirring with 90 mL of ethanol (96%) at 30°C for a time of 72 h. The final extracts were centrifuged at 20°C at 3,000 rpm for 15 min, the supernatant was filtered and concentrated in a rotary evaporator (BÜCHI R-114, Switzerland) at 40°C to dryness, and re-dissolved in water for antigenotoxic assay at starting concentration of 20.0 mg/mL (9).

Experimental design

The animals were randomly assigned to 6 groups (G):

G1: Negative control (6 mice) were given phosphate-buffered saline (PBS).

G2: Positive control (6 mice) were treated with 40 $\mu\text{g}/\text{kg}$ CP for 24 h. The animals were euthanized 2 h after the treatment.

G3: Mice (6 mice per dose; 24 mice total) were treated with four doses of *P. ostreatus* extract (150, 200, 250, and 300 mg/kg) delivered orally for 14 days. The animals were euthanized 2 h after the treatment.

G4- Pre-CP treatment : Mice (6 mice per mushroom extract dose; 24 mice total) were treated with four doses of *P. ostreatus* extract (150, 200, 250, and 300 mg/kg) delivered orally for 14 days. The animals were euthanized 2 h after the treatment.

G5- post-CP treatment : Mice (6 mice per mushroom extract dose; 24 mice total) were given CP (40 mg/kg) once daily orally, followed by four doses of *P. ostreatus* extract (150, 200, 250, and 300 mg/kg), delivered orally daily for 14 days. The animals were euthanized 2 h after the treatment.

G6- co-treatment with CP : Mice (6 mice per mushroom extract dose; 24 mice total) were given CP (40 mg/kg) once daily orally, simultaneously with four doses of *P. ostreatus* extract (150, 200, 250, and 300 mg/kg), delivered orally daily for 14 days. The animals were euthanized 2 h after the treatment.

Micronucleus (MN) test

The right and left femurs of the mice were dissected and fetal calf serum was used to flush out the bone marrow from the femoral cavity. Bone marrow cells were dispersed by gentle pipetting and pelleted through centrifugation at 1,000 rpm for 10 min. The cell pellet was re-suspended in a small volume of fetal calf serum that allowed cell smears to be prepared on microscope slides. The smears of cells were stained with Giemsa after complete air-drying, while, 2,000 polychromatic erythrocytes (PCEs) from every animal were inspected to quantify micronucleated polychromatic erythrocytes (MN-PCEs) under $1,000\times$ magnifications using the light microscope. Furthermore, toxicity to bone marrow was evaluated using the PCE/ normochromatic erythrocyte (NCE) ratio in 500 erythrocytes from each animal (11). The amount of antimutagen effects was measured according to (15) as follows:

$$\text{Antimutagens \%} = \frac{\text{Positive control} - \text{Treatment Groups}}{\text{Positive control}}$$

Statistical analysis

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Least significant difference –LSD test (ANOVA) was used to significant compare between means in this study.

RESULTS AND DISCUSSION

Cumin seeds is using as food for oyster mushrooms due the role it plays in improving the nutritional and treasury properties ,Cumin seeds are nutritionally rich; they provide high amounts of fat (especially monounsaturated fat), protein, and dietary fibre. Vitamins B and E and several dietary minerals, especially iron, are also considerable in cumin seeds Table1.The literature presents ample evidence for the biological and biomedical activities of cumin, which have generally been ascribed to its bioactive constituents such as terpenes, phenols, and flavonoids (16).

Table 1. Biochemical composition of 100 g of seed *Cuminum cyminum L.*

Parameter	seed Cuminum
Carbohydrate	44.24 g
Protein	17.81 g
Dietary fiber	10.5 g
fat	22.27 g
water	8.06
Thiamine	0.628 mg
Riboflavin	0.327 mg
Niacin	4.571 mg
Iron	66.36 mg
Mg	366 mg
potassium	1788 mg
sodium	168 mg
zinc	4.8 mg
Vitamin B6	0.435 mg
Vitamin C	7.7 mg
Vitamin E	3.33 mg

The nutritional components of *P. ostreatus* (ID:MF065715.1), including the carbohydrate, dietary fiber, total polysaccharides content, and TPC are shown in Table 2. The results in Table 3 shows the results of MN assay in the bone marrow cells of mice from our various experimental groups. the results showed significant differences ($P>0.05$) between groups that were treated with *P. ostreatus* extract alone and positive controls, on the other hand, We found no significant difference ($P>0.05$) between groups that were treated with *P. ostreatus* extract alone and our negative controls , suggesting that *P. ostreatus* extract does not cause significant genotoxicity on its own.

Table 2. Biochemical composition of oyster mushroom *P. ostreatus*

Parameter	<i>P. ostreatus</i>
Carbohydrate (mg/g)	38.20
Protein (mg/g)	30.60
Dietary fiber (g/kg)	333.5
Total fat (g/kg)	37.0
Total phenolic content (mg gallic acid /g)	21.12
Total polysaccharides (%)	14.11

Table 3. Incidence of micronuclei (% MN)%in the cells of the bone marrow of mice that treated with different doses of *P. ostreatus* extract

Experimental groups	Dose (mg/kg)	Total number of polychromatic erythrocytes evaluated	Micronuclei% (MN)
Negative control	0.00	1,000	0.85 a
Positive control (CP)	40	1,000	7.15 b
	150	1,000	2.04 c
Aqueous extracts of oyster mushroom <i>P. ostreatus</i>	200	1,000	1.34 c
	250	1,000	0.86 a
	300	1,000	0.82 a

Differences a, b, c, d are significant ($P<0.05$) to comparison with positive control

Table 4 shows the MN percentage in the bone marrow cells of mice treated with different doses of the *P. ostreatus* extract (150, 200,250, and 300 mg/kg) and one dose of CP. There

was a significant increase in the frequency of MN in the mice treated with CP in comparison to the negative control group ($P<0.05$) Figure1

Table 4. Incidence of MN in the cells of the bone marrow of mice treated with different doses of aqueous extracts of oyster mushroom, *P. ostreatus* (150, 200, 250 and 300 $\mu\text{g}/\text{kg}$)

Treatment Groups	Dose (mg/kg)	Total number of polychromatic erythrocytes	Total number. of MNs %	The amount of antimutagens effects
Negative control	0.0	1,000	0.76 a	-
Positive control treated with CP only	40	1,000	7.14 b	-
Pre-aqueous extracts of oyster mushroom, <i>P. ostreatus</i>	150	1,000	3.25 c	54.48
	200	1,000	2.02 c	71.70
	250	1,000	1.12 a	84.31
	300	1,000	0.68 a	90.47
Co-aqueous extracts of oyster mushroom, <i>P. ostreatus</i>	150	1,000	4.12 c	42.29
	200	1,000	3.45 c	51.68
	250	1,000	1.25 a	82.49
	300	1,000	0.93 a	86.61
Post-aqueous extracts oyster mushroom, <i>P. ostreatus</i>	150	1,000	5.56 c	22.12
	200	1,000	4.42 c	38
	250	1,000	3.75 c	47.47
	300	1,000	2.48 c	65.26

Differences a, b, c, d are significant ($P < 0.05$) to comparison row

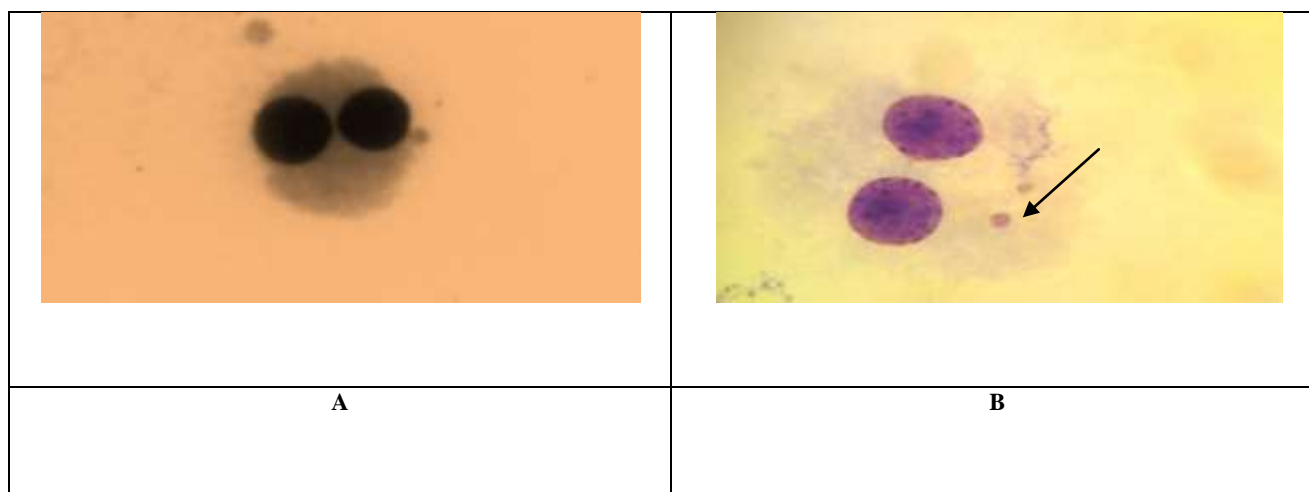


Figure 1. Micrographs images display the cells of bone marrow in treated male mice. A: “Normal” polychromatic erythrocytes; B: polychromatic erythrocytes with “micronucleus” examined at 1,000 \times .

Importantly treatment with *P. ostreatus* extract resulted in significant reduction ($P < 0.05$) in MN % with all the doses of the extract evaluated, compared with the MN % observed upon treatment with only CP ($P < 0.05$). Furthermore, there was no significant difference between the MN % observed with the *P. ostreatus* extract +CP in combination regimens, and the negative controls. These data strongly suggest that *P. ostreatus* extract mitigates the *in vivo* genotoxicity induced by CP. Chromosomal damage upon exposure to CP was the main cause underlying the appearance of MN in the bone marrow cells of mice treated with CP and this phenotype provided a robust readout for genotoxic insult to the nuclei (24). The current study aimed to evaluate the activity of *P. ostreatus* cultivated in growth medium (containing cumin extract), to alleviate the genotoxic side-effects of CP treatment. The anti-neoplastic drug CP is generally used as a chemotherapeutic agent for several autoimmune disorders and neoplastic diseases (25). Unfortunately, treatment with CP was found to encourage the incidence of secondary tumors that were treatment-related in human cancer survivors due to the drug's genotoxic effects on healthy somatic cells. Moreover, the toxic side-effects of CP were related to its metabolite acrolein (19). These adverse side-effects may have resulted from oxidative stress that may have diminished the antioxidant activities of key enzymes and caused a strong increase in free radicals and lipid peroxidation (30). The failure of cellular antioxidant defenses concomitant with a surge in oxidative stress due to ROS overproduction has been found to induce the formation of bone marrow MN and chromosomal breakage (5); therefore, co-administration of antioxidants and free radical scavengers may help reduce treatment-associated genotoxicity. More recently, anti-cancer treatment regimens involving CP are increasingly incorporating other protective and detoxifying agents (5). In this study, a new local strain of the oyster mushroom *P. ostreatus* that was cultivated in growth medium containing cumin extract was used and the ability of *P. ostreatus* extract to modulate the genotoxic effects of CP on murine bone marrow cells was evaluated. The obtained data showing that even the highest

dose of *P. ostreatus* extract did not cause any significant genotoxicity compared to the negative control group (Table 3) indicated that the *P. ostreatus* extract was neither mutagenic nor clastogenic; thus, the *P. ostreatus* extract can potentially be used as a protective agent for oxidative stress without any side-effects. Furthermore, administration of the *P. ostreatus* extract at doses of 150, 200, 250, and 300 mg/kg/day for 14 days prevented the formation of MN in the bone marrow cells of CP-treated mice in a dose-dependent manner, thereby revealing the anti-clastogenic effects of the mushroom extract as well as its ability to protect cells from the genotoxic effects of CP. Previous studies have noted that chemical composition of the same mushroom can differ between lineages (26). Hence, the conditions of growing, harvesting, preparing, and storing also have their influence on the composition and the mushroom's biological activity (17). Therefore, the cumin (*Cuminum cyminum* L.) extract was used to enhance the mushroom's antioxidant activity. Many researchers recorded that cumin which has a high phenolic content and an accepted antioxidant activity that can be used as a supplement for both nutritional purposes as well as for preservation of foods (9). Cumin's antioxidant effects may be associated with the presence of phenolic compounds, anthocyanin, and essential oils within cumin (2). Cumin seeds have anti-carcinogenic properties and can help prevent the development of stomach or liver tumors in laboratory animals (11).

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